Impact of green tea catechins on the oxidative stability of polyunsaturated fatty acid-rich oils

Caroline Waingeh Nain

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Promoteur : Prof. Yvan Larondelle (UCLouvain)

Président du jury : Prof. Michel Ghislain (UCLouvain)

Membres du jury : Prof. Philippe Baret (UCLouvain)
Prof. John Van Camp (UGent)
Prof. Cathy Debier (UCLouvain)
Prof. Marie-Louise Scippo (ULiège)

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bless His holy name (Ps 103:1).

Summary

Lipid oxidation is one of the major causes of deterioration in edible oils, negatively affecting the sensory and nutritional properties of foods and limiting product acceptability. To curb the oxidation of oils during storage and when incorporated into food products, we investigated the effect of green tea catechins on the oxidative stability of oils with a high PUFA content, including DHA-rich oil, linseed oil and sacha inchi oil. The effect of catechins was also evaluated in salad dressings, mayonnaise and crackers enriched with these oils. We showed that green tea extracts (GTEs) enhance the oxidative stability of these oils by limiting the formation of hydroperoxides and their breakdown into secondary oxidation products, decreasing the degradation of fatty acids and inhibiting or reducing the losses in tocopherols. The GTEs were also effective antioxidants in complex food systems enriched with ALA, such as salad dressings and mayonnaise. However, a pro-oxidant effect of GTE was observed in crackers enriched with ALA and DHA, most likely due to the cooking process and interaction of catechins with other compounds in the matrix. In addition, we demonstrated that the antioxidant activity of GTE in the DHA-rich oil was influenced by its catechins profile. GTE rich in epigallocatechin showed more potent antioxidant activity in DHA-rich oil than a GTE rich in epigallocatechin gallate. The efficiency of GTE was also shown to be dose-dependent. Thus, the concentration of catechins in the extract and the quantity of extract added to oil or food products is vital to achieving good antioxidant activity. The results obtained, therefore, indicate that green tea catechins are suitable for use as natural antioxidants in oils with a high PUFA content.

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Abbreviations

ALA	α-linolenic acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
DHA	Docosahexaenoic acid
DHAO	DHA-rich oil
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
GTE	Green tea extract
HPLC	High performance liquid chromatography
HPSEC	High-performance size exclusion chromatography
MDA	Malondialdehyde
MRO	Mayonnaise with rapessed oil
MRSIO	Mayonnaise with rapeseed and sachainchi oils
MUFA	Monounsaturated fatty acid
NMR	Nuclear magnetic resonance analysis
OSGT	Oil-soluble green tea extract
p-AV	p-anisidine value
PUFA	Polyunsaturated fatty acis
PV	Peroxide value
RO	Rapeseed oil
SFA	Saturated fatty acids
SIO	Sacha inchi Oil
TBARS	Thiobarbituric acid reactive substance
TBHQ	Tert-butylhydroquinone

General introduction

Oils and fats form an integral part of a healthy diet. Edible oils from vegetable and marine sources consist mainly of triglycerides (>90%), which are molecules composed of three fatty acids linked via an ester linkage to a glycerol backbone. The fatty acids that make up triglycerides may differ in chain length and number of double bonds (Cuvelier and Maillard, 2012). Thus, fatty acids can be classified with respect to the degree of unsaturation into saturated, with no double bonds; monounsaturated (MUFA), with one double bond and polyunsaturated (PUFA), with two or more double bonds. Polyunsaturated fatty acids (PUFAs) are of particular interest. They act as health modulators, either as such or as precursors of compounds such as eicosanoids and docosanoids, among which some modulate the risk of cardiovascular and inflammatory diseases, diabetes and neurological disorders, among others (Vázquez et al., 2019; Zárate et al., 2017). Among the PUFAs, linoleic acid (C18:2) and α-linolenic acid (C18:3, ALA) are essential fatty acids that can only be obtained from the diet and are precursors of the omega-6 and omega-3 fatty acid families, respectively. However, the conversion of ALA to its long-chain derivatives, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is substantially low in humans, estimated at less than 6% and 1%, respectively (Brenna, 2002). Consequently, EPA is considered 'semi-essential', and DHA is "essential" because a sufficient contribution from the diet is respectively useful or necessary to cover physiological requirements (SHC, 2016). Fats and oils are therefore indispensable in the diet as a source of these fatty acids, in addition to contributing to energy intake, acting as a carrier for fat-soluble vitamins and enhancing the organoleptic quality of foods (Hernandez, 2016).

The Belgian Superior Health Council recommends a total fat intake between 20 and 35% of daily calories, with the PUFAs expected to

contribute 5-10 % of the daily calories. In addition, an ALA intake of 2.2 and 2.8 g/day is recommended for adult women and men, respectively, based on daily energy requirements of 2000 kcal for women and 2500 kcal for men. On the other hand, a daily allowance of 250-500 mg/day is recommended for EPA + DHA, irrespective of gender (SHC, 2016). Nevertheless, the 2014 food consumption survey of the Belgian population revealed an average intake of 1.5 g/day of ALA and 190 mg/day of EPA + DHA (De Ridder et al., 2016). Hence, consuming food products and oils rich in these PUFAs is encouraged, which further fuels the interest in oils rich in ALA, such as linseed and sacha-inchi oils, as well as DHA-rich oil supplements.

Unfortunately, oils rich in PUFAs are highly susceptible to oxidative deterioration. Exposure to oxygen, light, heat and metal ions results in the formation of hydroperoxides, which further break down into secondary oxidation products, including aldehydes and ketones responsible for off flavours (Choe and Min, 2006). Thus, lipid oxidation in oils and oil-containing foods leads to decreased sensory quality, nutritional value and shelf life (Ahmed et al., 2016; Syed, 2016). In addition, some secondary oxidation products have potentially harmful effects on health. The oxidation rate of oils depends on the degree of unsaturation of their fatty acids and their content. Indeed, oils with a high PUFA content are more sensitive to oxidation, and the sensitivity increases with the number of double bonds on the fatty acids (Syed, 2016). Lipid oxidation, therefore, poses a real challenge in the handling, processing and storage of PUFA-rich oils. Hence, various strategies have been implemented to reduce or prevent lipid oxidation, including creating a physical barrier between the oil and the external environment, encapsulation and the incorporation of antioxidants, which is the commonly used strategy.

Antioxidants inhibit lipid oxidation by either scavenging free radicals, chelating transition metals, quenching singlet oxygen and/or inhibiting the activity of photoactivated sensitizers (Decker

et al., 2010). An antioxidant is "any substance which, when present in low concentration relative to that of an oxidizable substrate, significantly retards or inhibits the oxidation of that substrate"(Halliwell, 1995). Antioxidants have become an indispensable group of food additives, mainly because of their unique properties of extending the shelf life of food products by retarding lipid oxidation and also protecting the human body from the effects of free radicals and reactive oxygen species (Gulcin, 2020). Antioxidants used in food systems should be inexpensive, effective and non-toxic at low concentrations, easy to incorporate, of good solubility and able to partition favourably between the oil–air/water interfaces in bulk oil/emulsions, stable and able to survive processing, with no effect on odour, taste or colour of the product (Frankel, 2012a; Gulcin, 2020; Shahidi and Ambigaipalan, 2015).

Antioxidants naturally present in oils or added intentionally are determining factors in the oxidative stability of PUFA-rich oils. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are used in oils and food products to delay oxidation, but because of health risks associated with these compounds, their application is regulated in most countries (Lourenço et al., 2019). Hence, increasing interest is focused on natural antioxidants, especially with the surge in consumers' preference for natural products over synthetic ones (Scott et al., 2020). In this light, various plant extracts have been investigated for antioxidant activity, including those prepared from rosemary, green tea leaves, tomato peels, grape seeds, rambutan peel and apple peel (Elbadrawy and Sello, 2016; Kozłowska et al., 2019; Nieto et al., 2018; Phuong et al., 2020; Wolfe et al., 2003). Some of these extracts such as that from green tea leaves may exhibit similar or better antioxidant properties compared to synthetic antioxidants. Indeed, green tea extract (GTE) has shown better efficiency in delaying the oxidation of seal blubber and menhaden oils than

commonly used synthetic antioxidants like BHA and BHT (Wanasundara and Shahidi, 1998). Furthermore, a lower oxidation level has been reported for GTE-supplemented biscuits stored at 70°C for 30 days compared to those supplemented with BHT (Mildner-Szkudlarz et al., 2009). In addition, GTE showed more potent antioxidant activity in seal blubber oil, cooked meat, and table spreads than α-tocopherol (Dwyer et al., 2012; Tang et al., 2001; Wanasundara and Shahidi, 1998). Among the plant extracts, GTE appears quite strong. As an example, it has also shown a more prominent antioxidant activity than rosemary extract in margarine enriched with rapeseed oil (Fruehwirth et al., 2021) and in butter (Gramza-Michalowska et al., 2007). GTE, can thus be considered as a plant extract with a potent activity among the natural antioxidants, being thus a potential candidate for use in highly polyunsaturated systems.

Green tea is a beverage rich in polyphenols prepared from leaves of *Camellia sinensis* and widely consumed around the world. The interest in green tea is further incited by studies demonstrating its antioxidative (Yang et al., 2009), antihypercholesterolemic (Kim et al., 2011), anticarcinogenic (Gu et al., 2013), anti-obesity and antibacterial (Reygaert, 2018) properties through *in vitro*, animal and human studies. The beneficial effects of green tea are attributed to its catechins content, which accounts for about 15-27% of the dry weight of green tea leaves (Senanayake, 2013). The antioxidant activity of green tea extracts (GTE) has been demonstrated in various food systems, including baked products (Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009), meat (Tian and Huang, 2019), beverages (Boroski et al., 2020), table spreads (Dwyer et al., 2012), as well as bulk oils (Shen et al., 2021; Wanasundara and Shahidi, 1998).

Although green tea catechins are proven antioxidant agents, their activity depends on the system in which they are added. Not much literature is available on the antioxidant activity of GTE in oils with a high content of PUFAs, especially ω-3 fatty acids, which leads us

to the purpose of this thesis: to assess the impact of green tea catechins on the oxidative stability of oils with a high PUFA content such as DHA-rich oil, linseed oil, and sacha inchi oil. The research is based on the hypothesis that green tea catechins are potent antioxidants that can delay or slow down oxidation in these PUFA-rich oils. It has an applied dimension geared towards practical application in industries.

Thesis outline

The first part of this thesis is introductory, with the first chapter setting a background for the work by reviewing the literature on lipid oxidation, its mechanisms and consequences, as well as the antioxidant activity of green tea catechins and extracts in oils and oil-containing foods. A detailed overview of the objectives and scientific strategies then followed in Chapter 2. Then, the experimental work, written in the form of scientific papers, is presented in Chapters 3, 4 and 5. In Chapter 3, three studies are described, *investigating the ability of GTE to delay or slow the formation of oxidation products in PUFA-rich oils*. Chapter 4 discusses the effect of the catechins profile of GTE on its antioxidant activity in DHA-rich oil. *In Chapter 5, 3* studies investigate *the antioxidant activity of GTE in food matrices enriched with PUFA-rich oils.* A general discussion of the results, research prospects emanating from our study and general conclusions are then presented in Chapter 6.

Chapter 1. Literature review

1. Lipid oxidation in edible oils: mechanisms and consequences

1.1 Introduction

Lipid oxidation is an undesirable series of chemical reactions between fatty acids and oxygen, resulting in the quality deterioration of edible oils and fat-containing foods (Ahmed et al., 2016; Mozuraityte et al., 2016a). The oxidation of oils destroys essential fatty acids, and low-molecular-weight off-flavour compounds are produced, which makes the oil less acceptable to consumers as well as for industrial use (Choe and Min, 2006). Unsaturated fatty acids are more susceptible to lipid oxidation, as oxidation begins with the double bonds of fatty acids (Zamuz et al., 2022). In addition, the susceptibility and the rate of oxidation increase as the number of double bonds in the fatty acid increases (Syed, 2016). Lipid oxidation has always been one of the major issues in the food industry and, even more so now, as the recognition of the nutritional and health importance of PUFAs has led to an increase in the formulation of foods with high PUFA content (Schaich, 2020). Thus, understanding the lipid oxidation mechanism, its effects and how it can be prevented is necessary when working with PUFAs.

1.2 Lipid oxidation Mechanisms

Lipid oxidation proceeds through different pathways, including autoxidation (oxidation with $^3O_2$), photooxidation (oxidation with $^1O_2$) or enzymatic oxidation.

1.2.1 Autoxidation

Autoxidation is the most important process by which lipid oxidation takes place in edible oils or oil-based foods. It occurs via

a free radical chain mechanism in an autocatalytic manner (Mozuraityte et al., 2016a). Autoxidation corresponds to a sequence of chemical reactions that result from the interaction of unsaturated fatty acids with oxygen and leads to lipid breakdown and the formation of a wide range of oxidation products (Kubow, 1992; Wang et al., 2017). The autoxidation process occurs in three main steps: initiation, propagation and termination (Figure 1).

Initiation

$$RH \rightarrow R^{\bullet} + H^{\bullet}$$

Initiation is the step during which fatty acid radicals or alkyl radicals ($R^{\bullet}$) are formed. When a lipid substrate (RH) is exposed to an "initiator" such as heat, light or metal ions, a hydrogen atom is abstracted from the carbon atom adjacent to the double bond, resulting in the formation of a radical ($R^{\bullet}$) (Ahmed et al., 2016; Echegaray et al., 2022). The energy to extract hydrogen from the fatty acid depends on the position of the hydrogen in the molecule (Choe and Min, 2006). In the case of two double bonds separated by a carbon (dienes), the removal of the proton is accompanied by a rearrangement of the bonds, which then become conjugated (Cuvelier and Maillard, 2012). Thus, conjugated dienes or trienes can be formed. The ease by which fatty acid radicals are formed increases with an increased degree of unsaturation because the more double bonds there are around a hydrogen atom, the less energy for its extraction (Holman and Elmer, 1947; Wang et al., 2017).

Propagation

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

Propagation begins once the alkyl radical (R•) is formed. First, the alkyl radical reacts rapidly with $^3O_2$ and forms a lipid peroxyl radical (ROO•), another reactive radical (Choe and Min, 2006).

Once formed, the lipid peroxyl radical abstracts hydrogen from another unsaturated fatty acid to form a hydroperoxide (ROOH) and another alkyl radical (R•) (Choe and Min, 2006; Wang et al., 2017). Next, the new alkyl radical reacts with $^3O_2$ to give a peroxyl radical, creating a radical propagation chain. Because this step is slow and rate-determining, hydrogen abstraction from unsaturated lipids become selective for the weakly bound hydrogen. The susceptibility of lipids to autoxidation depends on the availability of allylic hydrogens and their relative ease of reacting with peroxyl radicals to form hydroperoxides (Frankel, 2012). It is estimated that when a radical initiates oxidation, about 25 fatty acid molecules are oxidised in the propagation phase (Cillard and Cillard, 2006). The length of the propagative cycle is proportional to the degree of unsaturation and is limited by the availability of lipid substrates and oxygen (Kubow, 1992).

Termination

$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$$

$$R^{\bullet} + ROO^{\bullet} \rightarrow ROOR$$

$$ROO^{\bullet} + ROO^{\bullet} \rightarrow ROOR + O_2$$

Termination is the final step of the autoxidation reaction, where two radicals react to produce a non-radical species or when an antioxidant donates a hydrogen atom to the lipid peroxyl radical, forming a non-radical species (Ahmed et al., 2016; Cillard and Cillard, 2006). Various termination reactions are possible, as shown in equations above. However, most common reactions occur through the combination of peroxyl radicals ($ROO^{\bullet}$) (Labuza and Dugan, 1971). The termination reactions result in the formation of peroxyl-linked dimers (ROOR), ether-containing dimers (ROR) or carbon-carbon-linked dimers (R-R) (Frankel, 2012). Also, hydroperoxides are unstable and further decompose into secondary oxidation products (Wang et al., 2017).

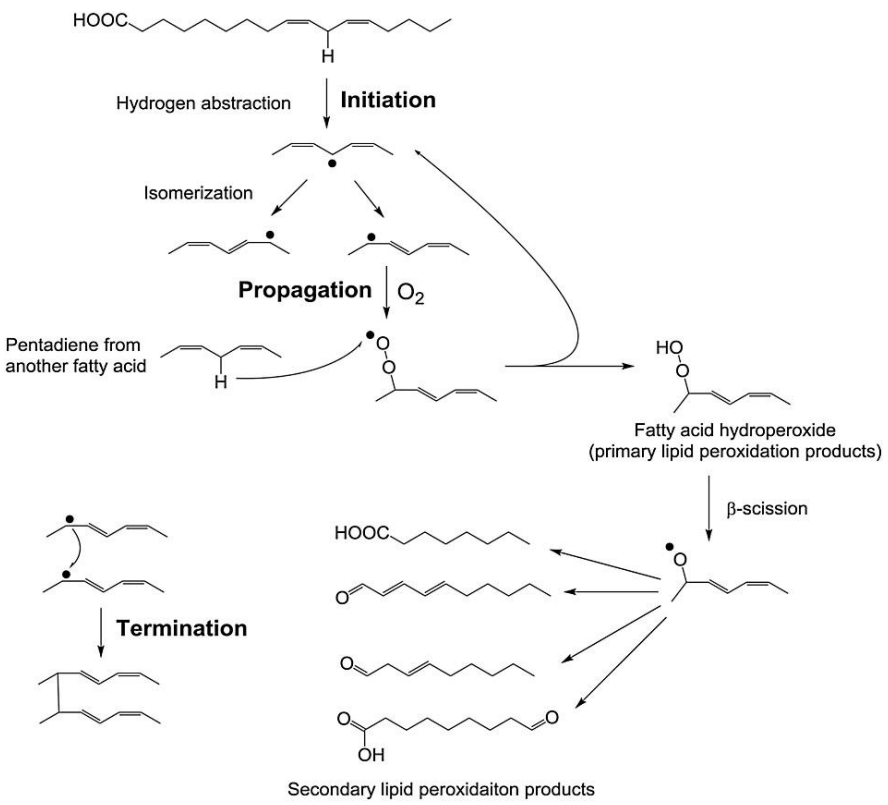


Figure 1. A simplified scheme of lipid autoxidation (Wang et al., 2017)

1.2.2 Photooxidation

Photooxidation, or simply light-induced oxidation, occurs in the presence of light, oxygen, and photosensitizers. Photosensitizers are molecules that enter an excited state by absorbing light in the visible or near-UV range and can convert triplet oxygen into reactive singlet oxygen (Frankel, 2012; Mozuraityte et al., 2016a). Examples of photosensitizers are haemoglobin, myoglobin, pheophytin, and chlorophyll (Choe and Min, 2006; Zamuz et al., 2022). The oxidation of lipids by photooxidation can proceed via two pathways: Type I and Type II (Figure 2). In Type I photooxidation, the excited triplet photosensitizer interacts directly with unsaturated fatty acids to generate alkyl radicals, which then react with triplet-state oxygen to form peroxyl radicals.

In type II photooxidation, the excited photosensitizer reacts with molecular oxygen in the triplet state to form singlet oxygen molecules (Madhujith and Sivakanthan, 2019; Zamuz et al., 2022). The reactive singlet oxygen then reacts with the unsaturated fatty acid to form hydroperoxides that decompose into secondary oxidation products. The rate of lipid oxidation mediated by singlet oxygen is much higher than the rates in autoxidation. For instance, the reaction rates of linoleic acid with singlet oxygen and triplet oxygen are 1.3 x 10^5 and 8.9 x 10^1 $M^{-1}S^{-1}$, respectively (Choe and Min, 2006; Mozuraityte et al., 2016a).

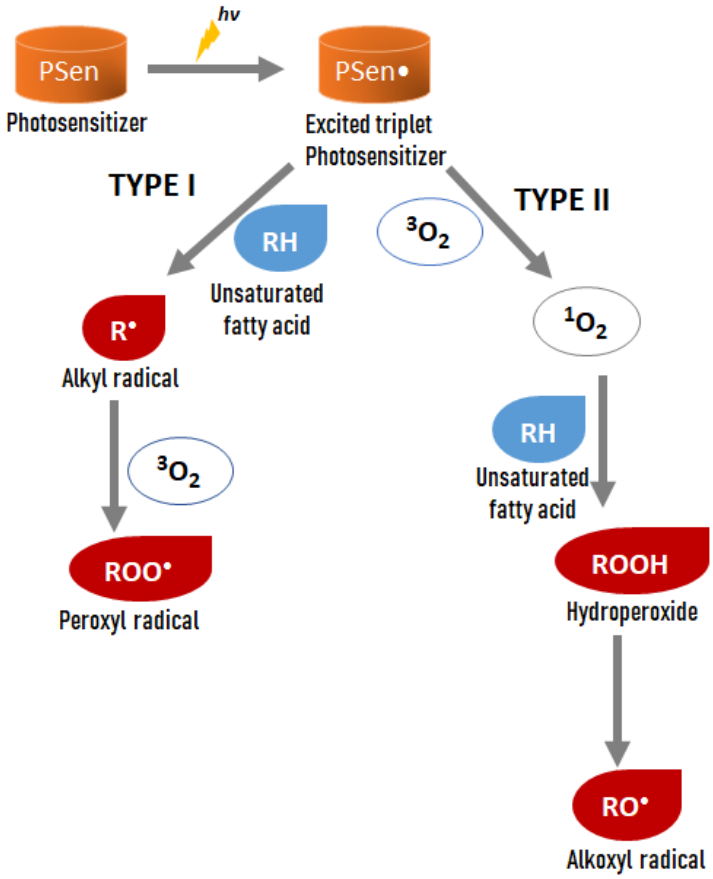


Figure 2. Lipid photooxidation pathways (Zamuz et al., 2022)

1.2.3 Enzymatic oxidation

Lipid oxidation can also result from the activity of enzymes. Lipoxygenase is the main enzyme involved in the enzymatic oxidation of lipids, catalysing the reaction of unsaturated fatty acids with oxygen to form hydroperoxides (Tayeb et al., 2017; Wang and Hammond, 2010). Lipoxygenase is an enzyme with iron in its active centre (Choe and Min, 2009). Lipoxygenase in the ferrous state (Fe^{2+}) is inactive with fatty acids but is oxidised to an active ferric (Fe^{3+}) form by fatty acid hydroperoxides or hydrogen peroxide (Madhujith and Sivakanthan, 2019; Zheng and Brash, 2010). Lipoxygenase in the ferric state abstracts hydrogen from a 1,4-pentadiene fatty acid moiety to form a pentadienyl radical, which further reacts with molecular oxygen to form a peroxyl radical, leading to the formation of hydroperoxides (Ahmed et al., 2016; Wąsowicz et al., 2004). The favoured substrates for oxidation by lipoxygenase are oils containing linoleic acid, ALA, arachidonic acid, EPA and DHA (Choe and Min, 2009; Hsieh and Kinsella, 1986). Figure 3 illustrates the enzymatic oxidation of lipids by lipoxygenase.

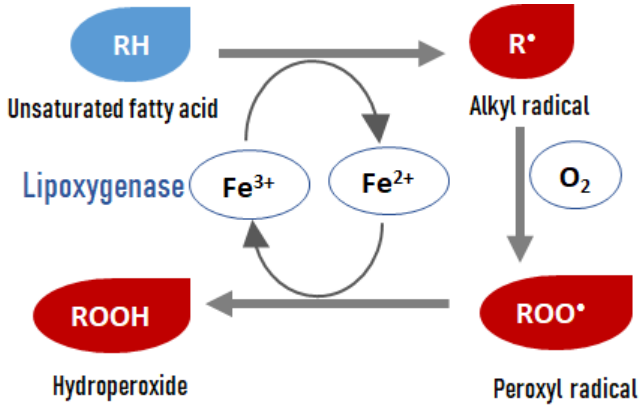


Figure 3. Lipid oxidation catalysed by lipoxygenase (Zamuz et al., 2022)

1.2.4 Decomposition of hydroperoxides

The hydroperoxides, formed during autoxidation, photooxidation or enzymatic oxidation, are unstable and further break down to form secondary oxidation products (Wang et al., 2017). The decomposition of hydroperoxides is catalysed by metal ions such as Fe^{2+} and Cu^{+}, high temperatures or ionising radiations (Johnson and Decker, 2015; Nawar, 1984). The initial step in the decomposition of hydroperoxides is the formation of peroxyl and alkoxyl radicals by homolytic cleavage of an O-H or O-O bond, respectively (Kubow, 1992). In the presence of metal ions, the reaction is much faster (Ahmed et al., 2016).

a) $Fe^{3+} + ROOH \rightarrow ROO^{\bullet} + Fe^{2+} + H^{+}$ (slow reaction)
b) $Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-}$ (fast reaction)

The alkoxy radical then undergoes homolytic *β*-scission of the carbon-carbon bond and produces oxo-compounds and saturated or unsaturated alkyl radicals (Matthäus, 2010). Furthermore, following electron rearrangement, the addition of hydroxyl radical, or hydrogen transfer, secondary lipid oxidation products formed are mostly low molecular-weight aldehydes, ketones, alcohols, carboxylic acids and short-chain hydrocarbons (Choe and Min, 2006). Most of these secondary oxidation products are responsible for the off-flavour in oxidised edible oil and are characterised by very low perception thresholds (Madhujith and Sivakanthan, 2019). Cyclisation and addition reactions also arise from alkoxyl radicals resulting in the formation of epoxides and polymers. Thus, lipid oxidation is highly complex, as shown in Figure 4 below.

The formation of the secondary oxidation products is directly conditioned by the initial fatty acid composition of the oil (Choe and Min, 2006; Echegaray et al., 2022). The decomposition of hydroperoxides becomes significant at advanced levels of linoleic acid oxidation and earlier stages of oxidation of ALA and fatty acids containing more than three double bonds (Frankel, 2012).

Although three successive steps (initiation, propagation and termination) classically describe oxidation, the timing of these steps is more intricate because secondary oxidation compounds could form in the first steps of lipid oxidation, simultaneously with the primary oxidation compounds (Dridi, 2016).

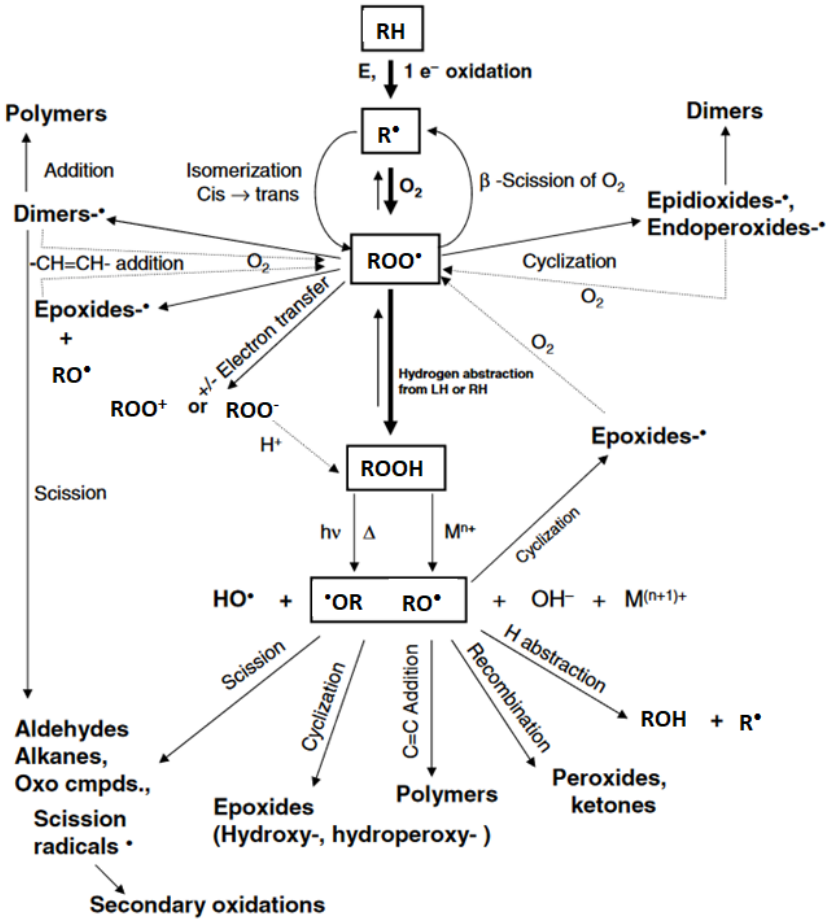


Figure 4: Integrated scheme of the main reactions involved in lipid oxidation (Schaich, 2020).

1.3 Factors affecting the oxidation of oils

Many factors influence the rate at which lipid oxidation occurs in an oil or oil-containing food. These factors are related to both the inherent characteristics of the oil itself (intrinsic factors) and to elements that are external to it (extrinsic factors) (Figure 5).

Figure 5. Factors influencing lipid oxidation of edible oils (Echegaray et al., 2022)

1.3.1 Fatty acid composition of oil

As mentioned earlier, the susceptibility of a fatty acid to oxidation increases with the number of double bonds (Ahmed et al., 2016; Labuza and Dugan, 1971; Wang et al., 2017). As a result, unsaturated fatty acids are highly susceptible to oxidation, and edible oils that are more unsaturated are oxidised more quickly than less unsaturated oils (Choe and Min, 2006). This is because the dissociation energy of the C-H bond at bis-allylic methylene groups present in PUFAs is lower than at allylic methylene groups in MUFAs (Gómez-Cortés et al., 2015; Johnson and Decker, 2015). When the relative oxidative stabilities of typical PUFAs are compared, DHA (22:6n-3) is most rapidly oxidised, followed by EPA (20:5n-3), arachidonic acid (20:4n-6), ALA (18:3n-3), and linoleic acid (18:2n-6) (Miyashita, 2014, 2019). The autoxidation

rates of some PUFAs based on oxygen absorption showed that linoleate was 40 times more reactive than oleate, linolenate was 2.4 times more reactive than linoleate, and arachidonate was 2.0 times more reactive than linolenate (Frankel, 2012).

1.3.2 Presence of pro-oxidants

Pro-oxidants are compounds that initiate, facilitate, or accelerate lipid oxidation and are efficient at very low concentrations (Mozuraityte et al., 2016a). These compounds include transition metals, haemoproteins, chlorophylls and enzymes.

Transition metals such as copper, iron, manganese, and nickel are naturally present in vegetable oils, absorbed by plants during growth and can also get into oil during processing (Madhujith and Sivakanthan, 2019). They act as pro-oxidants by reacting directly with lipids to form lipid alkyl radicals or by reacting with hydroperoxides, resulting in their decomposition into secondary oxidation products (Ahmed et al., 2016; Frankel, 2012). The effect of metal ions can be decreased by using chelating materials that form strong complexes with metals.

In animal-derived lipids, free iron and/or heme proteins are the two primary forms of iron responsible for the oxidation of fatty acids (Amaral et al., 2018). Haemoglobin and myoglobin are the most abundant heme proteins in animal tissues, particularly in blood-rich organs (Mozuraityte et al., 2016a). Free iron can be released by destroying the heme group in haemoglobin and myoglobin. On the other hand, the oxidation of heme-proteins results in products that can react with PUFAs and degrade hydroperoxides into peroxyl and alkoxyl radicals (Domínguez et al., 2019; Zamuz et al., 2022)

Chlorophyll is a plant pigment naturally occurring in oils. This compound and compounds derived from its degradation (pheophytin and pheophorbide) act as sensitizers to produce

singlet oxygen, which triggers photooxidation reactions (Matthäus, 2010). In this way, they strongly accelerate the oxidation of vegetable oils. On the contrary, in the absence of light, chlorophylls can act as antioxidants by donating hydrogen to the free radicals present in the oil (Choe and Min, 2006).

Enzymes such as lipoxygenase favour oxidation in oils by catalysing the reaction between molecular oxygen and a lipid substrate to form hydroperoxides (Tayeb et al., 2017).

1.3.3 Oil processing

The degree of processing applied to an oil affects its oxidative stability. Crude oils generally contain non-triglyceride components such as free fatty acids, partial acylglycerols, pigments (gossypol, chlorophyll), vitamins, protein fragments, trace metals, and mucilaginous materials (Madhujith and Sivakanthan, 2019). There are two different methods of oil production (with and without refining), which affect oxidative stability in different ways. Thus, a distinction is made between refined and virgin oils.

In the case of refined oils, the raw plant material is mechanically and then chemically extracted with organic solvents (usually hexane), resulting in very high extraction yields (Echegaray et al., 2022). A refining process is then carried out to eliminate the undesirable compounds generated and make the oil suitable for the food industry (Gharby, 2022). However, although the refining process removes impurities, it also removes antioxidant compounds in the oil, reducing oxidative stability (Choe and Min, 2006).

Virgin oils, on the other hand, are obtained only by physical processes. Thus, the extraction is done by pressing followed by filtration, decantation or centrifugation. In this way, oils with high oxidative stability are obtained because although pro-oxidant substances are not eliminated, for the most part, the oils contain

high concentrations of bioactive compounds with antioxidant activity (Echegaray et al., 2022). However, if the starting raw material does not have a good oxidative status or if the physical conditions of extraction and/or storage are inadequate, the virgin oil obtained may have lower oxidative stability than that obtained by refining techniques (Matthäus, 2010).

1.3.4 Light exposure

Although light is a known catalyst for oxidation, its effect is more important in photooxidation than in autoxidation. Photosensitizers are electronically excited when they absorb light and subsequently lead to the formation of singlet oxygen or react directly with a lipid substrate (Mozuraityte et al., 2016a). Light of shorter wavelengths has more detrimental effects on oil than longer wavelengths, with ultraviolet light being more harmful than visible light (Choe and Min, 2006; Sattar et al., 1976). It was observed that singlet oxygen reacts about 1000–10,000 times faster than oxygen in a triplet ground state at ambient temperature (Velasco and Dobarganes, 2002). This is because singlet oxygen can react directly with lipids, whereas triplet oxygen reacts with lipid radicals (Dridi, 2016). Therefore, storing oils away from light is important to enhance oxidative stability.

1.3.5 Oxygen exposure

Oil oxidation occurs when oil, oxygen and catalysts are in contact. Therefore, oxygen is a necessary condition for oxidation to occur and a source of reactive oxygen species that can react with alkyl radicals and/or directly with fatty acids (Choe and Min, 2006; Echegaray et al., 2022). Both the concentration and type of oxygen affect the oxidation reaction of oils and oil-containing foods (Choe and Min, 2006). The most abundant and stable form of oxygen, triplet oxygen, can be present as a dissolved gas in an oil or food matrix, as undissolved gas in the headspace above the food, or as

undissolved gas trapped in a solid or semi-solid food matrix (Johnson and Decker, 2015). Dissolved oxygen has been correlated with oxygen disappearance, generation of free radicals, and formation of volatile lipid oxidation products in food (Johnson and Decker, 2015). The oxygen concentration in oil is conditioned by the partial pressure of oxygen in the headspace of the oil container (Madhujith and Sivakanthan, 2019). In concrete terms, high partial pressures of oxygen cause the proportion of dissolved oxygen in the oil to increase, which triggers oxidation reactions (Min and Wen, 1983; Mozuraityte et al., 2016b). The oxygen partial pressure can influence the oxidation kinetics as follows:

- At high oxygen partial pressures (i.e. oxygen is not limiting), the oxidation rate depends on the substrate concentration, the propagation phase is rapid, and all free radicals are converted to peroxyl radicals, regardless of oxygen concentration.
- At low partial pressures (i.e. oxygen is limiting), if the substrate concentration is high, the oxidation rate depends directly on the oxygen partial pressure, and the substrate concentration remains almost unchanged.

Although headspace oxygen has less surface area in contact with lipids than dissolved oxygen, it can be easily dissolved in the food by diffusion or mechanical agitation and thus constitutes a real oxygen reservoir for oxidation (Johnson and Decker, 2015).

1.3.6 Temperature

Temperature is one of the main factors influencing lipid oxidation rate during food processing and storage (Dridi, 2016). Various studies have reported an increase in the rate of autoxidation and decomposition of hydroperoxides with temperature increase (Aidos et al., 2002; Choulitoudi et al., 2021; Liu et al., 2014; Mohanan et al., 2018). Conversely, when the temperature is low or moderate, the formation of oxidation products is slow, which corresponds to a long induction phase (Choe and Min, 2006). Šimon

and Kolman (2001) showed that raising the temperature from 100°C to 125°C decreases the induction time from about 11 hours to less than 2 hours for rapeseed oil and from 7 hours to 1 hour for sunflower oil. Fish oil samples stored at -18°C were shown to have a shelf life twice as long as samples held at 4°C, highlighting the effect of storage temperature on the storage stability of fish oil (Boran et al., 2006).

Unlike autoxidation, the temperature has little effect on reactions that occur during photooxidation due to the low activation energy necessary for the reaction between singlet oxygen and fatty acids (Choe and Min, 2006). However, in general, the increase in temperature leads to an acceleration in oxidation, which follows the Arrhenius law (Sathivel et al., 2008):

$$k = Ae^{-\frac{Ea}{RT}}$$

where k is the reaction rate constant, A is the pre-exponential factor or frequency factor, Ea is the activation energy (kJ mol^{-1}), R is the molar gas constant (8.314 J K^{-1} mol^{-1}), and T is the absolute temperature (K).

1.3.7 Presence of antioxidants

An antioxidant is usually defined as "any substance that, when present at low concentrations relative to that of an oxidisable substrate, significantly retards or prevents the oxidation of the substrate" (Cillard and Cillard, 2006; Mozuraityte et al., 2016b). Most edible oils naturally contain antioxidants such as tocopherols, tocotrienols, carotenoids, phenolic compounds, and sterols, which serve as endogenous protection against oxidation (Cuvelier and Maillard, 2012). However, the amounts are sometimes insufficient to protect the oil during prolonged storage, and many food processing operations can also destroy these natural antioxidants. Adding exogenous antioxidants is a solution often used to overcome this and extend the shelf life of edible oils (Decker et al., 2010). Antioxidants can be synthesised naturally or chemically.

Although chemically synthesised antioxidants are effective, in recent years, the focus has been on natural antioxidants, which are preferred by consumers (Blasi and Cossignani, 2020; Taghvaei and Jafari, 2015). Generally, antioxidants (natural and synthetic) are classified into two groups: primary and secondary.

Primary antioxidant

Primary antioxidants (AH) can donate hydrogen to peroxyl (ROO•) or alkoxyl radicals (RO•), blocking their reaction with fatty acids and thus acting as free radical scavengers (Mozuraityte et al., 2016a). The antioxidant thus becomes the radical species, but it is stable and less reactive than the peroxyl and alkoxyl radicals (Choe and Min, 2009). This scavenging mechanism is more efficient with peroxyl radicals, which contain less energy than alkoxyl radicals (Decker et al., 2010). The protection of Fatty acids by free radical scavengers increases the induction period, thereby decreasing the oxidation rate (Labuza and Dugan, 1971).

a) $ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$
b) $RO^{\bullet} + AH \rightarrow ROH + A^{\bullet}$

The major free radical scavengers are monohydroxy or polyhydroxy phenolic compounds because the phenolic ring gives them great stability, and the radical formed is stabilised by resonance delocalisation in the benzene ring structure (Choe and Min, 2006; Cuvelier and Maillard, 2012). The more resonance forms the phenolic radical has, the more stable it is and the stronger its antioxidant power (Labuza and Dugan, 1971).

The ability of the primary antioxidant to react more efficiently with free radicals than with unsaturated fatty acids is of key importance. This ability is related to the bond dissociation energy between the hydrogen and the free radical scavenger; the lower this energy, the more efficient the transfer of hydrogen to the free radical (Decker et al., 2010). The rules of redox reactions also come into play. Any compound whose reduction potential is lower than a free radical

can donate a hydrogen atom to this radical unless the reaction is kinetically unfavourable. Primary antioxidants generally have reduction potentials of about 500 mV or less, while alkyl, peroxyl, and alkoxyl radicals have reduction potentials of 600, 1000, and 1600 mV, respectively (Choe and Min, 2006). Other factors also influence the kinetics of the interaction between the free radical and the antioxidant, such as the steric barrier to hydrogen transfer or the solubility of the compound (Decker et al., 2010).

The efficiency of primary antioxidants can be affected by the concentration and time at which it is added to the lipid matrix. Primary antioxidants usually also react very slowly with peroxides to form alkoxyl radicals, but when their concentration is increased beyond a certain limit, the reaction is enhanced. Also, if the level of hydroperoxides is high at the time of antioxidant addition, this will mask its effectiveness (Labuza and Dugan, 1971).

Synthetic free radical scavengers

Synthetic antioxidants are deliberately added to products to prevent or delay the onset of lipid oxidation during the processing and storage of fats, oils, and lipid-containing foods. The synthetic antioxidants have different polarity, and the most used are BHT and BHA, both non-polar; TBHQ, which has an intermediate polarity; and propyl gallate, which is the most polar (Decker et al., 2010).

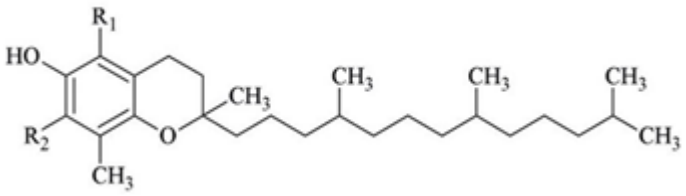
The antioxidant mechanism of synthetic phenolics leads to the formation of phenolic radicals that react only modestly with other PUFAs and oxygen to form hydroperoxides (Shahidi et al., 1992). Phenolic radicals can also react with other peroxyl radicals in termination reactions, leading to non-radical products (Decker et al., 2010). In addition, oxidised synthetic phenols undergo numerous degradation reactions to form compounds that still contain active hydroxyl groups and can therefore continue to scavenge free radicals (Decker et al., 2010; Shahidi et al., 1992). Synthetic antioxidants are strictly regulated because of safety

concerns, and the concentration is usually limited to 0.02% (Gulcin, 2020).

Naturally occurring free radical scavengers

The majority of natural antioxidants are phenolic compounds, and the most important groups of natural antioxidants are tocopherols, flavonoids and phenolic acids (Gulcin, 2020).

Tocopherols, also known as vitamin E, are among the most important natural fat-soluble antioxidants in many plants and their food derivatives. For example, sacha inchi oil, soybean oil, sunflower oil, canola oil, and corn oil contain relatively high amounts of tocopherols (Gliszczyńska-Świgło and Sikorska, 2004; Liu et al., 2014). The differences in tocopherols isomers are in their degree of methylation (Decker et al., 2010), and the four structural isomers are illustrated in Figure 6.



α-tocopherol: R1 = CH3 and R2 = CH3; β-tocopherol: R1 = CH3 and R2 = H

γ-tocopherol: R1 = H and R2 = CH3; δ-tocopherol: R1 = H and R2 = H

Figure 6. Structure of tocopherol

The antioxidant effectiveness of tocopherols depends on the concentration and isomers of tocopherol present (Choe and Min, 2006). The free radical scavenger activity of tocopherols is classified in the decreasing order δ > γ > β > α (Choe and Min, 2006). Meanwhile, α-tocopherol is generally the most reactive and less stable, followed by β-, γ-, and δ-tocopherols, which is related to their bond dissociation energies of 79.1, 80.2, 80.1, and 82.2 kcal/mol, respectively (Decker et al., 2010). The concentration of

tocopherol necessary for effective antioxidant activity varies with respect to the lipid matrix. For example, the optimal concentration of α-tocopherol as an antioxidant was reported as 50 ppm in sunflower oil (Blekas et al., 1995), 100 ppm in olive oil (Kamal-Eldin, 2006) and 250–500 ppm in corn oil emulsions (Huang et al., 1996). At high concentrations, tocopherols, especially α-tocopherol, act as pro-oxidants in vegetable oils via the propagation of free radicals, depending on the hydroperoxide concentration (Choe and Min, 2006).

Phenolic acids, widely distributed in plants, are hydroxy derivatives of aromatic carboxylic acids, which arise from either the benzoic acid group or the cinnamic acid group (Gulcin, 2020). The hydroxycinnamic acids include coumaric, ferulic, caffeic, chlorogenic, and sinapic acids, and the hydroxybenzoic acids include ellagic, gallic, salicylic, and vanillic acids (Choe and Min, 2009). The antioxidant activity of phenolic acids and their derivatives is influenced by the number and position of the hydroxyl groups bound to the aromatic ring, the binding site and the type of substituents (Gulcin, 2020; Sroka and Cisowski, 2003).

Flavonoids are major plant polyphenols, derivatives of diphenylpropanes and a heterocyclic 6-membered ring with oxygen, which act as very effective antioxidants (Gulcin, 2020). They include flavanols (catechins, naringin), flavanones (hesperidin, naringenin), flavones (apigenin, luteolin), flavonols (kaempferol, quercitrin, myricetin, quercetin) and anthocyanins (Choe and Min, 2009). Flavonoids may act as antioxidants by scavenging radicals such as lipid peroxyl radicals (Dugas et al., 2000), superoxide anion radicals (Fazilatun et al., 2005; Robak and Gryglewski, 1988) and hydroxyl radicals (Treml and Šmejkal, 2016), and may also act as singlet oxygen quenchers and metal ion chelators (Mira et al., 2002).

In general, the antioxidant ability of flavonoids depends on the presence of hydrogen or electron-donating substituents able to reduce free radicals and the ability to delocalize the unpaired

electron leading to the formation of a stable phenoxy radical. In addition, these compounds have metal-chelating potential, strongly dependent on the arrangement of hydroxyls and carbonyl groups around the molecule (Gulcin, 2020). Thus, some flavonoids act both as primary and secondary antioxidants.

Secondary antioxidants

Secondary antioxidants prevent or delay oxidation by indirect mechanisms, including metal chelation and singlet oxygen quenching, to limit the oxidation phenomenon (Mozuraityte et al., 2016a). They are called preventive antioxidants because they affect the initiation rate of oxidation by controlling the production of free radicals at the source before the propagation step (Labuza and Dugan, 1971). Thus, they complement other means of preventing oxidation or work in synergy with free radical scavengers (Cuvelier and Maillard, 2012).

Metal chelation

Transition metals are arguably the most important pro-oxidants in foods because of their presence in virtually all biological tissues and their ability to directly extract hydrogen from fatty acids to form alkyl radicals and to break down hydroperoxides into free radicals (Decker et al., 2010). Metal chelators form insoluble complexes with metals such as iron or copper ions, thereby inhibiting their redox cycle and preventing metal-catalysed lipid oxidation (Cillard and Cillard, 2006). They can also act by sterically hindering the formation of complexes between metals and lipid hydroperoxides or occupying all metal coordination sites (Decker et al., 2010). Chelating agents are generally water soluble, but some, such as citric acid, have been made lipid soluble, favouring the inactivation of metals in the lipid phase. The activity of chelators is pH-dependent since the chelator must be ionised to be active (Decker et al., 2010). The most commonly used metal-chelating agents are ethylenediaminetetraacetic acid (EDTA) and citric acid. Some plant polyphenols also have this ability due to

their orthodiphenolic groups. This is the case, for example, of quercetin, catechins and carnosic acid (Cuvelier and Maillard, 2012).
Chelators can also act as pro-oxidants by reducing the redox potential of metals, making redox cycling easier and favouring oxidation. The pro-oxidant or antioxidant properties depend on the concentration of metals and chelators. For instance, EDTA is prooxidative when the EDTA/iron ratio is <1 but antioxidative when EDTA/iron is >1 (Decker et al., 2010).

Singlet oxygen quenching

Singlet oxygen is typically formed in food by photosensitizers such as riboflavin and chlorophyll in the presence of light. It is a very reactive pro-oxidant because of its ability to directly add to the double bonds of unsaturated fatty acids, leading to the formation of hydroperoxides with further decomposition to secondary oxidation products (Decker et al., 2010). Singlet oxygen can be inactivated by chemical or physical quenching.

Chemical quenching involves a reaction between an antioxidant (quencher) and $^1O_2$, in which the antioxidant is oxidised to form its breakdown and oxidation products (Choe and Min, 2009). Chemical quenching of singlet oxygen typically occurs by compounds with double bonds, such as tocopherols and carotenoids (Decker et al., 2010). Carotenoids are particularly effective quenchers, with lycopene being the most reactive with $^1O_2$, showing a reaction rate ($31 \times 10^9\ M^{-1}S^{-1}$) six times higher than β-carotene (Cillard and Cillard, 2006). On the other hand, tocopherols, thiols, and ascorbic acid are less efficient towards the deactivation of $^1O_2$. Nevertheless, these compounds have high concentrations in biological systems and contribute equally with carotenoids to the deactivation of $^1O_2$. In addition, amino acids (such as histidine, tryptophan, and methionine), peptides, proteins and phenolic compounds are also good singlet oxygen quenchers (Choe and Min, 2009).

Physical quenching is a more effective pathway for singlet oxygen inactivation. In this case, the quencher deactivates singlet oxygen to the ground state triplet oxygen by either energy transfer or charge transfer without any chemical change and, therefore, no oxidation of the antioxidant (Choe and Min, 2006; Cillard and Cillard, 2006). This is possible when the energy level of a quencher is very near or below that of singlet oxygen (Choe and Min, 2009). Carotenoids with nine or more conjugated double bonds are good singlet oxygen quenchers by energy transfer.

1.3.8 Simplified kinetics of lipid oxidation

The kinetics of lipid oxidation can be summarised in three main stages (Figure 7).

Lag or induction phase (A): there is a decrease in the concentration of active antioxidants, while oxidation is relatively slow with the formation of small quantities of free radicals. The duration of this phase depends on factors such as the degree of unsaturation of fatty acids, the amount of antioxidants initially present in the matrix, the presence of pro-oxidants, the availability of oxygen and the temperature to which the lipid is exposed (Dagmey, 2020; Semb, 2012).

Production and accumulation of hydroperoxides (B): the time needed to reach the maximum level of hydroperoxides is related to the degree of unsaturation and happens fast in highly unsaturated lipids because their hydroperoxides decompose more easily.

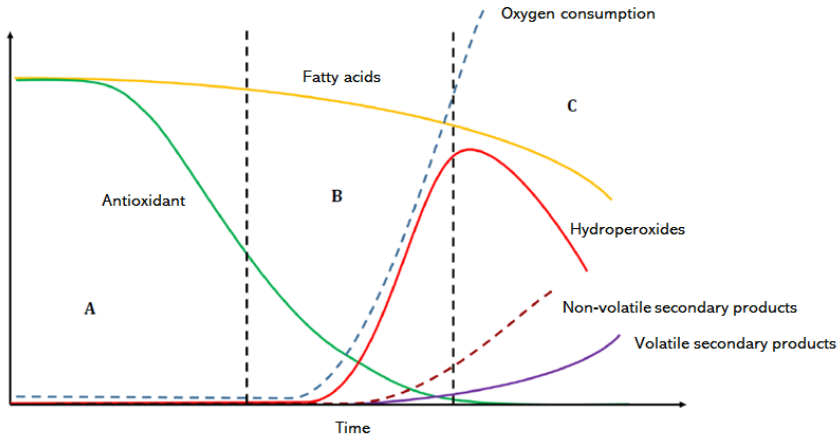


Figure 7. Scheme of the evolution of lipid oxidation over time (Dagmey, 2020)

Decomposition of hydroperoxides (C): at this stage, the hydroperoxides' decomposition rate surpasses formation rate (Shahidi and Wanasundara, 2002). In theory, this means that the primary oxidation products will dominate in the early stage, and secondary oxidation products will be more in the later stages of oxidation (Semb, 2012). The oxidation rate is, however, influenced by the intrinsic and extrinsic factors to which the oil is subjected.

1.4 Consequences of oxidation

Lipid oxidation reactions produce off-flavour compounds that make the oil less acceptable or unacceptable to consumers or for industrial application as a food ingredient (Choe and Min, 2006). Apart from losses in sensory characteristics, losses in nutritional value due to the degradation of essential fatty acids, vitamins and other bioactive compounds are also observed, as well as the formation of products that may be harmful to human health (Madhujith and Sivakanthan, 2019).

Loss in sensory properties

The oxidation of edible oils decreases sensory quality since oxidation reactions cause changes in the odour, flavour, colour, and

texture (Gómez-Cortés et al., 2015). The formation and accumulation of volatile products derived from lipid oxidation, such as alkanals, 2-alkenals, and trans, trans-2,4-alkadienals, with low odour threshold values contribute to the rancid flavour of oxidised oil (Choe and Min, 2006). Other oxidation compounds in edible oils that affect the aroma are 2,4,7-decatrienal, which produces a fishy off-flavour during storage or heat treatment, as well as 1-penten-3-one and 1-octen-3-one which are also associated with the "fishy" descriptor in oils (Echegaray et al., 2022; Matthäus, 2010). However, other compounds derived from lipid oxidation have been identified with pleasant odours. For example, 2,4-decadienal is considered one of the most important contributors to the flavour of deep-fried foods (Matthäus, 2010). Aldehydes and ketones are the main volatile compounds generated through lipid oxidation

In oil-containing foods such as emulsions, secondary lipid oxidation products (mainly aldehydes) can covalently or non-covalently bind to proteins, thus influencing the product texture and functionality and forming brown Maillard products (Bayram and Decker, 2022; Berton-Carabin et al., 2014). Free radicals originating from lipid oxidation also result in co-oxidation of colour pigments, e.g. oxidation of myoglobin negatively affects the colour of fresh meat (Wang et al., 2022).

Oils that contain ALA and long-chain omega-3 fatty acids result in volatile secondary oxidation products that are more damaging to the aroma, as would be the case of the 2,4,7-decatrienal (Matthäus, 2010).

Loss in nutritional quality

During the oxidation of the oils, there is a loss of unsaturated fatty acids (some being essential fatty acids) and vitamins and a degradation of beneficial antioxidants (Decker et al., 2010). In addition, in PUFA-containing foods, the reaction of lipid oxidation

products with proteins and amino acids leads to the loss of essential amino acids (Frankel, 2014).

Formation of toxic compounds

The oxidation of PUFAs leads to secondary oxidation products, which might have toxic effects. These potentially harmful lipid oxidation products include the following compounds.

- 4-hydroxy-2-trans-nonenal and 4-hydroxy-2-trans-hexenal are hydroxylated α,β-unsaturated aldehydes, which are non-volatile compounds and of interest due to their possible implication in the onset of diseases such as diabetes, atherogenesis, Alzheimer's disease, and cancer (Ma et al., 2020). Their toxicity is blamed on high chemical reactivity with nucleophiles such as proteins, DNA, and phospholipids, which results in structural damage and functional alterations *in vivo* (Ayala et al., 2014; Esterbauer et al., 1991; Long and Picklo, 2010).
- Malondialdehyde is also an oxidation product of interest observed to be mutagenic owing to its ability to react with the DNA base guanine and to exert cancer-initiating effects (Vieira et al., 2017).
- Acrolein (2-propenal) is an unsaturated aldehyde formed from lipids during high-temperature cooking and is acknowledged as toxic. Acrolein has been found to have adverse effects on the nervous system, respiratory tract, and cardiovascular organs (Zirak et al., 2019). Varying biological impact was observed in mice fed daily with acrolein, including myocardial oxidative stress, cardiomyopathy, and blood vessel dysfunction. The amount of acrolein fed to mice was established by considering the daily acrolein intake of an average human, thus suggesting a possibly harmful effect on the human body (Ismahil et al., 2011).
- Crotonaldehyde (2-butenal) is an unsaturated aldehyde mainly produced by the incomplete combustion of fats and can also be formed during the storage of lipids (Wang et al., 2022).

Crotonaldehyde led to liver damage and hepatic tumours in rats by forming propanodeoxyguanosine adducts in DNA (Chung et al., 1986). It can cause oedema-type lung damage and inflammation of bronchial cells (Dwivedi et al., 2018).

1.5 Methods for monitoring lipid oxidation

The assessment of lipid oxidation in oils and foods is necessary, given the effect of oxidation products on the sensory and nutritional properties of foods and human health. A variety of methods are used to determine lipid oxidation based on the appearance of primary oxidation products (conjugated dienes, hydroperoxides), secondary products (polymers, volatile compounds), and the consumption of oxygen or unsaturated fatty acids, among others (Table 1). To obtain reliable and complete information on a food's oxidation status, measuring both primary and secondary oxidation compounds is recommended (Dridi, 2016; Dwivedi et al., 2018; Shahidi and Wanasundara, 2002).

Expensive methods have also been developed to determine the degree of lipids oxidation, such as nuclear magnetic resonance (NMR), electron spin resonance (ESR), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) and Raman spectroscopy (Litwinienko, 2005; Wang et al., 2022). For most of these methods, there is no need to perform complicated processing on the sample (e.g. extraction of oil); thus, the properties of the sample are better maintained (Siciliano et al., 2013). However, these methods require sophisticated equipment.

Table1. Methods for determining lipid oxidation in oils

Component measured		**Method**	**Principle**
Primary oxidation products	Hydroperoxides	Iodometry (peroxide value) (AOAC, 2000)	Reduction of hydroperoxides in the presence of iodide ion. Measurement of iodine formed by titration with sodium thiosulfate
		Ferrous oxidation methods	Measurement of the oxidation of ferrous ions to ferric ions in the presence of ammonium thiocyanate or xylenol orange
		FTIR	Measurement based on the stoichiometric reaction of triphenylphosphine with hydroperoxides to produce triphenylphosphine oxide, which exhibits a readily measurable absorption band at 542 cm^{-1}.
		Chemiluminescence	Detection of chemiluminescent products generated by the reaction of hydroperoxides with luminol and dichlorofluorescein
		GC-MS	Determination of alcohols generated by reduction of hydroperoxides
	Conjugated dienes/trienes	UV spectroscopy	Absorbance measurement at 233 nm (dienes) / 268 nm (trienes)
	Aldehydes	*p*-Anisidine value (AOCS, 1998)	Generation of a coloured complex between p-anisidine and aldehydes (ex : 2-decenal ; 2,4

			decadienal ; 2,4,7 decatrienal), measurement by spectrophotometry at 350 nm
Secondary oxidation products	Substances reacting with thiobarbituric acid	TBARS assay	Generation of a coloured complex by reaction between thiobarbituric acid and oxidation derivatives (ex: malondialdehyde)
	Volatile compounds	GC	Volatile oxidation products such as hexanal, propanal can be determined by GC
	Carbonyls	Carbonyl content	Total or specific carbonyls by different spectrometric and HPLC techniques
	Polymers	HPSEC	Separation and identification of substances based on their molecular weight by HPSEC
Substrates	Oxygen	Absorption + mass gain	Measurement of decrease in oxygen pressure by GC and increase in mass gained by oxygen fixation
	Fatty acids	GC	Measurement of decrease in unsaturated fatty acids by GC analysis

GC-MS = Gas chromatography-mass spectrometry; TBARS = thiobarbituric acid reactive substance GC = Gas chromatography, HPLC = High-performance liquid chromatography; FTIR = Fourier transform infrared spectroscopy; HPSEC = High-performance size exclusion chromatography

Measuring the oxidative state of oil at a given time does not provide information about its possible evolution. However, it is possible to measure its oxidative stability or resistance to oxidation by using accelerated tests and the information obtained permits shelf life prediction (Matthäus, 2010). Methods used in determining the oxidative stability include the oven storage test, which applies temperatures between 25°C and 80°C with 60°C recommended for most studies (AOCS, 1997), the Swift test or Active Oxygen Method (AOM), which subjects oils to constant aeration at 98°C until a peroxide value of 100 meq O_2/kg is obtained and more recent automated tests such as Rancimat and Oxidative stability instrument (OSI), which are automated versions of AOM (Cuvelier and Maillard, 2012; Echegaray et al., 2022). In these tests, the oil is subjected to conditions that favour oxidation reaction and extrapolation to real storage conditions is nevertheless delicate and depends on each matrix.

1.6 Concluding remarks

Lipid oxidation is one of the major causes of deterioration in oils and oil-containing foods, which negatively affects the sensory and nutritional properties of foods, limiting product acceptability by consumers. The rate of lipid oxidation in oils depends on the fatty acid composition of the oil, its antioxidant content and how it is handled or processed. Besides the effect on nutrients, lipid oxidation can result in compounds with potentially toxic effects. The oxidative quality and safety of PUFA-rich oils can be ensured by reducing exposure to factors that catalyse oxidation and by supplementing with antioxidants.

2. Green tea catechins as natural antioxidants in edible oils and oil-containing foods

2.1. Introduction

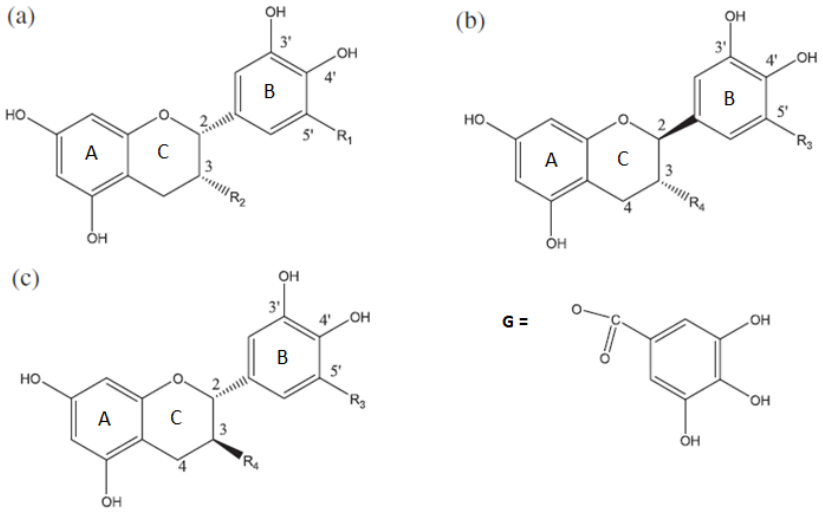
The tea plant (*Camellia sinensis*) is an evergreen shrub cultivated in over 30 countries worldwide, and the tea beverage is universally consumed (Vuong et al., 2010). The three major types of tea are green tea, oolong tea and black tea, based on the level of oxidation of polyphenols in tea leaves during processing. Green tea is obtained from fresh tea leaves by steaming or drying to limit the oxidation of polyphenolic components, especially catechins. Conversely, black tea is obtained by fermentation, which is a polyphenol oxidase-catalysed oxidation resulting in the loss of catechins. Oolong tea is a semi-fermented product with characteristics between black and green tea (Wang et al., 2000). Green tea has received a great deal of attention over the years, with studies suggesting its beneficial effect on health, including anticarcinogenic (Gu et al., 2013; Yang et al., 2009), antimicrobial and antiviral (Siriphap et al., 2022; Yang et al., 2021), antihypercholesterolemic (Kim et al., 2011), anti-obesity (Huang et al., 2014; Zang et al., 2021), anti-inflammatory (Donà et al., 2003) and antioxidant effects (Forester and Lambert, 2011; Peluso and Serafini, 2017). The beneficial effect of green tea is attributed to its catechins.

2.2. Green tea catechins

Green tea has a high polyphenol content, accounting for 25-35% of the dry weight of tea leaves, but this amount may vary depending on where the tea is grown, growth conditions, when it is harvested and how the leaves are processed (Reygaert, 2018; Senanayake, 2013). Catechins are the predominant polyphenolic compounds in green tea, making up 70-80% of the polyphenols (Liang et al., 1990;

Zheng et al., 2018). Catechins belong to the family of flavonoids and the subgroup flavan-3-ols, or flavanols. The major catechins in green tea leaves are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), the latter being the most abundant catechin in tea leaves (Luo et al., 2020). During processing and heat treatment of tea leaves, the epicatechins can be converted into their corresponding non-epistructured (or trans) catechins, i.e. catechin, gallocatechin, catechin gallate and gallocatechin gallate (Liu et al., 2021; Wang and Helliwell, 2000).

Catechins possess two benzene rings, the A and B rings, and a dihydropyran heterocycle or C-ring (Figure 8). The molecules have two chiral centres on carbons 2 and 3, leading to four diastereomers, two of which are in the cis configuration and the other two in the trans configuration (Senanayake, 2013). The structural features of catechins that significantly contribute to their antioxidant properties are the number and position of hydroxyl groups (or their substituents) on the rings and the presence/absence of a galloyl moiety (Botten et al., 2015). Thus, the structures involved in the antioxidant activity of catechins include the ortho-3'4'-dihydroxyl group or 3'4'5'-trihydroxyl group in the B-ring, a gallate group located at the 3 position of the C-ring, and hydroxyl groups at the 5 and 7 positions of the A-ring (Senanayake, 2013). In addition to catechins, green tea contains other polyphenols, such as gallic acid, quercetin, kaempferol, myricetin and their glycosides, but at lower concentrations (Baldi et al., 2020; Liu et al., 2020).



Catechins	Abbrev.	R_1	R_2	R_3	R_4
(-)-Epigallocatechin	(-)-EGC	OH	OH	-	-
(-)-Epicatechin	(-)-EC	H	OH	-	-
(-)-Epigallocatechin gallate	(-)-EGCG	OH	G	-	-
(-)-Epicatechin gallate	(-)-ECG	H	G	-	-
(-)-Gallocatechin	(-)-GC	-	-	OH	OH
(-)-Gallocatechin gallate	(-)-GCG	-	-	OH	G
(-)-Catechin gallate	(-)-CG	-	-	H	G
(+)-Catechin	(+)-C	-	-	H	OH

Figure 8. Chemical structures of green tea catechins: (a) epistructured catechins; (b) non-epistructured catechins and (c) (+)-catechin (Ananingsih et al., 2013).

2.3. Antioxidant activity of green tea catechins

Several antioxidant mechanisms have been considered for flavonoids, particularly catechins, in food and biological systems, including scavenging activity, metal chelation, and inhibition of free radical-generating enzymes, such as lipoxygenases (Banjarnahor and Artanti, 2015).

2.3.1. Radical scavenging activity

The antioxidant activity of catechins is due to the aromatic ring and hydroxyl groups that can bind and neutralise lipid free radicals in an effective way. Numerous studies have demonstrated that tea polyphenols and catechins are excellent electron or hydrogen donors and efficient scavengers of peroxyl radicals ($ROO^{\bullet}$), hydroxyl radicals ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet -}$), and singlet oxygen ($^1O_2$) (Frankel, 2014; Higdon and Frei, 2003; Senanayake, 2013). The ability of a compound to act as a free radical scavenger is partly related to its one-electron reduction potential, which measures the reactivity of antioxidants as hydrogen or electron donors (Higdon and Frei, 2003). The lower the reduction potential, the less energy is needed for hydrogen or electron transfer. For example, the reduction potential values of EC, EGC, ECG and EGCG are 570, 430, 550 and 430 mV, respectively, whereas the value is 480 mV for vitamin E (alpha-tocopherol) and 280mV for vitamin C (ascorbic acid) (Choe and Min, 2009). Thus, EGCG and EGC have lower reduction potentials than vitamin E, suggesting they are more effective donors than α-tocopherol, which is not the case for EC and ECG. However, vitamin C has a significantly lower reduction potential than all catechins and therefore has a stronger donation power. In addition to the ability to transfer its hydrogen or electron, the effectiveness of an antioxidant is also determined by its reaction rate with free radicals in a system (scavenging rate constant) and the stability of the resulting antioxidant radical (Higdon and Frei, 2003). Catechins scavenge lipid peroxyl radicals and become more stable phenoxy radicals, well known for their resonance stabilisation (Figure 9). The phenoxy radicals then undergo disproportionation and produce phenolic quinone and a dihydroxy phenolic compound (Choe and Min, 2006).

All catechins have at least one orthodihydroxyl group (o-3'4'-OH) in the B-ring, which participates in electron delocalisation and stabilises the radical form. Gallocatechins (EGC and EGCG) have a

trihydroxyl group in the B-ring (3'4'5'-OH), while catechin gallates (ECG and EGCG) have an esterified gallate moiety in position 3 of the C-ring, thus having three additional hydroxyl groups. The trihydroxyl group and the gallate moiety have been associated with increased antioxidant activity (Avila-Flores and Medellin, 2004; Colon and Nerín, 2015). Studies examining the oxidation products of gallocatechins with peroxyl radicals indicated that the 3'4'5'-OH group is the principal site of antioxidant reaction in EGC and EGCG (Valcic et al., 2000; Valcic et al., 1999) and the A ring was also shown to be a possible antioxidant site following the assessment of oxidation products from reactions of H_2O_2 with EGC and EGCG (Zhu et al., 2000).

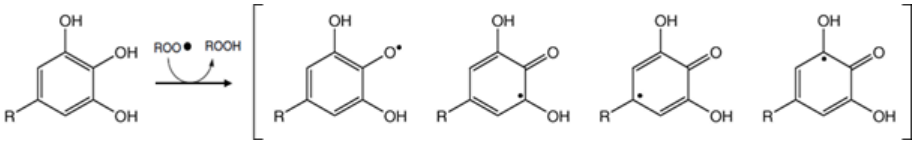


Figure 9. Resonance stabilisation of unpaired electrons by the gallate ring found in some tea catechins (Lambert and Elias, 2010).

2.3.2. Metal chelation

Catechins are able to chelate metal ions, such as iron and copper, due to the di/tri-hydroxy structure of the B ring and the gallate moiety (Botten et al., 2015). Chelating metal ions may contribute to the antioxidant activity of catechins by inhibiting transition metal-catalysed free radical formation and hydroperoxide decomposition reactions (Higdon and Frei, 2003). The phenolic-metal complexes appear to be more stable at neutral pH, while under acidic conditions, complexes formed are less stable, leading to the liberation of reduced metal ions and semiquinone radicals (Lambert and Elias, 2010).

2.3.3. Enzyme inhibition

Green tea catechins have been shown to inhibit the activity of lipoxygenases and cyclooxygenases *in vitro.* Lipoxygenases and cyclooxygenases catalyse the synthesis of leukotrienes and prostaglandins from long-chain polyunsaturated fatty acids like arachidonic acid. They are capable of co-oxidising other molecules through peroxidase activity, thus, potentially increasing oxidative stress or damage in some tissues. EC inhibited 15-lipoxygenase-mediated LDL oxidation more effectively than ascorbic acid or α-tocopherol at similar concentrations.

2.4. Pro-oxidant activity of catechins

Although catechins are antioxidants with the ability to scavenge radicals and chelate transition metals, they have been shown to act as pro-oxidants in some cases. Azam et al. (2004) compared the pro-oxidant properties of EC and EGCG and showed that EGCG was more effective as a pro-oxidant by reducing Cu(II) to Cu(I), which in turn can lead to the formation of reactive oxygen species and catechins are oxidised in the process (Figure 10). Furthermore, Huang and Frankel (1997) showed that the antioxidant activity of tea catechins varied depending on the lipid systems. For example, they observed that in corn oil oxidised at 50°C, catechins acted as antioxidants, but all showed pro-oxidant activities in corresponding corn oil-in-water emulsions. The authors suggested that in the emulsion system, trace metals present in the corn oil may be reduced more effectively by tea catechins (e.g., $Fe^{3+} \rightarrow Fe^{2+}$), and the reduced metal ions are capable of initiating phenolic oxidation, with reactive oxygen species generated in the process (Huang and Frankel, 1997). In addition, the reduced forms of transition metals are also known to catalyse lipid hydroperoxide decomposition to lipid alkoxyl ($RO^{\bullet}$) and hydroxyl radicals ($HO^{\bullet}$) (Lambert and Elias, 2010).

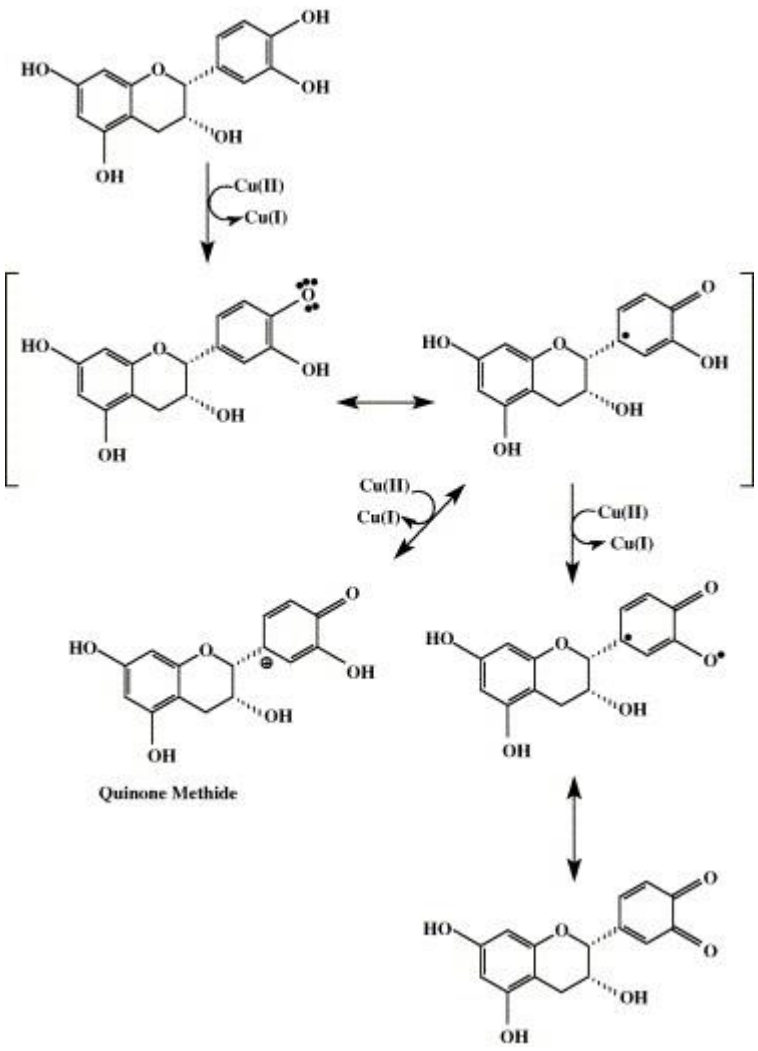


Figure 10. Reaction of Cu(II) with epicatechin (Azam et al., 2004).

2.5. Interaction of catechins with other antioxidants

The interactions among different antioxidant components can be synergistic (the combined effect of two components is much greater than the sum of the effects of each agent given alone), additive (the combined effect of two or more components is equal to the sum of the effect of each agent given alone) or antagonistic (opposite of synergism)(Sonam and Guleria, 2017). Synergistic, antagonistic or additive interactions have been reported among green tea catechins and between the catechins and other antioxidants (Colon and Nerín, 2015). Synergistic interactions take place when an antioxidant is regenerated by others, when there is a sacrificial oxidation of an antioxidant to protect another, and when two or more antioxidants show different antioxidant mechanisms (Choe and Min, 2009).

The best-known synergistic effect is between a metal chelator and a free radical-scavenger. These compounds demonstrate better antioxidant activities in oil when they are together than when used separately. In this interaction, the chelator acts at the initiation step of oxidation, whereas the free radical scavenger operates at the propagation step (Choe and Min, 2006). A strong synergistic effect was found for the combination of α-tocopherol and GTE in an oil-in-water emulsion of methyl linoleate and pure bulk oil [143]. Synergism was also observed in a ternary antioxidant system, including green tea polyphenols, α-tocopherol and ascorbic acid. Kinetic and mechanistic studies of the antioxidant process revealed that this synergism was due to the regeneration of the α-tocopherol by green tea polyphenols and the latter by ascorbic acid (Dai et al., 2008). Green tea polyphenols regenerate α-tocopherol by reducing the α-tocopheroxyl radical.

Colon and Nerín (2015) have reported on the interactions between catechins and other minor tea polyphenols, such as quercetin and kaempferol. The mixtures of quercetin + individual catechins did not show significant differences from the sum of their individual

effects (additive interaction), except for the combination of quercetin+EGCG that showed synergistic interactions. On the other hand, the mixtures of kaempferol + individual catechins showed additive interactions in all cases. However, care must be taken with combinations of antioxidants, as antagonistic effects are also possible. This has been observed between caffeic acid with various molecules, such as catechin, quercetin, or α-tocopherol (Choe and Min, 2009).

2.6. Factors that affect the activity of tea catechins

Important characteristics of catechins to consider for food applications are their sensitivity to high temperatures, their hydrophilicity and their pH-dependent stability. Indeed, catechins show good stability at a pH below 4, but when at alkaline pH, they degrade more rapidly (Senanayake, 2013; Wang et al., 2017). In addition, storage temperature significantly affects tea catechins' stability, with the rate of epimerisation and oxidation being higher at high temperatures (Ananingsih et al., 2013). The hydrophilicity or lipophilicity of a compound is measured as log P, with negative or very low values measured for hydrophilic compounds and vice versa (Gadkari et al., 2016). This value is defined as the logarithm of the ratio of the concentration of a chemical substance in a 2-phase system composed of 1-octanol and water ($\log([octanol]/[water])$). Table 2 shows the log P values for the major catechins found in tea measured by chromatography or predicted using computer software (Marczyński et al., 2018; Shibusawa et al., 2005). Although the values differ between different bibliographic sources, their order of hydrophilicity is conserved, allowing for qualitative interpretations. Overall, catechins are slightly soluble in lipids, with ECG being the most lipophilic, as its log P is the highest.

Table 2. Log P of the major tea catechins

Catechins	Log P* by HSCCC	Log P * by Pallas	Log P** by Chem Axon
EGC	-0.53	0.43	1.49
EC	0.10	0.86	1.80
EGCG	1.10	1.67	3.08
ECG	0.50	2.10	3.88

* HSCCC = high-speed Countercurrent Chromatography, Pallas = computer software (Shibusawa et al., 2005),
**ChemAxon = computer software (Marczyński et al., 2018)

2.7. Method of green tea extract incorporation into food

It is necessary to correctly incorporate GTE into food products to ensure that its antioxidant components are properly dissolved and/or evenly dispersed in the food matrix. Since only a small amount of GTE may be needed to extend the shelf life of foods, the method of application can determine antioxidant effectiveness and depends on the type of food products, processing methods, and the availability of equipment used to process the food (Senanayake, 2013). Commercial GTEs are mostly available as fine, water-soluble powder. Thus, the GTE can be dissolved in water before adding to foods, as is the case when GTE was added to some minced meat products (Mitsumoto et al., 2005; Tian and Huang, 2019). In formulations containing dry ingredients, GTE in powder form is often preferred as it can be easily blended with the dry ingredients. For example, to evaluate the effect of GTE on the quality of steamed Chinese bread, GTE in powder form was mixed with wheat flour prior to bread production (Ning et al., 2019). GTE can also be dissolved in a food-grade solvent such as ethanol to produce an oil-soluble extract to be directly added to oils and fats (Senanayake, 2013; Wanasundara and Shahidi, 1996). Besides water-soluble GTEs, oil-soluble green tea catechins (OSGT) have been developed

in recent years by the esterification of catechins with fatty acids, and their antioxidant activity has been investigated in different food systems (Drury et al., 2016; Myers et al., 2013; Shen et al., 2021).

2.8. Application of green tea extract as a natural antioxidant in food

The antioxidant activity of individual catechins and GTE have been investigated in a variety of food systems, including bulk oils, baked products and emulsions. The main areas of application of GTE are summarised in Table 3. Generally, GTE improved the antioxidant stability of most products to which it was added. However, GTE containing chlorophyll exhibited a pro-oxidant effect in seal blubber oil and menhaden oil, whereas, when dechlorophyllized, GTE showed excellent antioxidant activity in both oils at ≥ 200 ppm. In addition, the efficacy of GTE in the seal blubber and menhaden oils was higher than that of BHA, at 200 ppm, BHT, at 200 ppm, but less than that of TBHQ, at 200 ppm, which are commonly used synthetic antioxidants (Wanasundara and Shahidi, 1998). GTE (1% lipid wt.) also showed better antioxidant activity than BHT in biscuits stored at 70°C for 30 days (Mildner-Szkudlarz et al., 2009). In refined bleached and deodorised palm olein, the antioxidant activity of GTE (200-1800 ppm) was either comparable to or better than that of BHT (200 ppm). GTE also exhibited more potent antioxidant activity in some food products than other natural antioxidants. Indeed, tea catechins (200 and 400 ppm) inhibited lipid oxidation in raw beef stored for seven days at 4°C to a greater extent than vitamin C (Mitsumoto et al., 2005). The antioxidant activity of GTE was also more potent than that of α-tocopherol in marine oils (Wanasundara and Shahidi, 1998), cooked beef and chicken meat (Tang et al., 2001) and table spreads, where α-tocopherol acted as a pro-oxidant (Dwyer et al., 2012). In addition, GTE at 0.16% and 0.2% wt. showed a more prominent antioxidant activity than rosemary extract in margarine containing refined

palm oil, palm stearin and rapeseed oil (Fruehwirth et al., 2021). Gramza-Michalowska et al. (2007) also observed a higher induction time for summer butter supplemented with 0.02% wt. GTE (71.2h) as compared to the same butter supplemented with rosemary extract (42h).

Lipid-soluble catechins were shown to be effective in maintaining the desirable red colour of meat products and delaying the formation of oxidative by-products, compared to untreated meat products. The lipid-soluble catechins also lowered the accumulation of oxidative products during the storage of mayonnaise and ranch salad dressings prepared with soybean oil (Drury et al., 2016). In another study, supplementing canola oil with OSGT (2500 ppm) improved the oxidative stability of the oil. The accumulation of hydroperoxides in OSGT-supplemented oil was comparable to samples supplemented with TBHQ (200 ppm) when tested under ambient storage, while this accumulation was higher under accelerated storage at 60°C (Shen et al., 2021).

Table 3. Studies on the antioxidant activity of green tea extracts (GTE) in food products

Product	methods	Major findings	Reference
Marine oils (menhaden oil and seal blubber oil)	100-1000 ppm of GTE added, 6 days at 65°C compared with BHT, BHA, α-tocopherol. PV and TBARS monitored.	Excellent antioxidant activity of GTE at ≥ 200 ppm. Pro-oxidant effect detected for GTE containing chlorophyll.	Wanasundara and Shahidi (1998)
Sunflower oil	α-tocopherol in combination with GTE, or EC or (+)-catechin compared to samples with single compounds. Oxidative stability by Rancimat method.	A synergistic effect found between α-tocopherol and GTE; antagonistic or additive effects observed between α-tocopherol and EC and α-tocopherol and catechin.	Yin et al. (2012)
Refined bleached and deodorised palm olein	200-1800 ppm of GTE added, Rancimat method (110°C) and 30 days at 70°C compared with BHT (200 ppm). Changes in PV, p-AV and TOTOX measured.	GTE increased induction time better than BHT but had similar antioxidant activity to BHT in the oven storage test.	Womeni et al. (2016)
Canola oil	OSGT compared to TBHQ, rosemary extract and mixed tocopherols in canola oil; oven test at 60°C and at	Except for tocopherols, all other antioxidant ingredients improved the stability of the oils under all conditions; OSGT had comparable	Shen et al. (2021)

	room temperature (22-24°C); PV, aldehydes, antioxidant level analysed.	antioxidative performance to TBHQ under storage at 22-24°C	
Bread	50-150 mg GTE/100g of flour (500 - 1500 ppm), measurement of catechin stability during the preparation of dough and 4 days storage at room temperature.	Catechins were stable during dough preparation and storage for four days.	Wang and Zhou (2004)
Biscuits	GTE at 0.02%, 0.1% and 1% (lipid wt. basis), BHA (0.02%) baked at 205°C for 11 min; sensory analysis (Panel = 10); storage for 20 days at 60°C, with monitoring of PV and p-AV, fatty acid profile.	1% GTE had an excellent antioxidant effect reducing PV by up to 73% compared to BHT with 16% reduction; samples with GTE received higher scores for overall acceptability.	Mildner-Szkudlarz et al. (2009)
Sponge cake	GTE powder added to sponge cakes at concentrations of 0.5, 1.0, 1.5, and 2.0% (w/w wheat flour mass) and assessed for total polyphenol content, antioxidant activity, peroxide value, sensory acceptance, and microbial quantity	Sponge cake with 1.0% green tea extract powder obtained the best quality with low peroxide value and good microbial quality	Nhung et al. (2022)

Cooked beef and chicken meat	300 ppm of tea catechins or α-tocopherol (dissolved in 90% ethanol), oven cooked 160°C for 30 min, oxygen permeable package and stored at 4°C for 10 days in a cabinet or under light; TBARS measured.	Both tea catechins and α-tocopherol improved oxidative stability, but TC was more effective.	Tang et al. (2001)
Beef and chicken patties	200 and 400 ppm of tea catechins compared with vitamin C (both dissolved in water). Oven cooked 180°C, 15 mins; sealed in oxygen permeable package or under vacuum and stored for 7 days at 4°C under light.	Tea catechins caused discolouration in the patties, reduced lipid oxidation compared to the control and limited oxidation to a greater extent than Vitamin C.	Mitsumoto et al. (2005)
Minced duck meat	300 – 1000 ppm of tea catechins (dissolved in water); meat formed into patties and stored at 4°C under light for 10 days; Evolution of TBARS, catechin deposition	Tea catechins-treated meat had lower TBARS. The tea catechins residues first increased up to day 7 and decreased on day 10.	Tian and Huang (2019)
Table spreads	GTE at 750 ppm or α-tocopherol at 250 ppm, spreads containing omega-3 oils, storage at 5°C for 50 days.	Spreads with GTE had the lowest PV, whereas spreads with α-tocopherol had the highest. Particle size was not affected by antioxidant type.	Dwyer et al. (2012)

Margarine	Margarine samples supplemented with tocopheryl acetate, rosemary extract and GTE, as well as citric acid, β-carotene and NaCl and assessed for oxidative stability by PV, Rancimat and nuclear magnetic resonance analysis (NMR) after heating at 80°C for 1 h to accelerate oxidation.	Tocopheryl acetate promoted lipid oxidation, while rosemary and GTE led to the opposite. Whereas GTE alone showed antioxidant effect, combining GTE with citric acid, β-carotene or NaCl increased lipid oxidation.	Fruehwirth et al. (2021)
Butter	Butter samples enriched with GTE, rosemary extract, α-tocopherol and BHT at concentrations of 0.02% wt. of butter lipid content. Oxidative stability was conducted by Rancimat and Oxidograph test conditions (110°C).	GTE had the best antioxidant activity in Summer and winter butter, where the induction period was 71.2h for Rancimat and 81.2h and 66.5 h for Rancimat and 64.0 h for Oxidograph test, respectively. Also, rosemary extract and tocopherol showed strong antioxidant activity, although less potent than GTE.	Gramza-Michalowska et al. (2007)
Mayonnaise	Mayonnaise prepared with soybean oil was supplemented or not with rosemary extract (500 ppm), lipid-soluble catechins (250 ppm), EDTA	Samples supplemented with antioxidants had lower PV and alkenals accumulation than the mayonnaise without added	(Drury et al., 2016)

	(75 ppm) and monitored for PV alkenals during 4-month storage under ambient conditions	antioxidants. Also, the lipid-soluble catechins had a comparable effect to EDTA.

GTE = green tea extract; BHT = butylated hydroxytoluene; BHA = Butylated hydroxyanisole; PV = peroxide value; p-AV = p-Anisidine value, TBARS = thiobarbituric acid reactive substance; TOTOX = toal oxidation; EC = epicatechin; OSGT = oil-soluble green tea extract; TBHQ = tert-butylhydroquinone;

2.9. Safety of green tea catechins

Although GTEs are effective as antioxidants and flavourings, concerns have been raised regarding possible harmful effects associated with their consumption. These concerns involve cases of hepatotoxicity associated with the consumption of green tea catechins as dietary supplements. Because of these concerns, the European Food Safety Authority (EFSA) was asked to give a scientific opinion on the safety of green tea catechins from dietary sources. EFSA based its opinion on published scientific papers, including intervention studies on green tea preparations whereby effects on the liver were investigated by measuring serum transaminases, an increase being indicative of liver injury (EFSA, 2018).

In total, 49 intervention studies with green tea products stated to include data on liver parameters were retrieved from the literature by EFSA panel. Four of these studies were performed with green tea infusions, and the remaining studies were performed with GTEs. The studies varied considerably in terms of the dose and composition of green tea catechins administered, duration of treatment, number and health status of subjects, and results on liver parameters. Of the 49 studies, 9 reported elevated serum transaminases, with the incidence higher in the treated subjects than the controls in 7 of the studies (Table 4). The total number of treated participants in those studies was 724, varying from 7 to 513 per study, and the total number of cases with elevated serum transaminases in the treated subjects was 84. The daily dose of EGCG in the seven studies with a higher incidence of elevated serum transaminases in the green tea catechins groups compared to controls was 800 mg or above. Except for one study with 10 days duration where subjects were fasting at treatment (Ullmann et al., 2004), the duration of studies varied between 4 and 12 months, and the treatment was taken with food and usually divided into two daily doses.

Table 4. Intervention studies with green tea preparations (extracts) showing effects on liver parameters (transaminases)

Author, year	**Dose EGCG (mg/day)**	**Duration**	**Subjects treated/placebo**	**Liver effects (transaminases)**	**Odds ratio**
Garcia et al. (2014)	800	4 months	50/48	5 treated (Gr1 and 2) and 1 placebo (Gr3)	–
Lovera et al. (2015)	800	Phase I: 6 months, Phase II: 12 months	Ph1: 10/0; PhII: 7/5	Phase I: 1 (Gr3); Phase II; 4 in treated (2 Gr1; 1 Gr2; 1 Gr4) and 1 placebo (Gr1)	–
Dostal et al. (2015); Yu et al. (2017) *	843	12 months	513/508	36 in treated (22 Gr1, 7 Gr2, 6 Gr3 and 1 Gr4) and 4 placebo (Gr 1)	9.6 95% CI 3.4-27.0, $p < 0.0001$ (Dostal et al.); Grade 2 or and above 7.0 95% CI = 2.4–20.3, $p = 0.0002$ (Yu et al.)

Joe et al. (2015)	Poly E: 400, 800, 1,200; EGCG 50–75%	6 months	6, 7 and 20 received 400, 800 and 1,200, respectively/11	3 in treated (Gr1) and 3 placebo (Gr1)	–
Ullmann et al. (2004)	200, 400 and 800 pure EGCG	10 days	9 for each dose/9	1 in high dose (Gr1)	–
Crew et al. (2012)	800, 1200, 1,600	6 months	16, 11 and 3 received 800, 1,200 and 1,600, resp./10	1 in highest dose (Gr3); 2 in treated groups (Gr1)	–
Meshitsuka et al. (2017)	1,890	6 months	36/21	1 in placebo (Gr3–4)	–
Shanafelt et al. (2009)	8 doses; 800–4,000	Up to 6 months; Phase I	36; 3–6/dose; 33 at or above 800/0	11 in treated (Gr1)	–
Shanafelt et al. (2013)	4,000	6 months; Phase II	42/0	13 (Gr1); 6 (Gr2); 1 (Gr3)	–

* Two papers from the same study. Grade 1 (GR1) > ULN–3 x ULN; Grade 2 (GR2) : > 3–5 x ULN; Grade3 (GR3) : 3> 5–20 x ULN; Grade 4 (GR4) : > 20 x ULN . ULN : upper limits of normal ; Poly E : Polyphenon E, a decaffeinated green tea catechin mixture.

The doses of EGCG varied from 100 to 857 mg/day in the studies with no elevation of serum transaminases, and the duration of studies was from 10 days to 12 months.

Based on the available data, EFSA issued a scientific opinion that catechins in GTEs taken as a food supplement could pose health concerns for an intake of doses equal to or above 800 mg EGCG/day (EFSA, 2018). It is therefore recommended that individual portions of food supplemented with EGCG-containing GTE or food supplements for daily consumption should have less than 800 mg of EGCG. Traditionally, the mean daily intake of EGCG resulting from the consumption of green tea infusions ranges from 90 to 300 mg/day in Europe. Thus, no restriction is placed on green tea infusions prepared traditionally or reconstituted with an equivalent composition to traditional green tea infusions (EFSA, 2018).

2.10. Conclusion

The antioxidant activity of GTEs is attributed to their content in catechins, which limit oxidation by acting as radical scavengers, metal chelators or inhibiting the activity of enzymes. The extracts are commercially available as water-soluble powders and oil-soluble liquid extracts developed in recent years. The application of GTE as a natural antioxidant has shown to be effective in various food systems, with its antioxidant activity being comparable to or even better than some synthetic antioxidants. Also, GTE has shown better antioxidant activity than other commonly used natural antioxidants such as α-tocopherol, rosemary extract, and vitamin C. Thus, GTE is a potentially suitable antioxidant for products with high PUFA content.

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Chapter 2. Objectives and scientific strategy

Objectives and scientific strategy

The oxidation of PUFAs leads to quality deterioration in edible oils and fat-containing foods. The quality changes due to oxidation include nutritional losses, changes in flavour, odour and colour, and the formation of toxic secondary products. The higher the degree of unsaturation of fatty acids as well as their concentration in oil, the higher the susceptibility to oxidation. Therefore, inhibiting or delaying lipid oxidation is a major challenge for the food industry.

The oxidative stability of oils can be enhanced by the incorporation of antioxidants, which is a common strategy to retard lipid oxidation in edible oils (Decker et al., 2010). In recent years, much research has been carried out on the effect of supplementing edible oils with natural antioxidants extracted from plant materials. This is because natural antioxidants are considered a safer alternative to synthetic antioxidants. Among natural antioxidants investigated in edible oils and fat-containing foods, GTE has exhibited higher antioxidant activity compared to synthetic antioxidants such as BHA and BHT (Mildner-Szkudlarz et al., 2009; Wanasundara and Shahidi, 1998) and natural antioxidants like rosemary extract, vitamin C and α-tocopherol (Dwyer et al., 2012; Fruehwirth et al., 2021; Mitsumoto et al., 2005).

Although green tea catechins are proven antioxidant agents, their activity depends on the system in which they are added. Not much literature is available on the antioxidant activity of GTE in oils with high PUFA content, especially ω-3 fatty acids. Thus, this thesis assessed the impact of green tea catechins, when added as GTE, on the oxidative stability of oils with a high PUFA content. The research is based on the hypothesis that green tea catechins are potent antioxidants that can delay or slow down oxidation in these PUFA-rich oils**.** To achieve our objective, the PUFA-rich oils used in our research include DHA-rich oil (60% of DHA and >90% of PUFA), linseed oil (≈ 50% ALA) and sacha inchi oil, also a rich source of ALA (48%), but containing a higher level of linoleic acid

(33%) and total tocopherols. The specific objectives investigated and scientific strategies used are presented below.

Specific objective 1

To investigate if adding green tea extracts to PUFA-rich oils will delay or slow the formation of oxidation products. This is addressed in three separate studies presented in Chapter 3.

First, the antioxidant activity of GTE was evaluated in a DHA-rich oil compared to α-tocopherol (chapter 3A). Three doses of GTE (160, 400 and 1000 ppm) were chosen for this study based on concentrations that were effective in other matrices in previous studies without affecting the colour or taste of products (Wanasundara and Shahidi, 1998; Wang and Zhou, 2004; Womeni et al., 2016). GTE, which is hydrophilic, was compared with α-tocopherol, a commonly used lipophilic antioxidant naturally present in most unrefined edible oils. The concentrations of α-tocopherol (80, 200 and 500 ppm) used in this study were chosen from a range of concentrations (40-2000 ppm) that showed an antioxidant effect in previous studies (Fuster et al., 1998; Seppanen et al., 2010), although with some cases of pro-oxidant effects (Kim et al., 2007; Mohanan et al., 2018). The stability of the supplemented oil samples alongside the DHA-rich oil with no added antioxidants was determined by carrying out an oven storage test for accelerated ageing at 30°C. Temperatures between 25°C to 80°C accelerate oxidation while maintaining an oxidation mechanism similar to that at lower temperatures (AOCS, 1997). Although a temperature of 60°C is mostly recommended for oven storage tests, each accelerated test can be adapted to the type of fat, sticking as close as possible to the actual conditions in which the oxidation reactions occur (Frankel *et al*., 2005). Thus, 30°C, which is closer to ambient temperature, was used in this study because of the sensitive nature of the oil. The DHA-rich oil used in this study contained vitamin E added at the level of the company that supplied us with the oil.

In the second study, the effect of GTE was assessed on the oxidative stability of linseed oil (Chapter 3B). The oven storage test was carried out at both 30°C and 60°C. The concentration of GTE (1000 ppm) and α-tocopherol (200 ppm) chosen for this study were based on the results obtained with DHA-rich oil.

In the third study (Chapter 3C), the antioxidant activity of GTE (water-soluble) was compared to an oil-soluble green tea extract (OSGT) in sacha inchi oil. Adding GTE to oil requires prior dissolution in a solvent. Thus, to eliminate the need for solvents if results are translated to industrial settings, it was of interest to compare the antioxidant activity of GTE with three doses of an OSGT (1000, 2000 and 3000 ppm) based on the producer's recommendations and concentration of catechins in the extract. Another oil-soluble antioxidant formulation (Phytrox) containing tocopherols, rosemary extract and ascorbyl palmitate was also tested in this study based on recommendations from our industry partners. An oven temperature of 60°C was used to accelerate oxidation based on our previous results with linseed oil.

The oxidation process in these oils was monitored by measuring the peroxide and para-anisidine values (PV and p-AV). In addition, changes in fatty acids, catechins and tocopherol contents were also determined. The GTE used in all the studies in Chapter 3 had EGC as the most abundant catechin compound and was solubilised in ethanol before adding to oils.

Specific objective 2

To assess the effect of the catechins profile of GTE on its antioxidant activity in DHA-rich oil.

The proportion of catechins in green tea can vary with environmental conditions, variety, and agronomic conditions such as plucking time and leaf age (Lee et al., 2014; Zhang et al., 2018). Higher levels of epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) have been reported in young leaves (apical bud and 2

youngest leaves), whereas old leaves (8 to 10th leaf) were richer in epigallocatechin (EGC) and epicatechin (EC) (Chen et al., 2003). In addition, the extraction method and solvent used in preparing the GTE may also affect its catechins content (Banerjee and Chatterjee, 2015; Koch et al., 2020). With this possible variation in the catechins profile of GTE, it was of interest to investigate how this affects its effectiveness as an antioxidant. Thus, in this study presented in Chapter 4, we compared the antioxidant potency of two GTEs, one rich in EGC (used in the studies in Chapter 3) and the other rich in EGCG on a DHA-rich oil. To determine the contribution of the major catechins to the antioxidant effect of the GTE, the antioxidant activity of reconstituted mixtures of pure catechins in the same proportions as in the GTE's and individual catechins was also assessed. An oven storage test was carried out for 21 days at 30°C with the oxidative status, catechins content and fatty acid profile measured every 7 days. The DHA-rich oil used in this study was not supplemented with vitamin E by the company per our request.

Specific objective 3

To evaluate the antioxidant activity of green tea catechins when added to food matrices enriched with the PUFA-rich oils of interest.

As shown in the literature, the activity of an antioxidant may vary in different food matrices and can be affected by the processing methods (Frankel, 2012). Thus, it was important to evaluate the effect of GTE when the oils were added to more complex matrices. This objective was addressed in 3 studies. The matrices chosen were salad dressings prepared with linseed oil (Chapter 5A), mayonnaise enriched with sacha inchi oil (Chapter 5B), and crackers with the oil fraction containing linseed oil and DHA-rich oil (Chapter 5C). Salad dressings and mayonnaises generally have high oil content and are widely consumed. Therefore, they can serve as suitable matrices to promote the consumption of omega-3

PUFA-rich oils. The crackers were of interest as a more complex food on which heat treatment is applied.

The effect of GTE on the oxidative stability of linseed oil-based salad dressings (two-phase and stabilised emulsion) is presented in Chapter 5A. The antioxidant effectiveness was assessed under accelerated conditions (30°C, in open bottles) to determine the effective dose of GTE by measuring the PVs. The concentrations of GTE tested in this study were 400, 1000 and 2500 ppm to have a wider range from the effective concentration in oils (1000 ppm). The oxidative stability of dressings made with the most effective GTE dose was evaluated over a more extended period (49 weeks) at 20°C in closed bottles to determine GTE effectiveness during shelf storage. The oxidative stability of the dressings was also assessed under conditions of household use.

The mayonnaise was formulated after various preliminary trials to contain 9% of sacha inchi oil and 47% of rapeseed oil. The lower level of sacha inchi oil used was to minimize the characteristic nutty taste and herbal or green odour notes associated with volatile compounds in the sacha inchi oil (Gutiérrez et al., 2019; Ramos-Escudero et al., 2021). Rapeseed oil is one of the oils commonly used in the production of mayonnaises. Due to its low saturated fatty acid content it does not solidify at refrigeration temperature. In addition, the oil also contains ALA. The same antioxidants and concentrations evaluated in sacha inchi oil (Chapter 3C) were applied in this study.

In the final study, we investigated the effect of GTE on the oxidative stability of crackers enriched with omega-3 fatty acids. The crackers formulation used in this study was previously developed within the framework of a master's thesis and contained apples, carrots, quinoa, linseeds, egg whites and linseed oil (Birchall et al., 2021). DHA-rich oil was added to the original crackers formulation to obtain 250 mg of DHA/ serving of 50 g of crackers (250 mg of DHA being the daily recommendation of DHA). The crackers were

supplemented with GTE at a concentration of 0.1% and 1% based on previous investigations in baked products (biscuits and sponge cake), whereby 1% of GTE exhibited the best protective effect (Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009).

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Chapter 3. Oxidative stability of oils rich in Omega-3 polyunsaturated fatty acids

Foreword

Polyunsaturated fatty acids (PUFA) are very susceptible to oxidation, which leads to rancidity in oils. As shown in Chapter 2, green tea catechins have antioxidant activity, but this has not been exploited in oils with a high omega-3 PUFA content. Therefore, in this chapter, we investigated the antioxidant activity of GTE (water and oil-soluble, rich in EGC) in oils with a high omega-3 PUFA content through oven storage tests. The tests were conducted at 30°C (close to room temperature) and/or 60°C for shorter storage periods. First, we evaluated the effect of 3 doses of GTE in a DHA-rich oil while comparing it with α-tocopherol, an antioxidant naturally present in most edible oils (Chapter 3A). The results of this study have been published in *Antioxidants*. Secondly, the effects of GTE and α-tocopherol were confirmed in linseed oil, rich in α-linolenic acid (Chapter 3B). Although GTE showed efficient antioxidant activity in oils, this extract requires dissolution in a solvent in order to add to oils, which would therefore limit its industrial use in bulk oils. In a third study, we, therefore, compared the antioxidant activity of the GTE powder with an oil-soluble GTE, which also showed a dose-dependent antioxidant activity in sacha inchi oil (Chapter 3C).

Chapter 3A. Green Tea Extract Enhances the Oxidative Stability of DHA-Rich Oil

Nain, C.W.; Berdal, G.; Thao, P.T.P.; Mignolet, E.; Buchet, M.; Page, M.; Larondelle, Y.

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Abstract

Docosahexaenoic acid (DHA) is one of the most important omega-3 polyunsaturated fatty acids, with proven health-promoting properties. However, oils with a very high content in DHA (DHAO) are extremely susceptible to oxidation, which affects shelf stability and limits incorporation in food products. Green tea extracts (GTE) are potential candidates for the protection of these oils, but their use in such oils has not been previously reported. This study investigated the effect of GTE (160 ppm, 400 ppm, 1000 ppm) and α-tocopherol (80 ppm, 200 ppm, 500 ppm) on the oxidative stability of a DHAO over a 9-week storage at 30°C. The oxidative status was monitored during storage by the measurement of peroxide value (PV) and *p*-anisidine value (*p*-AV). Changes in eicosapentaenoic acid (EPA) and DHA content, as well as in catechins and tocopherol contents, were also evaluated. The addition of GTE enhanced the oxidative stability of DHAO by reducing the formation of peroxides and secondary oxidation products, whereas α-tocopherol had no significant effect on the PV of oil during storage but led to a significantly higher *p*-AV. The EPA and DHA content of DHAO was stable in GTE-supplemented samples whereas a decrease was observed in the control and α-tocopherol-supplemented samples. GTE also delayed the degradation of tocopherols initially present in the oil, while catechins resulting from the addition of GTE decreased progressively during the storage period.

Keywords: oxidation; DHA-rich oil; natural antioxidants; alpha-tocopherol; catechins; green tea extract (GTE)

1. Introduction

Docosahexaenoic acid (DHA, C22:6n-3) is a long chain omega-3 fatty acid and an essential component of a healthy diet. Its consumption has been associated with a decrease in the risk of cardiovascular and inflammatory diseases, cancer and neurological disorders, among others (Vázquez et al., 2019; Zárate et al., 2017). DHA can be produced endogenously from alpha-linolenic acid (C18:3n-3) by humans but the rate of biosynthesis is low and insufficient to meet physiological demands (Tocher et al., 2019). Dietary sources are therefore required to meet the recommended daily intake of 250 mg DHA/day set for adults by the French Food Safety Agency (AFSSA, 2010). The traditional sources of long-chain omega-3 fatty acids, namely DHA and eicosapentaenoic acid (EPA, C20:5n-3) in the diet are oily fish and seafoods, which are naturally rich in these fatty acids. Microalgae are a primary source of DHA in the marine ecosystem and in recent years, the cultivation of microalgae for oil production has emerged as a new source of DHA. DHA-rich oils (DHAO), being highly unsaturated, are especially susceptible to oxidation and require special handling to prevent the development of off-flavours (Ismail et al., 2016).

Lipid oxidation is one of the causes of quality deterioration in oils and food. When unsaturated lipids are exposed to metal ions, heat or light, free radicals are formed, which in turn react with oxygen to form hydroperoxides (Ahmed et al., 2016). Hydroperoxides are very unstable and further break down into aldehydes, ketones and alcohols, which are responsible for the off flavours in oxidized oils (Choe and Min, 2006; Symoniuk et al., 2016). The rate of oxidation is positively correlated to the number of double bonds in the fatty acids used as building blocks for the lipids of a food item. Thus, oils with high polyunsaturated fatty acid (PUFA) ratios tend to oxidize easily (Syed, 2016). It is therefore essential to protect these oils from oxidation in order to improve product shelf life and stability. Incorporating antioxidants is one of the effective methods to achieve this protection.

Antioxidants are compounds that inhibit lipid oxidation by either scavenging free radicals, chelating transition metals, quenching singlet oxygen and/or inhibiting the activity of photoactivated sensitizers (Gulcin, 2020). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are added to oils to delay oxidation but, because of the health risk associated with these compounds, their usage is regulated in most countries (Lourenço et al., 2019). Hence, there is an increasing interest in natural antioxidants, especially with the surge in consumers' preference for natural over synthetic products (Scott et al., 2020).

Most natural antioxidants are derived from plant materials such as fruits, vegetables, herbs and spices (Lourenço et al., 2019; Xu et al., 2017). These antioxidants from plants are mainly polyphenols, carotenoids and vitamins (Jideani et al., 2021). Alpha-tocopherol, which is the major tocopherol in many edible oils such as almond, peanut, olive, wheat germ, cottonseed and sunflower oils, has been effective in delaying oxidation in some oils, although it acts as a pro-oxidant if used in high concentrations (Matthäus, 2010; Mozuraityte et al., 2016; Shahidi and de Camargo, 2016). The optimal effective level of α-tocopherol varies from one oil to another with optimal efficiency reported at 50 ppm for sunflower oil (Blekas et al., 1995), 100 ppm for olive oil (Kamal-Eldin, 2006) and 250–500 ppm for corn oil emulsions (Huang et al., 1996). Other natural antioxidants such as quercetin and boldine, as well as a mixture of supercritical extracts of rosemary and α-tocopherol, were shown to be effective in improving the oxidative stability of oils with high contents of omega-3 fatty acids (≥80% of total fatty acids) (Ganga et al., 1998; Martín et al., 2012). Additionally, investigated for antioxidant activity are extracts from grape seed, apple peel, tomato peels, rambutan peel and green tea leaves (Elbadrawy and Sello, 2016; Kozłowska et al., 2019; Nieto et al., 2018; Phuong et al., 2020; Wolfe et al., 2003); and some of these

extracts exhibit similar or better antioxidant properties compared to synthetic antioxidants.

Green tea extracts (GTE) are produced from green tea leaves by conventional solvent extraction, ultrasound-assisted extraction, microwave-assisted extraction or supercritical fluid extraction among others (Banerjee and Chatterjee, 2015). The antioxidant activity of GTE is associated with its content of catechins, which prevent oxidation by chelating metal ions or by donating hydrogen atoms to free radicals, thereby blocking or slowing down the free radical chain reaction (Seeram et al., 2006). The major catechins of green tea include epigallocatechin (EGC), epicatechin (EC), epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) (He and Shahidi, 1997). The antioxidant activity of GTE has been demonstrated in various food systems, including baked products (Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009), meat and pork (Tian and Huang, 2019), beverages (Boroski et al., 2020), table spreads (Dwyer et al., 2012), as well as bulk oils, including marine oils (Shen et al., 2021; Wanasundara and Shahidi, 1998). To our knowledge, however, the effect of GTE has never been evaluated on hypersensitive oils with an extremely high content in DHA.

This study investigates the effectiveness of GTE as a natural antioxidant in an oil with a very high DHA content. The antioxidant effect of GTE was compared to that of α-tocopherol, which were both incorporated into DHA-rich oil samples and tested for oxidation at 30°C. Peroxide and para-anisidine values were monitored during the oxidation process. We also measured changes in DHA and EPA levels, as well as catechin and tocopherol contents. The results indicate that GTE is an efficient alternative for the stabilization of DHA-rich oils. Owing to the importance of dietary DHA for health, the present study offers a new tool to allow for its inclusion in prevention programs against various diseases.

2. Materials and Methods

2.1. Materials

DHA-rich oil (DHAO) made from deep-sea fish oil concentrate was kindly provided by Atrium innovations Inc. (Antwerpen, Belgium). Green tea leaf extract (GTE) containing 20% catechins was a product of Naturex (Avignon, France); D-α-tocopherol (1000 IU/g) was purchased from Sigma-Aldrich (Schnelldorf, Germany). All other chemicals used in this study were either of GC-grade, HPLC-grade or analytical grade.

2.2. Sample Preparation for Oxidative Stability Test

The effect of GTE and α-tocopherol on the oxidative stability of DHAO was evaluated using the oven storage test method for accelerated ageing of oils (AOCS, 1997). Oil samples were prepared with added antioxidant or without antioxidant (control) according to the method of Douny et al. (2016), with some modifications. In short, different concentrations of GTE (1000, 400 and 160 ppm) and of α-tocopherol (500, 200 and 80 ppm) were incorporated into DHAO. The GTE and α-tocopherol were each dissolved in absolute ethanol, vortexed for 1 min and sonicated (70 W, 40 kHz) for 15 min. A limited amount of ethanol was used so as not to exceed 4% weight of the final oil mixture. The oil and antioxidant blends were vigorously mixed with a glass rod for 3 min and flushed with nitrogen for 10 min to evaporate the ethanol. The oil samples were then distributed (10 g) in amber bottles (30 mL, Ø 2.7 cm), and the uncapped bottles were stored in the dark at 30°C for 9 weeks. Bottles were removed from the oven after 3 weeks, 6 weeks and 9 weeks of storage and analysed for oxidative status, fatty acid content, catechins and tocopherols. Separate sample bottles were used for each sampling time. Samples for initial analysis (0 week) were not put in the oven. The oxidative status determined by peroxide value (PV) and *p*-anisidine value (*p*-AV) was evaluated on the sampling day, and the remaining oil was flushed with argon and stored at −20°C for subsequent analyses of the other parameters.

The oil samples supplemented with GTE at 1000 ppm, 400 ppm and 160 ppm were referred to as GTE1000, GTE400 and GTE160, respectively, whereas those supplemented with α-tocopherol at 500 ppm, 200 ppm and 80 ppm were referred to as Toco500, Toco200 and Toco80, respectively. All samples were prepared in triplicate.

2.3. Assessment of the Oxidative Status

2.3.1. Peroxide Value (PV)

The PV, which measures primary oxidation products in oil samples, was determined according to the AOAC official method 965.33 (AOAC, 2000) with some modifications. An oil sample (2.5 g) was dissolved in 15 mL of acetic acid–chloroform solution (3:2 v/v). Then, 0.25 mL of saturated potassium iodide (KI) solution was added to the mixture, shaken for 1 min, kept in the dark for 5 min and 15 mL of distilled water were added. Titration was carried out with 0.1 N sodium thiosulfate solution until the yellow colour almost disappeared. 0.5 mL of starch solution (1% w/v) was added and titration continued until the blue colour disappeared. PV was calculated using the formula below:

$$\text{PV (meq } O_2\text{/kg oil)} = \frac{(V - V_b) \times N \times 1000}{m}, \quad (1)$$

where V and V_b are volumes (mL) of sodium thiosulfate for sample and blank, respectively, N is the normality of sodium thiosulfate and m is the mass of oil (g).

2.3.2. Para-Anisidine Value (*p*-AV)

Secondary oxidation products in oil samples were measured as *p*-AV according to the AOCS official method Cd 18–19 (AOCS, 1998). An oil sample of 0.3–0.5 g (m) was dissolved in 25 mL of isooctane and absorbance was measured at 350 nm (A_b) using a quartz cuvette. One mL of *p*-anisidine solution (0.25% in glacial acetic acid (w/v)) was added to 5 mL of this mixture and after 10 min in the

dark, the absorbance was measured at 350 nm (A_s). A blank was prepared with isooctane and measured the same way as the samples. The following equation was used to calculate *p*-AV:

$$p - \mathrm{AV} = \frac{25 \times (1.2 \times A_s - A_b)}{m}, \quad (2)$$

where 25 is the volume of isooctane used to dissolve the oil and 1.2 is a correction factor for dilution of sample solution with 1 mL of *p*-anisidine solution.

2.4. Determination of Fatty Acid Composition

The method used was adapted from Mellery et al. (2016). DHAO sample (200 mg) was weighed and converted to fatty acid methyl esters (FAME) via a two-step methylation with nonadecanoic acid (C19: 0, Sigma-Aldrich) used as an internal standard. First, the oil was methanolysed with 10 mL of 0.1 M KOH in methanol at 70°C for 60 min, then cooled to room temperature. Second, an acid-catalysed methanolysis was carried out by adding 4 mL of 1.2 M HCl in methanol and incubating at 70°C for 20 min. The FAME were then extracted by the addition of hexane and Milli-Q water. The FAME were separated and quantified by running samples in a GC Trace 1310 (ThermoQuest, Milan, Italy) gas chromatograph equipped with an As Triplus autosampler (Thermo Electron, Milan, Italy), an RT2560 capillary column of 100 m × 0.25 mm internal diameter (Restek, Bellefonte, PA, USA) and a flame ionisation detector set at 255°C. Fatty acids in the samples were identified and quantified by comparison of retention time and peak area with those of pure FAME standards (Larodan, Solna, Sweden). The data were processed using the Chromquest 5.0 data processing software (ThermoFisher Scientific, Waltham, MA, USA), and results are expressed as mg/g of oil.

2.5. Determination of Tocopherol Content

The alpha-, gamma- and delta- tocopherol content of oil was determined by high-performance liquid chromatography (HPLC). To prepare samples for analyses, 2.5 g of oil was dissolved in 25 mL of HPLC-grade hexane. The tocopherol standards (Sigma-Aldrich, St. Louis, MO, USA) were also prepared at different concentrations (1, 2, 5, 10, 20, 50 and 100 ppm) in hexane. The samples and standards were filtered through 0.45 µm syringe filters into HPLC injection vials. The tocopherols were separated and quantified by injecting 50 µL of diluted standards and samples in a chromatograph equipped with an autosampler AS3000 (Oven temperature = 35°C, tray temperature = 15°C), a P1000 spectra system pump (Thermo separation products, San Jose, CA, USA), a Luna 5 µm normal phase column of 250 mm × 4.6 mm internal diameter (Phenomenex, Torrance, CA, USA) and an FP-2020 fluorescence detector (Jasco, Tokyo, Japan). The mobile phase used was 2% (*v*/*v*) tetrahydrofuran in n-heptane at a flow rate of 1.8 mL/min. The data were processed using the Chromquest 5.0 data processing software, and the results are expressed as µg/g of oil.

2.6. Quantification of Catechins in GTE and in Oil Samples

The EGC, EC, EGCG and ECG contents were determined for GTE and oil samples. GTE (0.3 g) and absolute ethanol (12 g) were weighed in a centrifuge tube. After vortexing for 1 min, the mixture was sonicated (70 W, 40 kHz) for 15 min at room temperature and centrifuged for 5 min at 1800× *g*. The ethanolic extract obtained was filtered through a 0.45 µm syringe filter for analysis by HPLC.

The catechins were extracted from oil samples supplemented with GTE using a method adapted from Pizarro et al. (2013). The oil sample (0.5 g) was weighed into a 1.5 mL Eppendorf tube to which 1 mL of 90% ethanol was added, vortexed for 1 min and centrifuged for 5 min at 20,000× *g*. A volume of 750 µL of the ethanolic phase was transferred into another Eppendorf tube (2 mL) and after eliminating the remaining ethanolic phase, a second extraction of

the remaining oil phase was carried out. After the second extraction, 750 µL of the ethanolic phase was again collected and pooled with the volume collected from the first extraction. Ethanol was evaporated in a SpeedVac concentrator Savant SPD121P (ThermoFisher Scientific, Asheville, NC, USA), and the residue was solubilised in 1 mL of 1% formic acid in water. The solution was filtered through a 0.45 µL syringe filter into an HPLC vial. The HPLC used was equipped with an AS1000 autosampler (oven at 40°C and tray at 4°C), a P4000 pump (Thermo Separation Products, San Jose, CA, USA), a Phenomenex kinetex 2.6µm PFP 100A column (150 mm × 4.6 mm) and a DAD UV 6000 LP diode array detector (DAD; Thermo Separation Products, San Jose, CA, USA). The mobile phase consisted of solvent A (water with 1% (v/v) formic acid) and solvent B (acetonitrile with 1% (v/v) formic acid), used for analysis according to the following elution protocol: 0 min, 3% B; 5 min, 9% B; 11 min, 10.5% B; 20 min, 25% B; 25 min, 100% B; 30 min, 100% B; 37 min, 3% B; 40 min, 3% B. The flow rate was set at 1 mL/min with a sample injection volume of 10 µL. The catechins were identified and quantified by comparing retention times and peak areas with those of pure standard compounds (Extrasynthese, Genay, France). The results are expressed in µg/g.

2.7. Statistical Analysis

All experiments were carried out in triplicate, and results are expressed as mean ± standard deviation. An analysis of variance (ANOVA) was carried out using JMP Pro 15.2.0 statistical package to evaluate the effect of treatments during storage and the differences among the treatments. Sample means were compared by Turkey's HSD (honestly significant difference) test, with differences considered statistically significant at $p \leq 0.05$.

3. Results and Discussion

3.1. Characteristics of DHA-Rich Oil

The DHAO had a PUFA content of 873 mg/g corresponding to 99% of total fatty acids in the oil, with the major fatty acid being DHA (626.7 mg/g = 71.1% of total fatty acids), followed by EPA (149.3 mg/g = 16.9% of total fatty acids) and omega-3 docosapentaenoic acid (54.7 mg/g = 6.2% of total fatty acids). Other fatty acids were only present in trace amounts (Table 1). The total tocopherol content of the oil was 2174.4 μg/g. The concentrations of α-tocopherol, γ-tocopherol and δ-tocopherol were 342.2 μg/g, 1380.9 μg/g and 448.3 μg/g, respectively. The oil had a good oxidative status with a PV of 2 meq O_2/Kg and a *p*-AV of 5.2, which complied with the PV limit of 5 meq O_2/Kg oil and the *p*-AV limit of 20 established by the Global Organization for EPA and DHA Omega-3s (GOED) and by the Codex Alimentarius (Codex, 2017; Ismail et al., 2016).

Table 1. Fatty acid composition, tocopherol content and oxidative status of DHA-rich oil (DHAO).

	Characteristics	DHAO
Fatty acid composition (mg/g)	C16:1cis9	2.0 ± 0.13
	C18:1cis9	1.4 ± 0.07
	C20:4c5,c8,c11,c14	6.1 ± 0.17
	C20:5c5,c8,c11,c14,c17 (EPA)	149.3 ± 4.4
	C22:5c4,c7,c10,c13,c16	15.4 ± 0.5
	C22:5c7,c10,c13,c16,c19	54.7 ± 1.3
	C22:6c4,c7,c10,c13,c16,c19 (DHA)	626.7 ± 16.1
	c24:5c9,c12,c15,c18,c21	1.5 ± 0.1
	c24:6c6,c9,c12,c15,c18,c21	11.1 ± 0.28
	Total SFA	3.4 ± 0.84
	Total MUFA	4.9 ± 0.57
	Total PUFA	873 ± 22.84
Tocopherol (μg/g)	α	342.2 ± 3.6
	γ	1380.9 ± 7.1
	δ	448.3 ± 0.7
	Total	2171.4 ± 10.0
Oxidative status	Peroxide value (meq O_2/kg)	2.0 ± 0.0
	p-anisidine value	5.2 ± 0.3

Values are means of three measurements ± SD. SFA = Saturated fatty acids, MUFA = Monounsaturated fatty acids, PUFA = polyunsaturated fatty acids.

3.2. Oxidative Status of Oil during Storage

The changes in PV and *p*-AV of oil samples over the 9-week storage at 30°C are presented in Figure 1. The PV of the oil samples ranged from 1.6 to 2 meq O_2/Kg oil at the start of the storage test (0 week) and increased to different degrees in all the samples during the storage period (Figure 1A). After 3 weeks of storage, a significant increase ($p \leq 0.05$) in PV was detected in all samples except GTE1000, in which a significant change was not detected. The PVs of control samples and tocopherol-supplemented samples increased 100-fold and were significantly higher ($p \leq 0.05$) than

values for the GTE-supplemented samples. This drastic increase in PV had been observed in fish and algae oils during accelerated storage at 40°C, whereby PVs of up to 287.9 and 343 meq O_2/kg were detected after 96 h of storage in some of the oils (Frankel et al., 2002). The PV of GTE1000, GTE400 and GTE160 was 85.8%, 60% and 39% less than that of the control, respectively. After 6 weeks of storage, the PV of GTE1000 samples remained significantly lower ($p \leq 0.05$) than the PV of all other samples with a 55% lower value than the control. There were no significant differences ($p > 0.05$) observed in the PV among samples after 9 weeks of storage.

The *p*-AV of oil samples also changed during storage (Figure 1B). The *p*-AV of the control and α-tocopherol-supplemented samples significantly increased ($p \leq 0.05$) throughout the storage period. Meanwhile, the *p*-AV of the GTE-supplemented samples were stable during the first 3 weeks of storage and remained significantly lower ($p \leq 0.05$) than those of the other samples throughout the storage period. After 6 weeks of storage, the *p*-AV of the samples supplemented with 200 ppm and 500 ppm of tocopherol were significantly higher ($p \leq 0.05$) than those of the control and GTE-supplemented samples, suggesting a pro-oxidant activity. The rate of increase in *p*-AV for GTE-supplemented samples was inversely related to the concentration of GTE in the samples and was in the order of GTE1000 < GTE400 < GTE160. The *p*-AV of GTE1000 was the lowest throughout the storage period and was 65% lower than the control at 9 weeks of storage. The lower increase in PV and *p*-AV of GTE-supplemented samples could be explained by the presence of catechins, which as free radical scavengers, react with free radicals formed during the primary oxidation phase, thus disrupting the oxidation process (Fan et al., 2017).

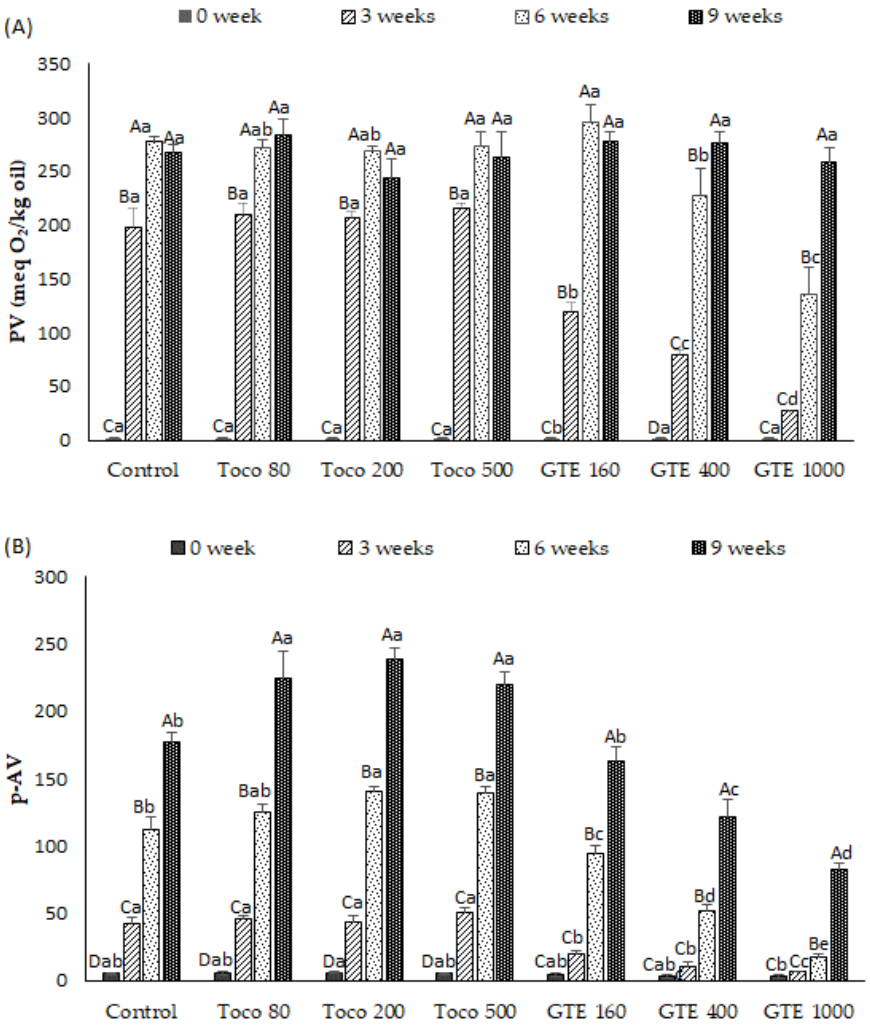

Figure 1. Peroxide value (PV) (**A**) and *p*-anisidine value (*p*-AV) (**B**) of DHA-rich oil (DHAO) with or without antioxidant during an oven storage test at 30°C. Values are mean ± SD (n = 3); Different capital letters (A–D) indicate significant differences ($p \leq 0.05$) during storage within a sample; different lower-case letters (a–e) among samples indicate a significant difference ($p \leq 0.05$) at a specific storage time. Control = DHA-rich oil without added antioxidant; Toco80, Toco200, and Toco500 = DHA-rich oil supplemented with 80 ppm, 200 ppm, and 500 ppm of α-tocopherol, respectively; GTE160, GTE400, GTE1000 = DHA-rich oil supplemented with 160 ppm, 400 ppm and 1000 ppm of green tea extract, respectively.

Highly unsaturated lipids, like those containing a high percentage of DHA, are especially prone to oxidation and require special handling to prevent the development of off-flavours (Ismail et al., 2016). The high oxidation rates of DHA and EPA and the instability of their hydroperoxides cause a rapid formation of secondary products. These secondary products include volatile aldehydes, ketones and other compounds, which, in turn, impart flavour reversion in fish oils (Bailey and Shahidi, 2005). The *p*-AV of GTE1000 at 6 weeks of storage, was still compliant with the *p*-AV limit of 20 established for concentrated fish oils (Codex, 2017; Ismail et al., 2016) but surpassed this limit by week 9. Supplementing DHAO with GTE improved its oxidative stability whereas the addition of α-tocopherol increased the formation of secondary oxidation products. The effectiveness of GTE as an antioxidant in DHAO is in agreement with previous results whereby GTE at concentrations of 200 ppm, 500 ppm and 1000 ppm improved the oxidative stability of refined menhaden oil (containing 10% of DHA) and seal blubber oil (containing 7.5% of DHA) more so than α-tocopherol at 500 ppm (Wanasundara and Shahidi, 1998). In another study, lipid fractions of sponge-fat cakes enriched with GTE (0.02%, 0.2% and 1%) also exhibited a greater resistance to oxidation than cakes without antioxidant (Kozłowska et al., 2019). The effect of tocopherols in oils is strongly concentration-dependent, varying with oxidation temperature and interactions with other molecules (Seppanen et al., 2010; Shahidi and de Camargo, 2016). Alpha-tocopherol has been shown to exert pro-oxidant effects in fish oil whereas binary or ternary antioxidant systems containing α- or γ-tocopherol concentrates, ascorbic acid (or ascorbyl palmitate), and lecithin, were successful in improving the oxidative stability (Hamilton et al., 1998; Mozuraityte et al., 2016). Similar to our results, the pro-oxidant effect of α-tocopherol (50, 100, 200 and 400 ppm) in linseed oil samples stored at 25, 40 and 60°C for over 30 days was reported by Mohanan et al. (2018) who confirmed their results in an accelerated test using the Rancimat technique. In another study, Fuster et al. (1998) reported

that both α- and γ-tocopherols within a concentration range of 40–200 ppm were effective in enhancing the oxidative stability of purified sunflower oil during 7 days of storage at 55°C, while neither α- nor γ-tocopherol displayed a pro-oxidant effect at concentrations as high as 2000 ppm.

3.3. The Fatty Acid Composition of DHAO during Storage

The EPA and DHA contents of DHAO samples on week 0 and after 9 weeks of oxidation are presented in Table 2. There was no significant difference ($p > 0.05$) in the fatty acid composition of samples at the start of storage (week 0). The mean contents of EPA and DHA in oil samples at week 0 were in the range of 138.7–151.3 mg/g and 583.5–635.7 mg/g, respectively. After 9 weeks of storage, a significant decrease ($p \leq 0.05$) in EPA and DHA was observed for the control, Toco80 and Toco200 samples. The addition of 500 ppm α-tocopherol also led to a visible decrease in both EPA and DHA, but this did not reach significance. In contrast, the addition of GTE had a clear protective effect on the degradation of EPA and DHA. Shi et al. (2020) observed a decrease in the EPA and DHA content of spray-dried matcha-tuna oil powders stored over 12 weeks at 40°C. An increase in the percentage of matcha (rich in catechins) in the spray-dried powders enhanced the protection of these fatty acids. In another study, the incorporation of tea catechins (1 mM) or α-tocopherol (1 mM) in an oleic and linolenic acid–rich diacylglycerol oil, had no effect on the fatty acid composition of oil during a 10-day storage at 60°C (Jung et al., 2014). Similar to the results observed in the present study for control and α-tocopherol-supplemented samples, Phung et al. (2020) reported an 8% decrease in EPA and DHA contents of hoki liver oil after 30 days of continuously bubbling the oil with oxygen gas and a 7% decrease when the oil was stored in an oven at 50°C for 30 days.

Table 2. The EPA and DHA content (mg/g) of DHA-rich oil at baseline (week 0) and after 9 weeks of storage at 30°C.

	EPA		**DHA**	
Treatment	Week 0	Week 9	Week 0	Week 9
Control	149.3 ± 4.4 ^{aA}	136.8 ± 5.0b ^{cB}	626.7 ± 16.1 ^{aA}	582.8 ± 20.3 ^{cdB}
Toco80	146.3 ± 1.9 ^{aA}	134.6 ± 4.3 ^{cB}	614.0 ± 7.5 ^{aA}	570.8 ± 18.2 ^{cdB}
Toco200	147.2 ± 0.9 ^{aA}	129.8 ± 3.0 ^{cB}	617.4 ± 3.4 ^{aA}	550.5 ± 12.0 ^{dB}
Toco500	141.6 ± 5.7 ^{aA}	132.7 ± 8.6 ^{cA}	593.9 ± 23.6 ^{aA}	563.4 ± 35.2 ^{cdA}
GTE160	138.7 ± 14.3 ^{aA}	140.1 ± 1.94 ^{abcA}	583.5 ± 58.3 ^{aA}	599.89 ± 7.8 ^{bcA}
GTE400	139.28 ± 7.3 ^{aA}	146.5 ± 1.5 ^{abA}	585.2 ± 28.2 ^{aA}	628.2 ± 7.1 ^{abA}
GTE1000	151.3 ± 8.8 ^{aA}	149.57 ± 2.12 ^{aA}	635.7 ± 36.9 ^{aA}	644.0 ± 9.0 ^{aA}

Values are mean ± SD (n = 3). Different superscript capital letters ($^{A \text{ or } B}$) indicate a significant difference ($p \leq 0.05$) between week 0 and week 9 (row) for the same fatty acid; different superscript lower-case letters ($^{a–d}$) within a column indicate significant differences ($p \leq 0.05$) among samples at the specific storage time. Control = DHA-rich oil; Toco80, Toco200 and Toco500 = DHA-rich oil supplemented with 80 ppm, 200 ppm and 500 ppm of α-tocopherol, respectively; GTE160, GTE400, GTE1000 = DHA-rich oil supplemented with 160 ppm, 400 ppm and 1000 ppm of green tea extract, respectively.

3.4. Losses in Catechins

The GTE used in this study had a total catechins content of 192.4 mg/g. The major catechin was EGC (115.1 mg/g) followed by EGCG (54.1 mg/g), EC (13.5 mg/g) and ECG (8.4 mg/g). The content of catechins in samples supplemented with GTE was proportional to the quantity of GTE added, with a total content of 39.4 μg/g, 99.4 μg/g and 224.8 μg/g in GTE160, GTE400 and GTE1000 samples, respectively (Table 3). After 9 weeks of storage, the total catechins content had reduced to 19–22 μg/g, with no significant differences observed among treatments. Additionally, the percentage losses in EGC, EC and EGCG were similar within a treatment, with a loss of 54–55% for GTE160, 85–86.6% for GTE400 and 92–95% for GTE1000. The losses in ECG were lowest in each treatment, with no loss observed for GTE160 samples, 15.5% for GTE400 and 43.54% for GTE1000 samples. Residual amounts of EGC, EC, EGCG or ECG among samples after 9 weeks did not differ. Based on these results, there seemed to be a minimum level beneath which the loss of catechins was no longer possible. This could explain why there was no significant difference in the PV among samples at this point.

Table 3. Catechins content (μg/g) of DHA-rich oil supplemented with green tea extract after 9 weeks of storage at 30°C.

		EGC	EC	EGCG	ECG	Total
Control	T = 0 week	0.72 ^{d}	0.07 ^{d}	0.91 ^{d}	0.31 ^{d}	2.1 ^{d}
GTE160	T = 0 week	22.7 ± 0.02 ^{c}	2.8 ± 0.06 ^{c}	9.7 ± 0.14 ^{c}	3.6 ± 0.02 ^{c}	39.4 ± 0.1 ^{c}
	T = 9 weeks	10.3 ± 0.19 ^{a}	1.3 ± 0.04 ^{a}	4.4 ± 1.4 ^{a}	3.9 ± 0.5 ^{a}	22 ± 2.3 ^{a}
	% loss	54.72	54.00	55.03	0.0	44.21
GTE400	T = 0 week	60.2 ± 1.5 ^{b}	7.4 ± 0.20 ^{b}	25.9 ± 0.56 ^{b}	4.8 ± 0.06 ^{b}	99.4 ± 2.1 ^{b}
	T = 9 weeks	8.1 ±0.62 ^{b}	1.1 ± 0.12 ^{a}	3.6 ± 0.09 ^{a}	4.1 ± 0.15 ^{a}	19.1 ± 1.1 ^{a}
	% loss	86.56	85.34	86.01	15.55	80.84
GTE1000	T = 0 week	138.9 ± 5.3 ^{a}	16.8 ± 0.5 ^{a}	59.5 ± 2.3 ^{a}	7.6 ± 0.39 ^{a}	224.8 ± 7.5 ^{ab}
	T = 9 weeks	9.5 ± 1.22 ^{ab}	1.2 ± 0.49 ^{a}	3.02 ± 0.2 ^{a}	4.3 ± 0.16 ^{a}	20.1 ± 2.14 ^{a}
	% loss	93.19	92.77	94.93	43.54	91.05

% loss = (amount at T = 0–amount at T = 9 weeks) × 100/amount at T = 0 week. Values are presented as mean ± SD (n = 3). Different superscript letters (^{a}–^{d}) within a column indicate significant differences ($p \leq 0.05$) among samples at the same storage time. EGC = epigallocatechin, EC = epicatechin, EGCG = epigallocatechin gallate, ECG = epicatechin gallate. GTE160, GTE400, GTE1000 = DHA-rich oil supplemented with 160 ppm, 400 ppm and 1000 ppm of green tea extract, respectively.

All the catechin compounds in GTE1000 significantly ($p \leq 0.05$) declined during 6 weeks of storage and there was no significant change ($p > 0.05$) in the content of ECGC and ECG, thereafter (Figure 2). The depletion was higher for EGC and EGCG than for the other catechins during the first 3 weeks of storage with a percentage loss of 66.6% and 85.1%, respectively. This significant loss in catechins also corresponded with a lesser change in PV, which could imply the depletion of catechins was due to its antioxidant activity. Catechins are known to delay oxidation by inhibiting the formation of free radicals or blocking the propagation of the free radical chain reactions, as well as chelating metal ions (Brewer, 2011; Chen and Chan, 1996). Catechins, donate hydrogen from the phenolic-OH group, thus reducing free radicals. The resulting unpaired electron in the phenolic compound becomes delocalized within the ring structure giving a relatively unreactive free radical (Duthie, 1999). The depletion of catechins (% loss) was observed in the order EGCG > EGC > EC > ECG and this is similar to the order of antioxidant activity observed for catechins in mackerel muscle and oil-in-water emulsions (Almajano et al., 2007; He and Shahidi, 1997).

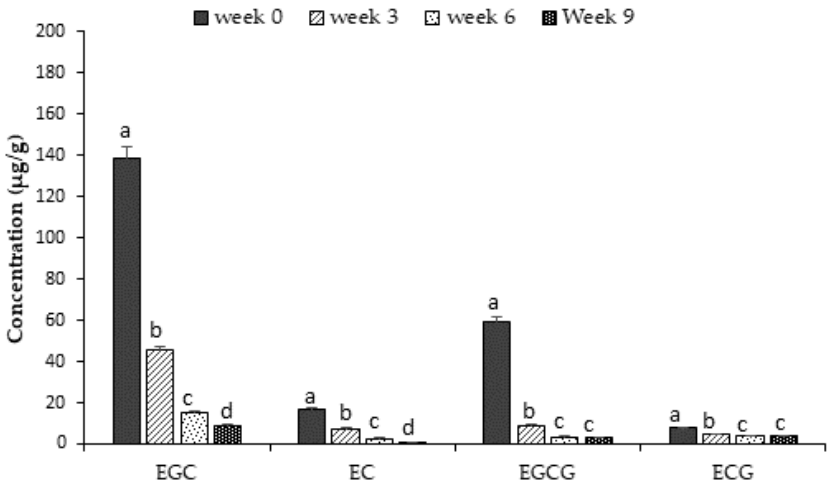

Figure 2. Catechins in DHA-rich oil supplemented with green tea extract at 1000 ppm and stored over 9 weeks at 30°C. Values are presented as mean ± SD (n = 3). Different letters (a–d) for each catechin indicate a

significant difference ($p \leq 0.05$) during storage. EGC = epigallocatechin,EC = epicatechin, EGCG = epigallocatechin gallate, ECG = epicatechin gallate.

3.5. Changes in Tocopherol Content of Oil during Storage

The α-tocopherol, γ-tocopherol and δ-tocopherol content of DHAO were 342.2 μg/g, 1380.9 μg/g and 448.3 μg/g, respectively. The oil was used without further purification and the effect of GTE-supplementation on the degradation of these tocopherols was evaluated (Figure 3A–D). A greater degradation rate of all tocopherol compounds was observed for the control sample. During the first 3 weeks of storage, α-tocopherol, γ-tocopherol and δ-tocopherol content of the control sample reduced by 60%, 45% and 17%, respectively and after 9 weeks, γ-tocopherol was the most degraded with a reduction of 96%, followed by α-tocopherol with 81% reduction and δ-tocopherol with a reduction of 67%. On the other hand, the α-tocopherol content of the GTE1000 samples remained stable during the first 3 weeks of storage while content of γ-tocopherol and δ-tocopherol remained stable for up to 6 weeks of storage. Losses in α-tocopherol, γ-tocopherol and δ-tocopherol of 65%, 72% and 28% were recorded for the GTE1000 sample after 9 weeks of storage. Thus, the incorporation of GTE in the oil reduced the depletion of the tocopherols with degradation levels ranked in the order of α-tocopherol > γ-tocopherol > δ-tocopherol. The differences in the rate of degradation of tocopherols are associated with their bond dissociation energy, which is in the order α < γ < δ and thus, α-tocopherol has a higher antioxidant activity followed by γ-tocopherol and then δ-tocopherol (Kmiecik et al., 2019; Wright et al., 2001). Accordingly, the rate of degradation was higher for α-tocopherol than for the γ-isomer in an oleic and linolenic acid-rich diacylglycerol oil, indicating a lesser stability for α-tocopherol (Jung et al., 2014). The degradation of tocopherols in GTE1000 began after most of the catechins had been depleted. This result seems to corroborate the result of Jung et al. (2011), which demonstrated that GTE was a better antioxidant

than α-tocopherol for protection against singlet oxygen oxidation of linoleic acid. Prabsangob and Benjakul (2018) also observed a delay in the frying-induced degradation of tocopherols in a soybean oil/tea seed oil blend when EGCG and EC were incorporated in the oil.

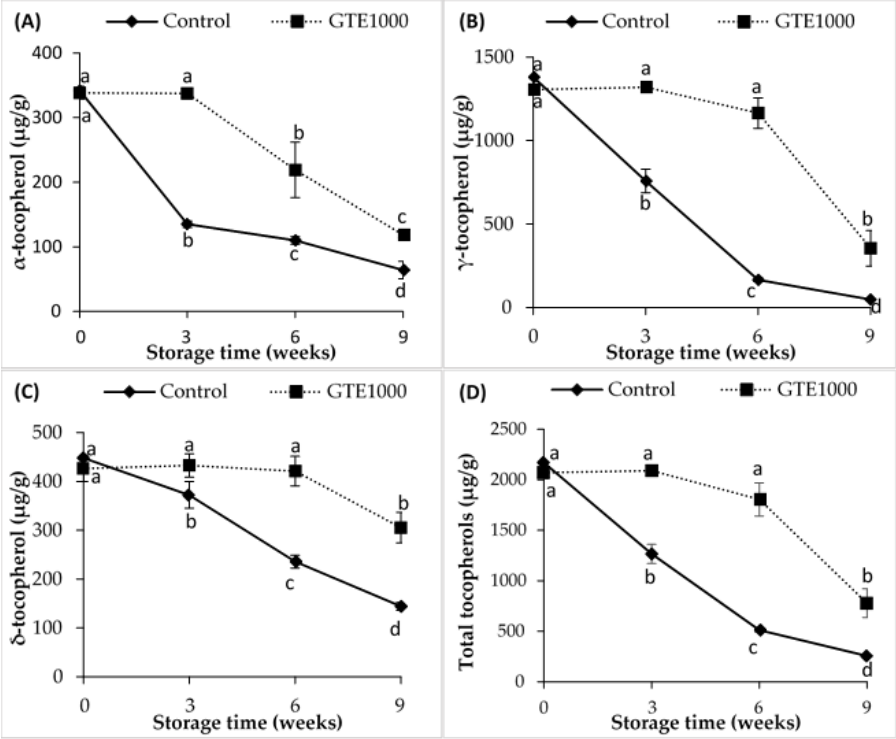

Figure 3. Tocopherol content of DHA-rich oil during 9 weeks of storage at 30°C. (**A**) α-tocopherol; (**B**) γ-tocopherol; (**C**) δ-tocopherol; (**D**) Total tocopherols. Values are presented as mean ± SD (n = 3). Different letters (a–d) indicate a significant difference ($p \leq 0.05$) during storage for each sample.

4. Conclusion

Supplementing DHAO with GTE significantly improved the oxidative stability of DHAO by delaying the formation of primary and secondary oxidation products as well as degradation of EPA, DHA and tocopherols. In contrast, the addition of α-tocopherol increased the formation of secondary oxidation products in the oil, therefore exhibiting a pro-oxidant tendency. The depletion of catechins matched the increase in PV, suggesting a predominant role of catechins in enhancing the DHAO oxidative stability. GTE could therefore serve as an alternative natural antioxidant to improve the shelf life of DHAO.

This study shows that hypersensitive oils with an extremely high content in PUFAs could be protected from oxidation by the incorporation of GTE. The oxidation of DHAO in this study was carried out at 30°C and in open bottles, although refrigeration temperatures are recommended for the storage of such hypersensitive oils. It is, therefore, of interest to repeat this experiment at refrigeration temperatures and to evaluate the oxidation products obtained in the oil. In addition, the stabilizing impact of GTE should also be evaluated in complex food matrices supplemented with DHA-rich oils.

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Chapter 3B. Effects of green tea extract and α-tocopherol on the oxidative stability of linseed oil

Nain, C.W., Thao, P.T.P., Larondelle, Y.

Abstract

Oils rich in polyunsaturated fatty acids are prone to oxidation, which affects sensory quality and storability. In view of improving the shelf stability of linseed oil, this study investigated the effect of green tea extract (GTE, 1000 ppm) and α-tocopherol (200 ppm) on its oxidative stability. Oil samples with or without antioxidants were prepared, and accelerated ageing tests were carried out at 30°C and 60°C. The oxidative status was monitored during storage by measuring the peroxide and *p*-anisidine values. GTE was effective in reducing the formation of peroxides and secondary oxidation products, whereas α-tocopherol showed pro-oxidant activity. Based on these results, GTE could serve as an alternative natural antioxidant to delay the oxidation of linseed oil.

Keywords: Oxidation; linseed oil; natural antioxidants; green tea extract; α-tocopherol; peroxide value; *p*-anisidine value

1. Introduction

Linseed (*Linum usitatissimum L.*) contains about 36-45% of oil, the major fatty acid being ALA, present at levels higher than 50 % of total fatty acids, which makes the oil one of the richest in ALA among edible oils (González-Mañán et al.; Piva et al., 2018). AlA is an essential fatty acid that must be obtained from the diet and a precursor of the omega-3 long -chain fatty acids. The consumption of omega-3 fatty acids is necessary for many physiological reasons and has been associated with a lower incidence of different types of illnesses, including inflammatory and cardiovascular diseases (Vázquez et al., 2019). However, due to its high levels of polyunsaturated fatty acids (PUFA), linseed oil is highly susceptible to oxidation, leading to the production of hydroperoxides which are further broken down to aldehydes and ketones or polymerise to form high molecular products (Choe and Min, 2006; Symoniuk et al., 2016). The accumulation of these oxidation products alters the nutritional and sensory quality of the oil. Most edible oils naturally contain antioxidants such as tocopherols, tocotrienols, carotenoids, phenolic compounds, and sterols, which provide endogenous protection against oxidation (Cuvelier and Maillard, 2012). However, the amounts present are sometimes insufficient to protect the oil during prolonged storage. In addition, processing operations can destroy these natural antioxidants. Therefore, supplementation with exogenous antioxidants is necessary to maintain the quality and oxidative stability of edible oils (Decker et al., 2010). Although synthetic antioxidants are effective in preventing PUFA oxidation, in recent years, the focus has been on natural antioxidants to meet the consumers' continuous demand for natural and healthy ingredients or food (Michotte et al., 2011).

In a previous study, green tea extract (GTE) was effective in protecting DHA-rich oil from oxidation, whereas α-tocopherol exhibited pro-oxidant activity (Nain et al., 2021). In the present study, we investigated the effect of supplementation with green tea

extract and α-tocopherol on the oxidative stability of linseed oil during ageing at 60°C and 30°C. The level of lipid oxidation was evaluated by determining the peroxide value (PV) and *p*-anisidine value (*p*-AV) during the ageing tests.

2. Materials and methods

2.1. Material

Cold pressed linseed oil (first pressure) without any added antioxidants was kindly provided by Vandeputte (Mouscron, Belgium). Green tea leaf extract (GTE) containing 20% catechins was a product of Naturex (Avignon, France), D-α-tocopherol (1000IU/g) was purchased from Sigma-Aldrich (Schnelldorf, Germany). All other chemicals used in this study were of analytical grade.

2.2. Fatty acid profile determination

The method used was adapted from Mellery et al. (2016) Each oil sample (1 g) was weighed and converted to fatty acid methyl esters (FAME) via a two-step methylation. First, the oil was hydrolysed with 10 ml of 0.1 M KOH in methanol at 70°C for 60 min, then cooled to room temperature. Secondly, an acid-catalysed methanolysis was carried out by adding 4 ml of 1.2 M HCl in methanol and incubating at 70°C for 20 min. The fatty acid methyl esters (FAME) were then extracted using hexane. The FAME were separated and quantified by running samples in a GC Trace (ThermoQuest, Milan, Italy) gas chromatograph equipped with a GC PAL autosampler, an RT2560 capillary column of 100 m x 0.25 mm internal, (Restek, Bellefonte, PA, USA) and a flame ionisation detector. Fatty acids in the samples were identified and quantified by comparison of retention time and peak area with those of a pure standard mixture of FAME. The data were processed using Chromquest 5.0 data processing software and results are expressed as percentage of

total identified fatty acids. Each oil sample was analysed in duplicate.

2.3. Sample preparation and accelerated storage

Oil samples were prepared with GTE (1000 ppm), α-tocopherol (200 ppm) and without added antioxidants (Control) as described previously (Nain et al., 2021). The oil samples were then distributed (20 g) in amber bottles (100ml, Ø 4 cm) and stored opened for 6 days at 60°C (accelerated ageing) or 96 days at 30°C (mildly accelerated ageing). Periodically, samples were taken out of the oven and analysed for primary and secondary oxidation products. All experiments were performed in triplicate.

2.4. Peroxide value and p-anisidine value

The peroxide value (PV), which measures primary oxidation products in oil samples, was determined according to the AOAC official method 965.33 (AOAC, 2000). Secondary oxidation products in oil samples were measured as p-anisidine value (*p*-AV) according to the AOCS official method Cd 18-19 (AOCS, 1998).

2.5. Statistical analysis

To evaluate the effects of treatments on PV and p-AV over time, a one-way analysis of variance (ANOVA) was performed and sample means were compared by the Turkey's HSD test with differences considered statistically significant at $p < 0.05$. The JMP 14.3 software was used to perform all statistical analysis.

3. Results

3.1. Fatty acid profile of oils

The fatty acid compositions of LSO in Table 1. The LSO used in this study contained 70.9% of PUFA and, as expected, the major fatty acid was ALA (54.97 %). These results are in agreement with literature, which reports an ALA content > 50 % of fatty acids in linseed oil (Golmakani et al., 2020; Michotte et al., 2011; Piva et al., 2018). Yoshime et al. (2018) had similar results; LSO contained 5 main fatty acids: C16:0 (6.46 %), C18:0 (5.54 %), C18:1 ω-9 (19.2 %), C18:2 ω-6 (14.22 %) and C18:3 ω-3 (54.59 %).

Table 1. Fatty acid profile, tocopherol content and oxidative status of linseed oil

Characteristic		**Linseed oil**
Fatty acid content (wt. %)	C16:0	5.37
	C18:0	4.43
	C18:1c9	17.87
	C18:2c9,c12	15.26
	C18:3c9,c12,c15	54.97
	ΣSFA	10.38
	ΣMUFA	18.67
	ΣPUFA	70.95
Tocopherol (μg/g)	α	4.6 ± 0.6
	γ	426.7 ± 60
	δ	3.1 ± 0.4
	Total	434.5 ± 61
Oxidative status	PV (meq O_2/kg oil)	1.1 ± 0.2
	p-AV	0.0

Values represent the mean of two determinations (fatty acids) and mean of three measurments ± standard deviation for tocopherols and oxidative status

3.2. Oxidative stability of oils

The oxidative status of each oil sample was assessed by measuring the PV and p-AV. The PV measures the amount of hydroperoxides, which are the primary products formed during lipid oxidation, and the p-AV gives a measure of secondary oxidation products, especially aldehydes. p-AV is therefore a complementary indicator to PV, as highly oxidized products may have a low PV when the primary oxidation products are largely metabolized to secondary products (De Boer et al., 2018; Ismail et al., 2016).

3.2.1. Peroxide value

At 30°C, very minimal changes in PV were detected during the first 32 days of storage, with a significant PV increase observed after 48 days of storage (Figure 1A). There was no significant difference ($p > 0.05$) between the control and treatments at the start of the experiment but this changed during storage. After 72 days of storage, the limit of PV recommended by Codex Alimentarius for cold pressed oils (15 meq O_2 / Kg oil) was exceeded by the control (17.67 meq O_2 / Kg oil) and α-tocopherol (51.36 meq O_2 / Kg oil) supplemented samples, whereas the GTE-supplemented sample was at a PV of 6.92 meq O_2 / Kg oil. At the end of the storage period (96 days), the highest PV was recorded for Toco200 (156.47 meq O_2 / Kg oil), followed by the control with a PV of and 82.7 meq O_2 / Kg oil and then the GTE-supplemented sample with the lowest PV of 19.7 meq O_2 / Kg oil, which was 76.2 % less than that of the control. The PV of α-tocopherol supplemented sample was 89 % greater than that of control, therefore, exhibiting pro-oxidant activity.

At 60°C, after 6 days of storage, the PV of GTE-supplemented oil (9.5 meq O_2 / Kg oil) was significantly less than all other treatments, and specifically 79.6 % less than the control. The PV of samples with α-tocopherol (50.5 meq O_2 / Kg oil) was significantly

higher than that of the control with a PV of 46.5 meq O_2 / Kg oil (Figure 1 B).

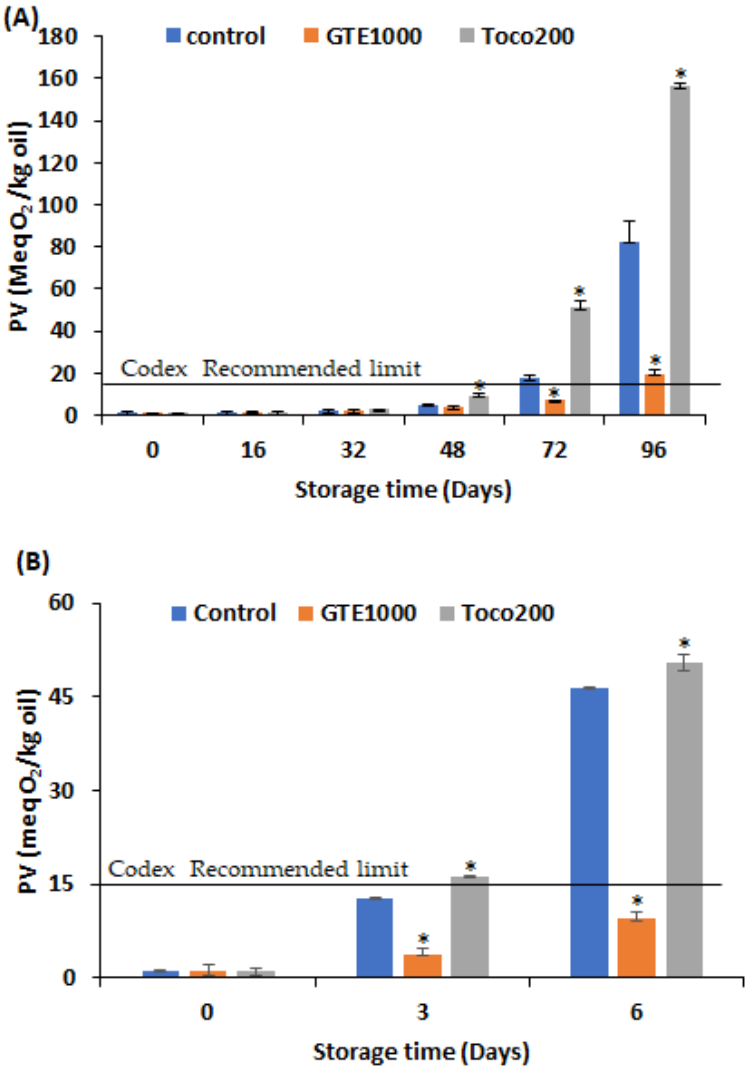

Figure 1. Changes in peroxide value (PV) of linseed oil submitted to an oven storage test at 30°C (A) or 60°C (B). Values are mean ± SD with n=3. Control = linseed oil without added antioxidants; GTE1000 and Toco200 = linseed oil supplemented with 1000 ppm green tea extract, and 200 ppm of α-tocopherol. * indicates significant difference ($p < 0.05$) from the control within the same storage time.

3.2.2. *p*-Anisidine value

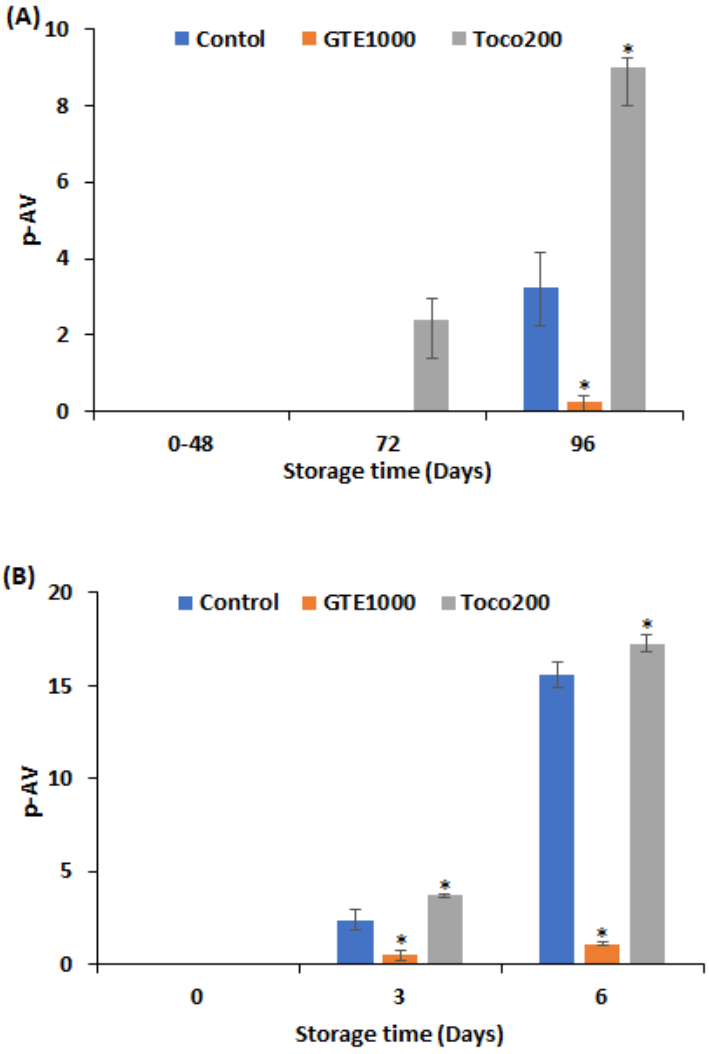

Figure 2. Changes in *p*-anisidine value (p-AV) of linseed oil submitted to an oven storage test at 30°C (A) or 60°C (B). Values are mean ± SD with n=3. Control = linseed oil without added antioxidants; GTE1000 and Toco200 = linseed oil supplemented with 1000 ppm green tea extract, and 200 ppm of α-tocopherol. * indicates significant difference ($p < 0.05$) from the control within the same storage time.

The p-AV of all samples stored at 30°C (Figure 2A) were below the limit of quantification for up to 48 days of storage for control samples, and up to 72 days of storage for samples supplemented with GTE. At the end of 96 days of storage, the p-AVs of the control samples and those supplemented with GTE and α-tocopherol were 3.3, 0.3 and 9 respectively. The pattern of p-AV build-up was similar to the pattern of PV increase in the samples. At 60°C, the p-AV of all treatments was below the limit of quantification on day 0 and increased progressively during storage (Figure 2B). On day 6, the p-AV of the GTE sample was 93 % less than that of control, while the α-tocopherol treatment had a p-AV significantly higher than that of the control.

4. Discussion

The results obtained for the ageing tests at 30°C or 60°C both showed that the addition of GTE (1000 ppm) significantly delayed the oxidation of linseed oil whereas α-tocopherol (200 ppm) enhanced oxidation. A similar effect of GTE and α-tocopherol was previously observed in DHA-rich oil stored at 30°C (Nain et al., 2021). The activity of antioxidants has been associated with their polarity. Polar antioxidants tend to be more effective at counteracting lipid oxidation in bulk oil whereas non-polar antioxidants tend to be more effective in emulsions (Frankel, 2012b). This theory is referred to as the polar paradox. GTE contains catechins (polar in nature) as its active antioxidant agents, which may partly explain its effective antioxidant activity in linseed oil. Polar antioxidants, in this case antioxidant agents in GTE, are more protective in bulk oils by being oriented in the air-oil interface, which is the site of oxidation while lipophilic α-tocopherol will remain in solution in the oil phase and will be less protective (Frankel, 2012a; Nahas and Berdahl, 2013).

As expected, oxidation products accumulated faster for the oven storage test at 60°C than 30°C, which had a long induction period.

However, the formation of primary and secondary oxidation products remained low under both storage conditions for the GTE-supplemented oil samples.

To the best of our knowledge, the effect of linseed oil supplementation with GTE has not been reported in previous studies, but natural antioxidants like myricetin, (+)-catechin, tannic acid, and ascorbyl palmitate, have been shown to enhance the oxidative stability of linseed oil (Douny et al., 2016; Michotte et al., 2011; Mohanan et al., 2018). Śpitalniak-Bajerska et al. (2018) reported the ability of dried apple pomace to improve the stability of vacuum-packed linseed oil stored at room temperature. The antioxidant activity of GTE (> 200 ppm) has also been reported for refined menhaden and seal blubber oils stored at 65°C (Wanasundara and Shahidi, 1998). In another study, lipid fractions of sponge-fat cakes enriched with GTE (0.02 %, 0.2 % and 1 %) also exhibited a greater resistance to oxidation than the cakes without antioxidant (Kozłowska et al., 2019).

Supplementing linseed oil with α-tocopherol resulted in a pro-oxidant effect, evidenced by higher PV and p-AV in the toco200 samples than in the control. Similar to our results, the pro-oxidant effect of α-tocopherol (50, 100, 200 and 400 ppm) in linseed oil stored at 25, 40 and 60°C for over 30 days has been reported by Mohanan et al. (2018) and they had similar results in an accelerated test using the Rancimat method. Tocopherols act as antioxidants by donating hydrogen atoms to lipid radicals producing a relatively stable radical $A^{\bullet}$, which reacts very slowly with the lipid substrate. At high antioxidant concentrations α-tocopherol can become pro-oxidant and a chain carrier by regenerating peroxyl radicals by reaction (-1) and abstracting hydrogen from the lipid substrate, thus generating new radicals (2) (Frankel, 2012a).

$$ROO^{\bullet} + AH \rightleftharpoons ROOH + A^{\bullet} \qquad (1/-1)$$

$A^{\bullet} + RH \rightarrow AH + R^{\bullet}$ (2)

The effect of tocopherols in oils is shown to be strongly concentration-dependent, varying with oxidation temperature and interactions with other molecules such as metals (Mozuraityte et al., 2016; Shahidi and de Camargo, 2016). Contrary to our results, Fuster et al. (1998) reported that both α- and γ-tocopherols within a concentration range of 40-200 ppm were effective in enhancing the oxidative stability of purified sunflower oil during storage for 7 days at 55°C and neither α- nor γ-tocopherol exhibited a pro-oxidant effect at concentrations as high as 2000 ppm. The sunflower oil used by these researchers was purified from endogenous antioxidants, trace metals and pigments. Based on the results, the authors suggested that α-tocopherol may not be a prooxidant *per se* but may act as a prooxidant synergist (or co-prooxidant) when present at high concentrations together with known prooxidants such as transition metal ions, lipid peroxides, or other oxidizing agents (Fuster et al., 1998).

Conclusion

Supplementing linseed oil with GTE significantly improved the oxidative stability of the oil by delaying the formation of primary and secondary oxidation products. In contrast, the addition of α-tocopherol (200 ppm) promoted oxidation of the oil. Green tea extract could therefore serve as an alternative natural antioxidant to improve the shelf life of linseed oil.

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Chapter 3C. The effect of oil-soluble green tea extract on the oxidative stability of sacha inchi oil

Nain C.W., Foncea P., Ide A., Larondelle Y.

Abstract

Green tea catechins have good antioxidant activity in highly polyunsaturated oils. However, being hydrophilic, adding catechins or green tea extract (GTE) powders to bulk oil usually requires prior solubilisation in a solvent. In recent years, oil-soluble green tea extracts (OSGTs) have been developed, which permit direct incorporation into the oil. In this study, we compared the antioxidant activity of three doses (1000 ppm, 2000 ppm and 3000 ppm) of an OSGT formulation in sacha inchi oil (SIO) to GTE (powder, 1000 ppm) and an oil-soluble mixed antioxidant formulation containing tocopherols, ascorbyl palmitate and rosemary extract (Phytrox, 1000 ppm). The oxidation of SIO without added antioxidant (control) and the five SIO treatments were monitored under accelerated storage at 60°C for 12 days by measuring the peroxide value (PV), p-anisidine value (p-AV) and the fatty acid content of the oil. The addition of the different extracts in SIO resulted in a PV and p-AV 5-72% and 12-64% lower than the control after 12 days, respectively. The overall effect on reducing the formation of oxidation products was in the decreasing order GTE > OSGT3000 > Phytrox > OSGT2000 > OSGT1000 after 12 days. Although, Phytrox showed a more potent antioxidant effect during the first six days of storage. The fatty acid content of SIO did not significantly change for any of the samples during the oxidation period. The degradation of tocopherols in SIO was inhibited or reduced with antioxidant supplementation, except for the treatment with OSGT1000. All three antioxidants tested in this study improved the stability of SIO. However, the effect of OSGT was dose-dependent, with better results obtained for SIO supplemented with 3000 ppm OSGT.

Keywords: Oil-soluble green tea extract, catechins, Phytrox, sacha inchi oil, alpha-linolenic acid.

1. Introduction

Sacha inchi *(Plukenetia volubilis L.)* is a plant native to the Peruvian amazon but nowadays also widely cultivated in southeast Asia as a promising valuable crop (Rawdkuen et al., 2016). The seeds of sacha inchi were traditionally used as food by the people of the amazon rainforest and contain 35-60% of oil, 25-30% of proteins with essential amino acids, minerals and tocopherols (Chirinos et al., 2013; Gutiérrez et al., 2011; Wang et al., 2018). Sacha inchi oil (SIO) is rich in unsaturated fatty acids, which represent 90-93% of the fatty acids (Herman et al., 2020; Liu et al., 2014; Maurer et al., 2012). SIO is of particular interest due to its high content of α-linolenic acid (ALA, 43-53%) and linoleic acid (33-40%), both essential in the diet. Other fatty acids in minor amounts include oleic acid, stearic acid and palmitic acid (Gutiérrez et al., 2017; Gutiérrez et al., 2011; Liu et al., 2014). The ALA content of SIO is comparable to that of flaxseed (52-60%) and Chia seed oil (58-63%). SIO has approximately twice the amount of ω-6 fatty acids as in flaxseed oil. With an ω-6/ω-3 fatty acid ratio in the range of 0.77-1.12, SIO is a good source of ω-3 and ω-6 fatty acids (Ramos-Escudero et al., 2019; Wang et al., 2018). A balanced ratio of ω-6/ω-3 fatty acids (4:1 to 1:1) is considered optimal for human health (Simopoulos and DiNicolantonio, 2016). However, there is an imbalance in the ratio of ω-6/ω-3 provided in a typical western diet reaching 15:1 to 16.7:1, and such a high ω-6/ω-3 ratio may increase the risk of chronic diseases such as cardiovascular diseases, inflammation, cancer, obesity and autoimmune diseases (Cárdenas et al., 2021; Simopoulos, 2006). The incorporation in the diet of oils rich in ALA, such as SIO, could therefore help in improving the ω-6/ω-3 ratio.

The high content of unsaturated fatty acids in SIO makes it susceptible to oxidation, negatively affecting the oil's sensory and

nutritional quality. Lipid oxidation is one of the causes of quality deterioration in oils and food. The exposure of unsaturated lipids to metal ions, heat or light leads to the formation of free radicals, which react with oxygen to form hydroperoxides (Ahmed et al., 2016). Hydroperoxides are unstable and further break down into aldehydes, ketones and alcohols, which are responsible for the off flavours in oxidised oils (Choe and Min, 2006). Liu et al. (2014) noted a steady increase in the peroxide value in SIO stored at different temperatures (4°C, 20°C and 30°C). Various approaches have been studied to improve the oxidative stability of SIO, such as roasting of seeds before oil extraction, whereby an increase in phenolic compounds was observed with an increase in roasting intensity (Cisneros et al., 2014), encapsulation (Suwannasang et al., 2021), supplementation with plant extracts such as tara (*Caesalpinia spinosa*) pod extracts or extracts from mashua (*Tropaeolum tuberosum*) (Chirinos et al., 2015; Herman et al., 2020).

In recent years, there has been an increase in the assessment of plant extracts as antioxidants in oils and fat-rich foods, especially with consumers' increasing preference for natural products (Scott et al., 2020). The antioxidants in these plant extracts are predominantly phenolic compounds, carotenoids and vitamins E and C (Jideani et al., 2021). Among the plant extracts with antioxidant effects in oils is the green tea extract (GTE), whose antioxidant activity is attributed to the presence of catechins (Nain et al., 2021; Shen et al., 2021; Wanasundara and Shahidi, 1998). However, catechins are hydrophilic; thus, GTE is generally solubilised in a solvent (*e.g*. ethanol) before addition to oil, which is not very applicable in an industrial milieu because of the quantities of solvent that may be required. Oil-soluble green tea extracts (OSGT) have thus been developed by esterifying green tea catechins with fatty acids, producing adducts that partition into the lipid phase (Drury et al., 2016; Myers et al., 2013). OSGT has been shown effective in delaying the formation of oxidation products in

canola, peanut, soybean and sunflower oils (Drury et al., 2016; Huang, 2015; Shen et al., 2021).

We previously showed the antioxidant activity of GTE in a DHA-rich oil (Nain et al., 2021). The present study aims at comparing the antioxidant effectiveness of OSGT to that of GTE in SIO under accelerated oxidation. Phytrox, an oil-soluble antioxidant containing a mixture of natural antioxidants, is used as a positive control. The antioxidant activity of the extracts is determined by measuring the peroxide value (PV), p-anisidine value (*p*-AV), and change in ALA and linoleic acid content of oil during storage. To the best of our knowledge, it is the first time that the effects of GTE, OSGT and Phytrox are investigated in SIO. Therefore, our results will provide helpful information about the use of these antioxidants in SIO and the dose of OSGT that is effective in this oil.

2. Materials and methods

2.1. Materials

Sacha inchi seeds and oil: Sacha inchi seeds from San Martin, Peru, were imported and provided for this study by Prellencia (Gembloux, Belgium). A preliminary test to extract oil from seeds was carried out using a manual hydraulic press (TecTake, China) and an automatic press (CGOLDENWALL-1500 W, China), which is preheated for five minutes prior to the pressing of seeds. The hydraulic press led to the production of cold-pressed oil. Meanwhile, the automatic press resulted in the production of oil at a temperature of 86°C, which was directly col down after production. The automatic press was more efficient with respect to the short time needed and better oil yield. No significant difference was observed in the oxidative stability of these oils during an oven storage test for 6 days at 60°C. Therefore, the oil used in the present study was obtained through the automatic press and centrifuged to eliminate particles.

***Natural antioxidants*:** The antioxidant ability of three antioxidants to improve the oxidative stability of SIO was tested. An oil-soluble green tea extract (OSGT) containing 14 % of active antioxidant molecule (epigallocatechin (EGC) esters), was kindly provided by Kemin industries (Herentals, Belgium). A water-soluble green tea leaf extract (GTE) was a product of Naturex (Avignon, France) and contained 25% of major green tea catechins: EGC (147.2 mg/g), epigallocatechin gallate (EGCG, 76.4 mg/g), epicatechin (EC, 20.0 mg/g), and epicatechin gallate (ECG, 10.0 mg/g). The third antioxidant used was Phytrox LTR15-IP MB (Phytrox), a product of Jan Dekker (Amsterdam, the Netherlands). Phytrox is a liposoluble liquid formulation containing a mixture of vegetable-derived tocopherols, ascorbyl palmitate and rosemary extract blended in lecithin and vegetable oil.

2.2. Sample preparation for accelerated ageing of sacha inchi oil

To evaluate the effect of Phytrox, GTE and OSGT in SIO, oil samples were prepared with 1000 ppm Phytrox, which is the dosage recommended by the producer, 1000 ppm GTE, which was the most effective concentration in our previous study (Nain et al., 2021) and 3 concentrations (1000, 2000 and 3000 ppm) of OSGT. GTE was added to the oil as an ethanolic extract, followed by evaporation of the ethanol under a stream of nitrogen gas (Nain et al., 2021). Phytrox and OSGT being liposoluble, they were each added directly to the oil. The antioxidant formulations were precisely weighed in a beaker to which SIO was added to obtain the desired concentration. The solutions were mixed with a glass rod for five minutes to ensure homogeneity. Oil samples were then distributed (10 g) in amber bottles (30 ml, 2.7 cm diameter) and stored opened in an oven at 60°C for 0, 3, 6 and 12 days. A control sample, without any added antioxidants, was also prepared. At each sampling point, the oil samples were analysed for oxidative status, while the fatty acid profile and tocopherol content analyses

were performed for all samples on day 0 and day 12. All treatments and control were prepared in triplicate.

2.3. Determination of oxidative status

The PV was determined by iodometric titration according to the AOAC official method 965.33 (AOAC, 2000). The *p*-AV was measured according to the AOCS official method Cd 18–19 (AOCS, 1998).

2.4. Analysis of fatty acid content

The fatty acid composition was analysed using the method previously described by Mellery et al. (2016). The fatty acids in oil were first converted to fatty acid methyl esters (FAME) via a two-step methylation with KOH and HCl. The FAMEs were then extracted in hexane and separated by gas chromatography. FAMEs were separated and quantified by running the samples in a GC Trace 1310 gas chromatograph (ThermoQuest, Milan, Italy) equipped with an As Triplus autosampler (Thermo Electron, Milan, Italy), an RT2560 capillary column of 100 m × 0.25 mm internal diameter (Restek, Bellefonte, PA, USA) and a flame ionisation detector set at 255°C [141]. Hydrogen was used as the mobile phase and was operated at a constant pressure of 200 kPa. Fatty acids in the samples were identified and quantified by comparison of retention times and peak areas with those of pure FAME calibration standards (Larodan, Solna, Sweden). Chromatograms were processed using Chromeleon 7 software (ThermoFisher Scientific, Waltham, MA, USA). The results are expressed in mg/g of oil.

2.5. Determination of tocopherol content

The alpha-, gamma- and delta-tocopherol content of the different oil treatments were determined by high-performance liquid chromatography (HPLC), as we previously described (Nain et al., 2021). To prepare samples for analysis, 1 g of oil was dissolved in 10 mL of HPLC-grade hexane. The tocopherol standards (Sigma-Aldrich, St. Louis, MO, USA) were also prepared at different concentrations (1, 2, 5, 10, 20, 50 and 100 ppm) in hexane. The samples and standards were filtered through 0.45 µm syringe filters into HPLC injection vials. The tocopherols were separated and quantified by injecting 50 µL of diluted standards and samples in a chromatograph equipped with an autosampler AS3000 (Oven temperature = 35°C, tray temperature = 15°C), a P1000 spectra system pump (Thermo separation products, San Jose, CA, USA), a Luna 5 µm normal phase column of 250 mm x 4.6 mm internal diameter (Phenomenex, Torrance, CA, USA) and an FP-2020 fluorescence detector (Jasco, Tokyo, Japan). The mobile phase used was 2% (v/v) tetrahydrofuran in n-heptane at a flow rate of 1.8 mL/min. The Chromquest 5.0 software (ThermoFisher Scientific, Milan, Italy) was used to process the chromatograph data, and the results are expressed as µg/g of oil.

2.6. Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation. The data for the PV, *p*-AV and fatty acid contents between treatments were subjected to an analysis of variance (ANOVA), and sample means were compared by Tukey-Kramer HSD (honestly significant difference) test. The fatty acid contents and tocopherol contents on day 0 and day 12 for each treatment, were compared by the Student's t-test. All statistical analyses were performed using JMP Pro version 16 (SAS institute inc., Cary, NC, USA), and the differences between the means were considered significant at $p \leq 0.05$.

3. Results

3.1. Characterisation of sacha inchi oil: fatty acid composition, tocopherol content and oxidative status

The fatty acid profile, tocopherol content and oxidative status of the freshly pressed SIO used in this study are shown in Table 1. The fatty acid analysis showed that SIO was made up predominantly of unsaturated fatty acids (90%), with a mean PUFA content of 81.5% and MUFA content of 8.99%. The major fatty acids detected in the oil in decreasing order were ALA (48.1%), linoleic acid (33.3%), oleic acid (8.4%), palmitic acid (3.8%) and stearic acid (2.7%). Vaccenic acid was also detected in the oil in a small quantity (0.55%). The total tocopherol content of SIO was 1592.2 ± 74.4 µg/g, with the most abundant isomers being γ-tocopherol and δ-tocopherol, representing 59.4 % and 40.4% of total tocopherols, respectively. A very low concentration of α-tocopherol was detected in the oil (0.2% of total tocopherols). The oxidative status of the fresh SIO was within acceptable limits with a *p*-AV of 1.8 and PV of 3.6 Meq O_2/kg, which was below the Codex Alimentarius limit 15 of Meq O_2/kg) for virgin oils (Codex, 1999).

Table 1. Fatty acid profile, tocopherol content and oxidative status of freshly pressed sacha inchi oil

Values are means of three determinations ± SD. SIO = freshly hot-pressed sacha inchi oil. MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. ND= not detected

Characteristics		SIO
Major fatty acids identified (mg/g)	Palmitic acid C16:0	38.3 ± 3.8
	Stearic acid C18:0	26.6 ± 2.6
	Oleic acid C18:1c9	83.5 ± 8.2
	Vaccenic acid C18: 1c11	5.5 ± 0.5
	Linoleic acid C18:2c9c12	333.4 ± 32.8
	A-linolenic acid C18:3c9c12c15)	480.8 ± 47.5
	Total SFA	68.7 ± 6.4
	Total MUFA	90.3 ± 8.4
	Total PUFA	815.3 ± 80.4
	Total UFA	905.6 ± 88.8
	ω6/ω3 ratio	0.69
Tocopherol content (µg/g)	Alpha	3.43 ± 0.1
	Gamma	945.3 ± 41.0
	Delta	643.42 ± 33.3
	Total	1592.2 ± 74.4
Oxidative status	Peroxide value (meq O_2/kg oil)	3.6 ± 0.01
	Para-anisidine value	1.8 ± 0.12

3.2. Effect of antioxidants on the oxidative status of oil during storage

3.2.1. Primary oxidation products

The PV of SIO (control) was initially 3.6 Meq O_2/kg at the start of the study (day 0). The addition of the different antioxidants resulted in a slight drop in PV and a significant difference ($p < 0.0001$) in the PV of treatments, with the OSGT and GTE-supplemented samples recording a significantly lower PV (1.6 meq

O_2 /kg) than the sample with Phytrox (2.03 meq O_2/kg). In the course of the 12-day storage at 60°C, a significant increase ($p < 0.0001$) in PV was observed in all treatments (Figure 1A), indicating the accumulation of hydroperoxides. The PV of the control increased steadily during the storage period and was significantly higher than the antioxidant-supplemented samples at all sampling points. SIO supplementation with antioxidants improved the oxidative stability of the oil to different degrees. The PV of the oil supplemented with Phytrox was stable during the first 6 days of storage, recording a significantly lower PV (2.29 meq O_2/kg oil) than the other treatments but a sharp, significant increase ($p < 0.0001$) was observed between day 6 and day 12. The oil supplemented with GTE showed a gradual increase in PV throughout the storage period. The OSGT showed a dose-dependent lowering effect on the oxidation of SIO, with the higher concentration of 3000 ppm being more effective. Apart from the control and OSGT1000 samples, the PV of all other treatments remained below the Codex Alimentarius recommended limit for virgin and cold-pressed oils (15 meq O_2/kg oil) during the first 6 days of storage. After 12 days of storage, the GTE-supplemented oil had the lowest PV (17.6 meq O_2/kg oil), followed by OSGT3000 (32.3 meq O_2/kg oil), Phytrox (38.5 Meq O_2/kg oil), OSGT2000 (42.0 meq O_2/kg oil), and OSGT1000 (59.4 meq O_2/kg oil) samples, which were significantly lower ($p < 0.0001$) than the control (62.8 meq O_2/kg oil) by 71.95%, 48.5%, 38.6%, 33.1% and 5.3%, respectively.

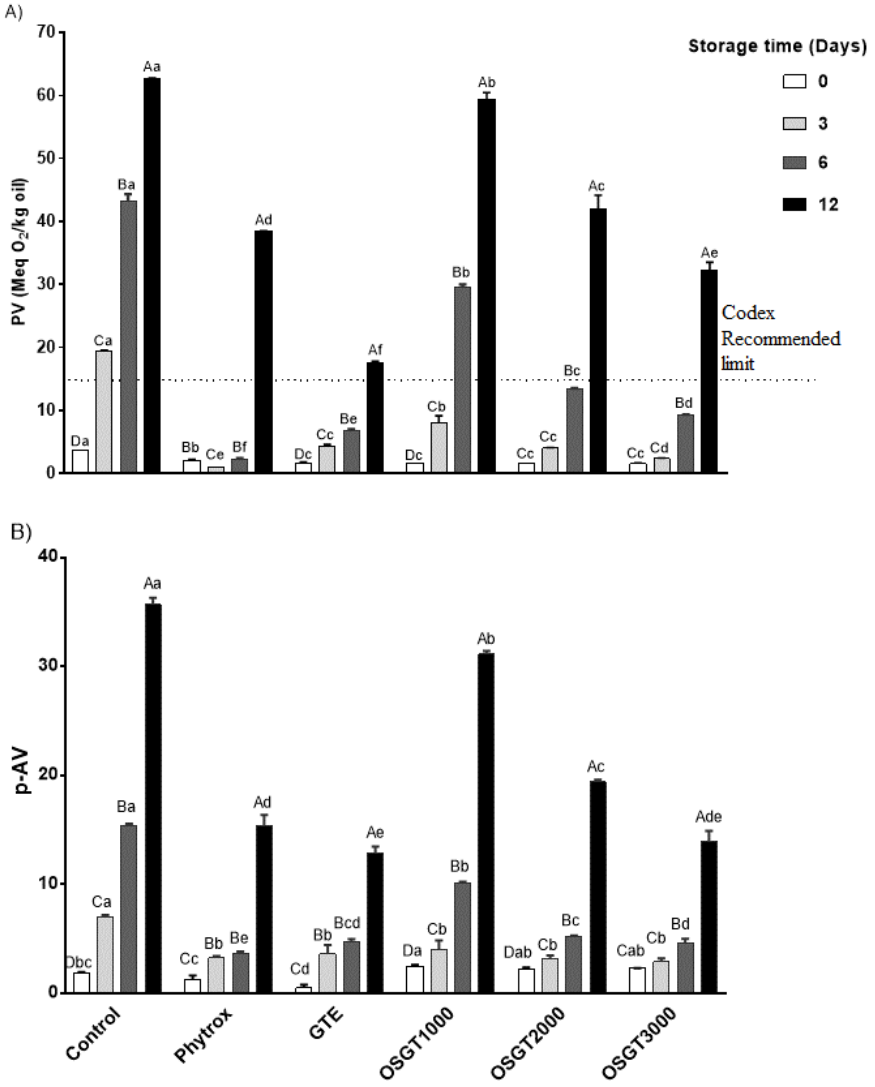

Figure 1. Evolution of peroxide value (A) and *p*-anisidine value(B) in Sacha inchi oil (SIO) stored in open bottles with and without added antioxidants for 12 days at 60°C. Values are mean ± SD (n = 3). Control = SIO without antioxidant; Phytrox = SIO +1000 ppm of Phytrox LTR15-IP MB; GTE = SIO + 1000 ppm of green tea extract (GTE), OSGT1000, 2000, 3000 = SIO + 1000, 2000, or 3000 ppm of an oil-soluble green tea extract (OSGT). Different capital letters (A-D) within a treatment indicate a significant difference ($p \leq 0.05$) during storage; no common lower-case letter (a-f) between the treatments at a specific time point indicates a significant difference ($p \leq 0.05$).

3.2.2. Secondary oxidation products

The secondary oxidation products were determined as *p*-AV, which measures aldehydes resulting from the breakdown of hydroperoxides. The *p*-AV of SIO before the start of the storage test was at 1.8, and the incorporation of antioxidants had a significant effect on its evolution (Figure 1B). The *p*-AV of treatments on day 0 ranged from 0.5 to 2.5, with the lowest recorded for the GTE-supplemented oil and the highest for the oil supplemented with OSGT1000. During the 12 days of storage at 60°C, a significant increase in *p*-AV was observed in samples. After 3 days of storage, the *p*-AV of the control was significantly higher, and no significant difference was observed between the oils with added antioxidants. After 6 days of storage, the *p*-AV of the control was still the highest (15.4), followed by treatment with OSGT1000 (10.1), and the other treatments had values lower than or equal to 5. At the end of the 12-day storage, the ability of the antioxidants to lower the *p*-AV was observed in the decreasing order GTE ≥ OSGT3000 ≥ Phytrox > OSGT2000 > OSGT1000, similar to the trend observed for the PV. However, there was no significant difference between the *p*-AV of GTE and OSGT3000 samples and between OSGT3000 and Phytrox-supplemented oils. The oils supplemented with GTE, OSGT3000, Phytrox, OSGT2000 and OSGT1000 had a *p*-AV, respectively, 63.9%, 60.9%, 56.9%, 45.6% and 12.8% lower than the control (*p*-AV = 35.67) on day 12.

3.3. Effect of antioxidants on the fatty acid content of sacha inchi oil during storage

To evaluate the effect of oxidation and antioxidant treatments on the fatty acid composition of SIO, oil samples were analysed on day 0 and after 12 days of storage at 60°C. The saturated and monosaturated fatty acids in the control and treatments remained stable during storage. Results obtained for the major PUFAs in SIO, linoleic acid and ALA are presented in Figure 2. The mean contents

of linoleic acid and ALA on day 0 ranged from 297 to 346 mg/g oil and 428.9 to 489.9 mg/g oil, respectively. After 12 days of storage, no significant change in fatty acid content was observed for any of the treatments or control, that is, the amount of fatty acids in the different oil samples remained stable during this period.

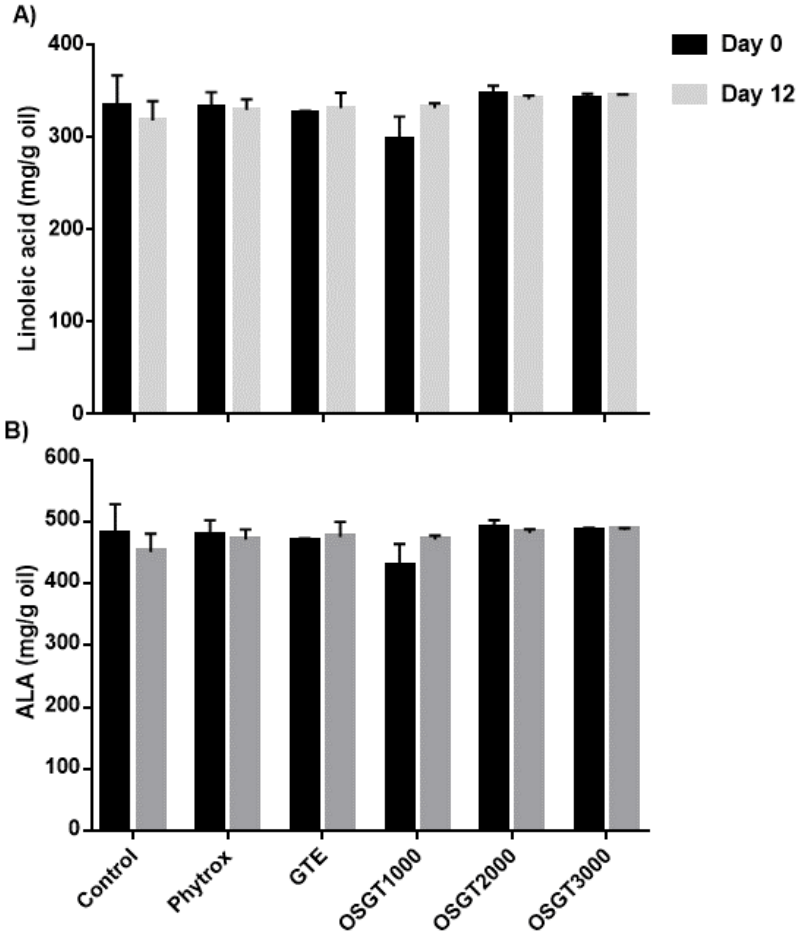

Figure 2. Linoleic acid content (A) and alpha-linolenic acid (ALA) content (B) in sacha inchi oil (SIO) with or without added antioxidants during accelerated storage at 60°C over 12 days. Values are mean ± SD (n = 3). No significant difference between treatments or between storage points. Control = SIO without antioxidant; Phytrox = SIO +1000 ppm of Phytrox LTR15-IP MB; GTE = SIO + 1000 ppm of green tea extract (GTE), OSGT1000, 2000, 3000 = SIO + 1000, 2000, or 3000 ppm of an oil-soluble green tea extract (OSGT).

3.4. Effect of antioxidants on the evolution of tocopherols in sacha inchi oil during storage

As shown in Table 1, SIO contains a high level of endogenous tocopherols. The tocopherol content of the different oil treatments was monitored during storage and presented in Figure 3. The fresh oil had an average total tocopherol content of 1592.2 μg/g, and the incorporation of antioxidants had no significant effect ($p = 0.2502$) on this concentration on day 0, with a mean total tocopherol content of the oil treatments ranging from 1404 to 1505.7 μg/g (Figure 3A). After 12 days of storage, a significant reduction of 59.2%, 52.5%, 29.9% and 14.5% in total tocopherols was observed for the control, OSGT1000, OSGT2000 and OSGT3000 treatments, with no significant difference between the control and OSGT1000 treatment. The GTE and Phytrox-supplemented samples showed no significant change in total tocopherols between days 0 and 12 and were also not significantly different ($p = 0.4$) from the OSGT3000 treatment.

Analysing the results with respect to individual tocopherol isomers, we observed that on day 0, the α-tocopherol content of the oil supplemented with Phytrox (20.8 μg/g) was significantly higher than that of the other samples (2.4 - 3.4 μg/g) (Figure 3B). This difference was expected since Phytrox contains tocopherols. The 12-day storage had no effect on the α-tocopherol content of GTE and OSGT3000 treatments, whereas a significant decrease was observed for the control (0.82 μg/g left of 3.4 μg/g) and treatments with Phytrox (14.26 μg/g left of 20.82 μg/g), OSGT1000 (0.9 μg/g left of 2.65 μg/g) and OSGT2000 (1.46 μg/g left of 2.62 μg/g).

The γ -tocopherol isomer was the most abundant tocopherol in the oil, and the changes observed in this isomer during storage are presented in Figure 3C. No significant difference ($p = 0.2361$) was observed in the γ-tocopherol content among the oil treatments, ranging from 890.8 to 945.3 μg/g of oil on day 0. After 12 days of

storage, a significant depletion of 78.3%, 71.3%, 42.6%, 28.0% and 17.9% γ-tocopherol was observed in the control, OSGT1000, OSGT2000, OSGT3000 and Phytrox treatments. There was no significant change ($p = 0.5089$) in the γ-tocopherol content of the GTE-supplemented oil during the storage period.

The average δ-tocopherol content in the oil treatments on day 0 was 594.0 to 643.5 µg/g, with no significant difference ($p = 0.2422$) observed between treatments. The δ-tocopherol content was stable during storage for the GTE, Phytrox and OSGT3000 treatments, whereas significant depletion was observed for the control (443.5 µg/g), OSGT1000 treatment (450.7 µg/g) and OSGT2000 treatment (543.4 µg/g).

Overall, the incorporation of OSGT1000 had no significant effect on the depletion of tocopherols, whereas the other treatments significantly improved the stability of total tocopherols in the decreasing order GTE ≈ Phytrox ≈ OSGT3000 > OSGT2000.

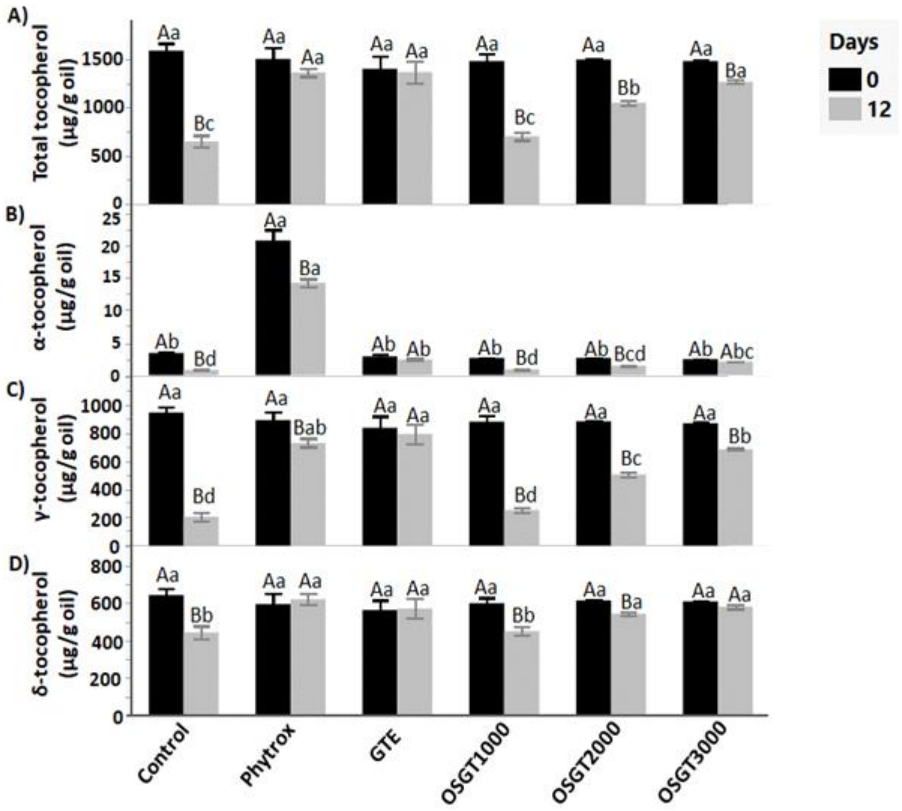

Figure 3. Total tocopherol (A), alpha-tocopherol (B), gamma-tocopherol (C) and delta-tocopherol (D) content of sacha inchi oil (SIO) with or without added antioxidants during an accelerated oxidation test at 60°C for 12 days. Values are mean ± SD (n = 3). Control = SIO without antioxidant; Phytrox = SIO +1000 ppm of Phytrox LTR15-IP MB; GTE = SIO + 1000 ppm of green tea extract (GTE); OSGT1000, 2000, 3000 = SIO + 1000, 2000, or 3000 ppm of an oil-soluble green tea extract (OSGT). Different capital letters (A-D) within a treatment indicate a significant difference ($p \leq 0.05$) during storage; no common lower-case letter (a-f) among treatments indicates a significant difference ($p \leq 0.05$) at a specific storage time.

4. Discussion

The importance of SIO is linked to its content in unsaturated fatty acids, especially ALA, as well as its content in other bioactive components, such as tocopherols. The unsaturated fatty acid (90 mg/g) and ALA (48.1%) contents of the SIO extracted and used in this study were similar to those reported by Cisneros et al. (2014) and Gutiérrez et al. (2017). Meanwhile, Herman et al. (2020) measured a lower amount of ALA (40.99 %) in cold-pressed SIO from roasted or unroasted seeds. SIO is known to contain a high level of total tocopherols (>1900 µg/g) that is higher than that of oils from flaxseed (377.1 µg/g), rapeseed (505.7 µg/g), corn (815.8µg/g), peanut (224.1 µg/g), soybean (846.0 µg/g), sunflower (625.3 µg/g) and olives (174.6) (Gliszczyńska-Świgło and Sikorska, 2004; Ramos-Escudero et al., 2019). The mean total tocopherol content (1592.2 µg/g) of the lab-pressed oil used in this study was lower than the minimum recommended by the Peruvian regulations, which is 1900 µg/g (Ramos-Escudero et al., 2019). However, these regulations are valid for extra virgin oil, which is different from the hot-pressed oil used in this case. Some tocopherols could have been degraded during the hot-pressing process (86°C) as these molecules are sensitive to heat. Cisneros et al. (2014) also reported lower tocopherol levels of 832 µg/g in SIO pressed under hot conditions (100-110°C). The PV of freshly extracted SIO (3.6 Meq O_2/kg oil) was within the Codex recommended limit and similar to the PV reported for SIO by Maurer et al. (2012), lower than the 4.34 Meq O_2/kg of SIO reported by Chirinos et al. (2015) and higher than the PV of SIO (1.85 and 2.16 meq O_2/kg) obtained by cold pressing (Herman et al., 2020; Liu et al., 2014).

The oxidation of oils rich in PUFAs follows a three-step process of initiation, propagation and termination, leading to the formation of oxidation products. The use of antioxidants retards lipid oxidation by scavenging free radicals, chelating prooxidative metals, and/or

quenching singlet oxygen and photosensitisers (Choe and Min, 2006). As seen from our results, adding Phytrox, GTE or OSGT to SIO lowered the formation of oxidation products compared to the control without added antioxidants. The antioxidant effectiveness was observed in the decreasing order GTE > OSGT3000 > Phytrox > OSGT2000 > OSGT1000. Phytrox prevented oxidation during the first six days of storage, whereas the GTE and OSGT3000 had a more persistent effect in retarding the oxidation process. The antioxidant effectiveness of GTE has been reported in different food matrices, including pastry, meat products, table spreads, food emulsions and bulk oils (Dwyer et al., 2012; Kozłowska et al., 2019; Tian and Huang, 2019; Wanasundara and Shahidi, 1996). GTE is also effective in delaying the oxidation of highly polyunsaturated oils like DHA-rich oil (Nain et al., 2021). In a previous study, OSGT (2500 ppm and 5000 ppm) was shown to be effective in improving the oxidative stability of canola oil under three storage conditions (60°C, 22-25°C for ambient conditions and 22-25°C with air-purging), with comparable performance to TBHQ observed under ambient conditions (Shen et al., 2021). Similar to the observation in the present study, OSGT also showed a dose-response trend in canola oil, with the higher dosage (5000 ppm) being better in slowing down the generation of hydroperoxides (Shen et al., 2021). Using the oxidative stability index, OSGT was shown to be more effective than mixed tocopherols and rosemary extract in canola, sunflower, peanut and soybean oils but comparable to ascorbyl palmitate in soybean oil (Huang, 2015). The antioxidant effectiveness of a Phytrox formulation containing basically rosemary extract has been reported in two previous studies. Gramza-Michalowska et al. (2008) compared the antioxidant activity of tea extracts with well-known commercial antioxidants (BHT and Phytrox) in a linoleic acid emulsion stored at 37°C for 19 hours and found that the tea extracts (1000 ppm) resulted in a lower increase of conjugated dienes in the emulsion than Phytrox. In another study, Gramza-Michałowska (2008) assessed the radical scavenging activity of different tea leaf extracts compared to other

natural extracts (rosemary ethanolic extract and Phytrox) and found that tea extracts (1000 ppm) had a stronger DPPH radical scavenging activity.

The fatty acid content of SIO remained stable during the 12-day storage period for the control and supplemented samples. Similarly, Maurer et al. (2012) observed no changes in saturated, monosaturated and polyunsaturated fatty acids in SIO during an oxidation test at 65°C for 20 days. Cisneros et al. (2014) reported a slight decrease in the ALA content of SIO stored at 60°C for 30 days (an 8% decrease).

Besides the fatty acid content, SIO is also considered important because of its tocopherol content. The tocopherol content in the control significantly decreased during the storage period, which was similar to the observation of Cisneros et al. (2014) for SIO stored at 65°C for 20 days. Apart from OSGT1000, the incorporation of the extracts either inhibited the degradation of tocopherols or lowered the degradation rate. In our previous study, we observed that adding GTE in DHA-rich oil (stored at 30°C for 9 weeks) decreased the degradation rate of tocopherols (Nain et al., 2021). Prabsangob and Benjakul (2018) also reported a delay in the frying-induced degradation of tocopherols in a soybean oil/tea seed oil blend supplemented with EGCG and EC.

The antioxidant effect of GTE and OSGT is due to the presence of catechins, which delay oxidation by acting as radical scavengers and metal chelators. Phytrox, on the other hand, contains mixed tocopherols, ascorbyl palmitate and rosemary extract, which all have antioxidant activity (Yin et al., 2021). The main compounds in rosemary extract responsible for its antioxidant activity include carnosic acid, rosmarinic acid and carnosol. These compounds, as well as tocopherols, can donate hydrogen to free radicals, thus breaking the free radical chain reaction. In addition, it has been reported that carnosic acid and ascorbyl palmitate can regenerate tocopherols from tocopherol free radicals, thus, enhancing

antioxidant activity (Fang and Wada, 1993; Yin et al., 2021). This synergistic effect between the antioxidant compounds in Phytrox could explain its potent antioxidant activity during the first 6 days of storage. However, the stability of antioxidants seems to be an important factor for a prolonged antioxidant effect. Yin *et al.* (Yin et al., 2021) observed a complete depletion of rosemary extract (carnosic acid) in canola oil after 14 days of storage at 60°C, whereas OSGT was entirely consumed only at the end of 28 days of storage. The depletion rate of the active compounds in the SIO treatments could explain why in the present study, OSGT and GTE showed better antioxidant activity after 12 days of storage compared to Phytrox.

5. Conclusion

This study compared the effect of OSGT, GTE and Phytrox on the oxidative stability of SIO during accelerated storage at 60°C for 12 days in open bottles in the dark. Our findings demonstrate that Phytrox is very effective in inhibiting oxidation during the first six days, whereas GTE and OSGT3000 are better antioxidants over the 12-day storage period. Overall, the ability of the different treatments to lower the formation of oxidation products was observed in the decreasing order Phytrox > GTE > OSGT3000 > OSGT2000 > OSGT1000 after 6 days and GTE > OSGT3000 > Phytrox > OSGT2000 > OSGT1000 after 12 days at 60°C. In addition, the supplementation of SIO with these antioxidants also reduced the degradation of tocopherols in the oil. The results obtained in this study indicate that not only GTE but also OSGT (3000 ppm) and Phytrox (1000 ppm) could be effectively used as antioxidants to enhance the oxidative stability of SIO. Considering that accelerated storage conditions were applied in this study, it might be of interest to evaluate the effect of these antioxidants in SIO under ambient and cold storage, which are recommended storage conditions for this oil

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Additional Data

A) Comparing the level of oxidation and effect of antioxidants in the PUFA-rich oils studied

1. DHA-rich oil and linseed oil at 30°C (Chapter 3, A and B)

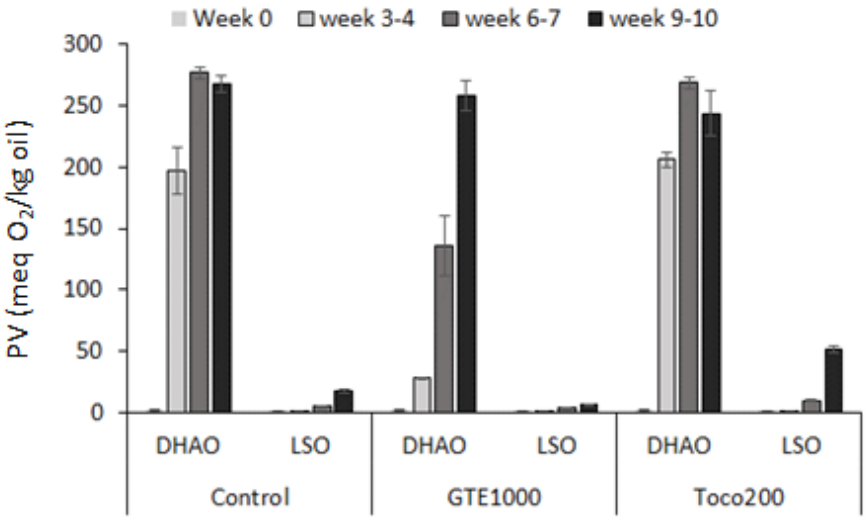

Figure 1S. Peroxide value (PV) of DHA-rich oil (DHAO) and linseed oil (LSO) with or without added antioxidants during storage at 30°C in open bottles in the dark. Values are mean ± SD with n=3. Control = LSO or DHAO without added antioxidants; GTE1000 = LSO or DHAO supplemented with 1000 ppm green tea extract; Toco200 = LSO or DHAO supplemented with 200 ppm of α-tocopherol.

Figure 1S compares the results obtained for the accelerated oxidation of DHA-rich oil and linseed oil in Chapters 3A and 3B, respectively. The formation of oxidation products was faster and higher in DHA-rich oil samples than in linseed oil stored at 30°C for up to 9 or 10 weeks. After 10 weeks of storage, the PV of the linseed oil samples were still within or just slightly above the 15 meq O_2/kg oil limit recommended by Codex Alimentarius. In contrast, DHA-rich oil treatments were far above this value after only 3 weeks of storage (Figure 1S). Secondary oxidation products measured as p-AV were not detected in the linseed oil samples except for α-

tocopherol supplemented samples after 10 weeks at 30°C (Table 1S). On the other hand, a remarkable increase in p-AV was observed for all treatments of DHA-rich oil. The antioxidant activity of GTE and the pro-oxidant effect of α-tocopherol were observed in both oils. The difference in the rate of oxidation in these oils is expected, first because of the difference in the degree of unsaturation of the fatty acids in the oils and, secondly, because of the amount of PUFAs. Linseed oil contained 70.9 wt.% of PUFAs, whereas the value reached 87.3 wt.% for the DHA-rich oil.

Table 1S. p-Anisidine value (p-AV) of DHA-rich oil (DHAO) and linseed oil (LSO) with or without added antioxidants during storage at 30°C in open bottles in the dark.

		***P*-AV**	
Treatment		**Week 0**	**Week 9-10**
	DHA	5.2 ± 0.3	176.9 ± 7.7
Control	LSO	0	0
	DHA	3.22 ± 1.1	82.1 ± 5.1
GTE1000	LSO	0	0
	DHA	6.1 ± 0.6	238.5 ± 9.0
Toco200	LSO	0	2.39 ± 0.5

Values are mean ± SD with n=3. Control = LSO or DHAO without added antioxidants; GTE1000 = LSO or DHAO supplemented with 1000 ppm green tea extract; Toco200 = LSO or DHAO supplemented with 200 ppm of α-tocopherol.

2. Linseed oil and sacha-inch oil at 60°C (Chapter 3, B and C)

Figure 2S compares the results obtained for the accelerated oxidation of linseed oil (LSO) and sacha in oil (SIO) at 60°C in chapters 3B and 3C. The PVs of LSO and SIO were similar after 6 days at 60°C (Figure 2S A). The p-AV of the control samples followed a similar pattern as PV, whereas for GTE-supplemented conditions, SIO had a higher p-AV (Figure 2S B). The slight

differences in the oxidation of these oils may be linked to their initial oxidative status. SIO had a higher PV (3.6 meq O_2/Kg oil) and p-AV (1.8) than LSO (PV, 1.2 meq O_2/Kg oil; p-AV = 0) at the start of the tests. In addition, although LSO and SIO contain ALA as major fatty acid, the total PUFA (70.9 wt.%) and total tocopherols (434.5 µg/g) in LSO were lower than in SIO, with 81.5 wt. % total PUFAs and 1592 µg/g total tocopherols.

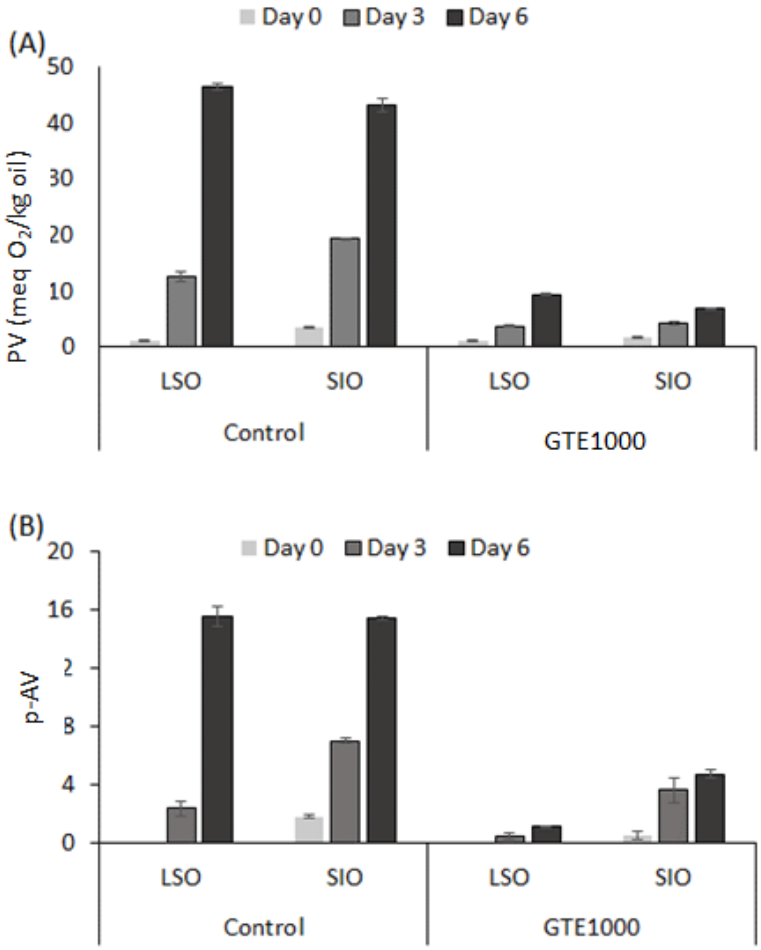

Figure 2S. Peroxide value (PV) (A) and *p*-anisidine value (*p*-AV) (B) of linseed oil (LSO) and sacha inchi oil (SIO) with or without green tea extract (GTE at 1000 ppm) during an oven storage test at 60°C. Values are mean ± SD (n = 3).

B) Oxidative stability of DHA-rich oil with or without added tocopherols at production

The DHA-rich oil used in Chapter 3A already contained high amounts of mixed tocopherols added at production. Therefore, to verify the effect of initial tocopherols content on the oxidation of oil and the antioxidant activity of GTE, we compared the oxidation of a DHA-rich oil with no added mixed tocopherols (total tocopherols, 218 μg/g) to that with added mixed tocopherols (total tocopherols, 2171μg/g).

The PV of the DHA-rich oil without added tocopherols was 29.9% higher than that with added mixed tocopherols after 21 days of storage at 30°C (Figure 3S). The mixed tocopherols, therefore, slightly enhanced the oxidative stability of the oil. Supplementing the oils with GTE significantly reduced the PV by 91% for the oil with no added tocopherols and 90% for oil with mixed tocopherols. This suggests that the amount of tocopherols in the oil does not affect the antioxidant activity of GTE.

The p-AV of DHA-rich oil with mixed tocopherols was 56.7% lower than oil with no added tocopherols after 21 days at 30°C. The secondary oxidation products were detected only at very low levels in the GTE-supplemented oils.

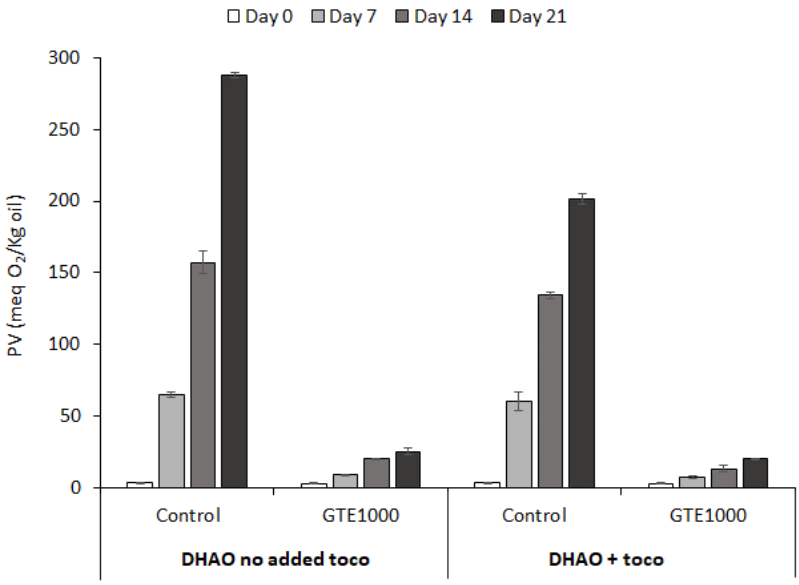

Figure 3S. Effect of initial tocopherol content in DHA-rich oil on its Peroxide Value (PV) during storage at 30°C for 21 days in open bottles in the dark. Values are mean ± SD with n=3. Control = DHA-rich oil with or without added tocopherols at production and with no GTE; GTE1000 = DHA-rich oil with or without added tocopherols at production and supplemented with GTE at 1000 ppm.

Table S2. Effect of Initial tocopherol content of DHA-rich oil on p-anisidine (p-AV) value during storage at 30°C in open bottles in the dark

		p-AV			
Oil	**Treatment**	**Day 0**	**Day 7**	**Day 14**	**Day 21**
No added tocopherol	Control	2.8 ± 0.5	15.9 ± 2.8	47.9 ± 1.5	100.6 ± 8.2
	GTE1000	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1
Added tocopherol	Control	3.8 ± 0.1	8.6 ± 1.7	18.6 ± 3.8	43.6 ± 1.8
	GTE1000	0.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 0.3

Values are mean ± SD with n=3. Control = DHA-rich oil with or without added tocopherols at production and with no GTE; GTE1000 = DHA-rich oil with or without added tocopherols at production and supplemented with GTE at 1000 ppm.

Chapter 4. The catechins profile of green tea extracts affects the antioxidant activity and degradation of catechins in DHA-rich oil

Nain, C.W.; Mignolet, E.; Herent, M.-F.; Quetin-Leclercq, J.; Debier, C.; Page, M.M.; Larondelle, Y.

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Foreword

In the previous Chapter, the antioxidant activity of GTE was shown in oils with high PUFA content. The GTE used in those studies was rich in EGC. However, the amount of different catechins in GTE can vary with respect to environmental conditions, variety, and agronomic conditions. Therefore, in this chapter, we investigated the effect of the catechins profile and individual catechins on the antioxidant activity of GTE. The results of this chapter give a better insight into the choice of GTE that will best delay oxidation in oils with high PUFA contents.

Abstract

This study investigated the effect of the catechins profile on the antioxidant activity of green tea extracts (GTEs) by comparing the antioxidant activity of an EGC-rich GTE (GTE1, catechin content: 58% EGC, 30.1% EGCG, 7.9% EC, and 3.9% ECG) and an EGCG- rich GTE (GTE2, catechin content: 60.6% EGCG, 17.7% EGC, 11.8% ECG, and 9.8% EC) in a DHA-rich oil. The effects of the individual catechins (EGC, EC, EGCG, and ECG) and reconstituted catechins mixtures (CatMix), prepared to contain the same amount of major catechins as in the GTEs, were also measured. All treatments (GTE1, CatMix1, GTE2, CatMix2, EGC250, EC250, EGCG250, and ECG250), each containing epistructured catechins at a concentration of 250 ppm, as well as the control (oil with no added antioxidant), were stored at 30°C for 21 days with sampling intervals of 7 days. The antioxidant activity was assessed by measuring the peroxide value (PV) and p-anisidine value (p-AV) of oils. Changes in fatty acid content and catechins content were also monitored. Both GTEs enhanced the oxidative stability of the DHA-rich oil, but GTE1 demonstrated a stronger antioxidant activity than GTE2. No significant difference was observed between the PV of treatments with GTE1 and CatMix1 during storage, whereas the PV of oil with GTE2 was significantly higher than that with CatMix2 after 21 days. Among the individual catechins, EGC was the strongest antioxidant. Overall, the antioxidant activities of the extracts and catechins were observed in the decreasing order GTE1 ≈ EGC250 ≈ CatMix1 > GTE2 > EGCG250 ≈ CatMix2 > ECG250 > EC250. A significant change in fatty acid content was observed for the control and EC250 samples, and the catechins were most stable in GTE1-supplemented oil. Our results indicate that the EGC-rich GTE is a more potent antioxidant in DHA-rich oil than the EGCG-rich GTE.

Keywords: green tea extract; catechins; antioxidant activity; oxidative stability; catechin degradation; DHA-rich oil

1. Introduction

Green tea, prepared from the leaves of *Camellia sinensis*, is a widely consumed beverage rich in polyphenols. The interest in green tea has been further incited over the years by studies demonstrating its anti-carcinogenic (Gu et al., 2013; Yang et al., 2009), anti-microbial and antiviral (Siriphap et al., 2022; Yang et al., 2021), antihypercholesterolemic (Kim et al., 2011), anti-obesity (Huang et al., 2014; Zang et al., 2021), anti-inflammatory (Donà et al., 2003), and antioxidant properties (Forester and Lambert, 2011; Peluso and Serafini, 2017). The beneficial effects of green tea are attributed to its high catechins content, which accounts for about 15–27% of the dry weight of green tea leaves (Lee et al., 2014; Liang et al., 1990; Zheng et al., 2018). The major catechins in green tea are: (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG), and (–)-epigallocatechin gallate (EGCG), the latter being the most abundant (Luo et al., 2020). The difference between these catechins is in the number of hydroxyl groups on the B-ring and the presence or not of a galloyl group on the C-ring (Figure 1). The major catechins can be converted during tea leave processing into their corresponding non-epistructured catechins, i.e., (±)-catechin, (–)-gallocatechin, (–)-catechin gallate and (–)-gallocatechin gallate (Liu et al., 2021; Wang and Helliwell, 2000). Apart from catechins, tea leaves also contain small amounts of other phytochemicals such as caffeine, phenolic acids, and flavonols (mainly quercetin, kaempferol, myricetin, and rutin) (Liu et al., 2021; Wang and Helliwell, 2000).

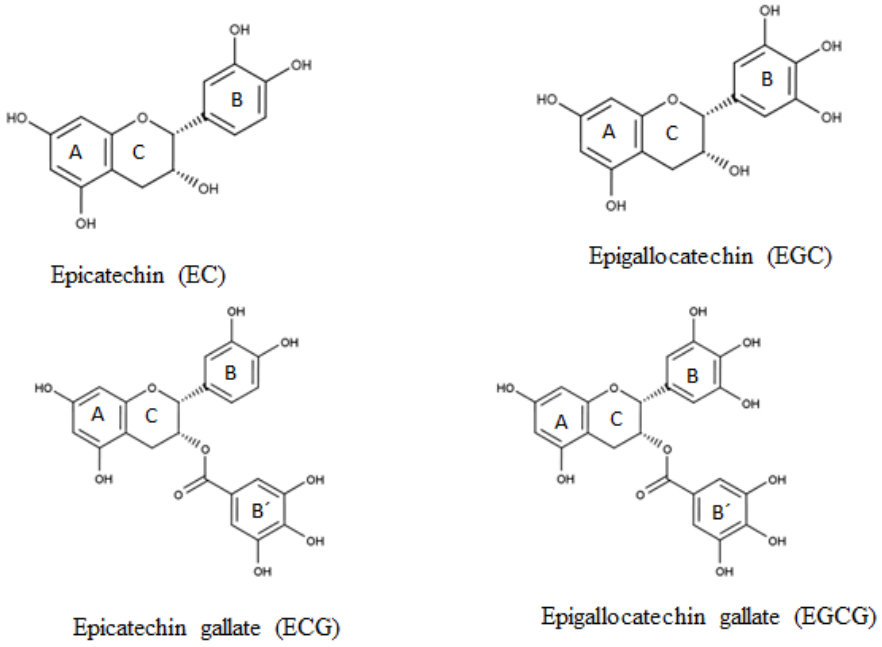

Figure 1. Chemical structure of major green tea catechins (Baldi et al., 2020; Yoshino et al., 2014).

The chemical structure of catechins seems to have an influence on their antioxidant activity, with the number and position of the hydroxyl groups in the molecule playing a substantial role (Senanayake, 2013). Gallocatechins (EGC and EGCG) have a trihydroxyl group in the B-ring (pyrogallol group), while catechin gallates (ECG and EGCG) have an esterified gallate moiety attached to position 3 of the C-ring. The trihydroxyl group on the B-ring and the gallate moiety have both been associated with increased free radical scavenging ability of catechins, thus increased antioxidant activity (Colon and Nerín, 2015). Various studies have reported a stronger antioxidant activity for esterified catechins. He and Shahidi (1997) observed the potency of catechins added at a concentration of 0.68 mmol/kg (200 mg EC equivalent/kg) in a fish meat system to be in the decreasing order EGCG ≈ ECG > EGC >> EC on the basis of peroxide value (PV). In another study, the antioxidant activity of catechins (200 ppm) in seal blubber and menhaden oils stored at 60°C was in the decreasing order ECG >

EGCG > EGC > EC [23]. In a β-carotene linoleate model system, the effectiveness was in the order ECG > EGCG = EC > EGC with respect to β-carotene bleaching (Amarowicz and Shahidi, 1995). However, in some studies, a stronger antioxidant activity was recorded for catechins containing the pyrogallol moiety. The antioxidant activity of tea catechins (200 ppm) in canola oil heated at 95°C for different times varied in the decreasing order EGC > EGCG > EC > ECG (Chen and Chan, 1996). Huang and Frankel (1997) observed varying trends in the antioxidant activity of tea catechins in different lipid systems. In corn oil triglycerides stored at 50°C, the antioxidant potency of catechins (140 μM) was observed in the order EGC ≈ EGCG ≈ ECG > EC. In contrast, all the catechins were prooxidants in a corresponding corn oil-in-water emulsion, whereas in soy lecithin liposomes also stored at 50°C, EGCG was the best antioxidant followed by EC, EGC, and ECG at 20 μM. These authors suggested that variation in the antioxidant activities among catechins could be partly explained by differences in their reducing potential, stability, and relative partition between phases in different lipid systems (Huang and Frankel, 1997).

Green tea extracts (GTEs) rich in catechins are produced from green tea leaves by different processes such as conventional solvent extraction, ultrasound-assisted extraction, microwave-assisted extraction, high-pressure processing, or supercritical fluid extraction, among others (Banerjee and Chatterjee, 2015). The amount of catechins recovered in an extract can vary depending on the extraction solvent, temperature, and duration (Hu et al., 2009; Koch et al., 2020). Green tea leaves contain as most abundant catechin EGCG (40–69%), followed by EGC (12–23%), ECG (13–21%), and EC (5–9%) (Amrouche et al., 2021; Reygaert, 2018; Zheng et al., 2018), but these proportions could also vary with environmental conditions, variety, as well as agronomic conditions such as plucking time and leaf age (Lee et al., 2014; Zhang et al., 2018). For example, Chen et al. (2003) measured higher levels of EGCG and ECG in young leaves compared to mature leaves, while

old leaves were richer in EGC and EC. This implies that catechins profiles of GTEs would vary with respect to the source and age of tea leaves and the extraction procedure, which could affect antioxidant effectiveness.

This study compares the effect of two GTEs with different catechins profiles, one rich in EGC (GTE1) and the other rich in EGCG (GTE2), on the oxidative stability of a DHA-rich oil. To ascertain that the antioxidant effect is a result of catechins (epistructured) and not due to other active compounds in the GTE, we also evaluated the antioxidant activity of reconstituted catechins mixtures in the proportions present in the GTEs, as well as the effect of the individual catechins. In addition, the fate of catechins in the oil was also assessed while assessing possible degradation products. This study contributes to a better understanding of the role of the catechins profile in the antioxidant activity of GTEs, with the ultimate aim to guide the choice of GTE that will better enhance the oxidative stability of oils and derived products enriched in highly valuable fatty acids.

2. Materials and Methods

2.1. Materials

The two GTEs were both products of Naturex (Avignon, France). Green tea leaf PE 50% Poly.UV/20% Catec.HPLC/7% EGCG (EA830365): powdered extract of green tea leaves, standardized to a minimum content of 50% polyphenols, 20% catechins, and 7% EGCG, referred to as GTE1 in this study and green tea extract PE 95% Poly.UV/60% Catec.HPLC/30% EGCG (ED840370): powdered extract of green tea leaves, standardized to a minimum content of 95% polyphenols, 60% catechins and 30% EGCG., referred to as GTE2.

DHA-rich oil with no added antioxidants was kindly provided by KD Pharma (Bexbach, Saarland, Germany). The oil had a total polyunsaturated fatty acid (PUFA) content of 906.8 mg/g with a

mean content of 661.5 mg/g being docosahexaenoic acid (DHA) and 137.4 mg/g of eicosapentaenoic acid (EPA).

The analytical standards, EC, EGC, ECG, and EGCG with the following respective HPLC purity: ≥99%, ≥98%, ≥97.5%, and ≥98%, were purchased from Extrasynthese (Genay, France). All other chemicals used in this study were either of GC-grade, HPLC-grade, or analytical grade.

2.2. Quantification of Major Catechins in GTE

The catechins profiles of GTE1 and GTE2 were determined by measuring their content in EGC, EC, EGCG, and ECG as previously described [34]. GTE (0.3 g) was weighed in a centrifuge tube to which 15 mL of absolute ethanol was added. After vortexing for 1 min to solubilize the extract, the mixture was sonicated (70W, 40 kHz) for 15 min at room temperature and centrifuged for 5 min at 1800× g (Eppendorf Centrifuge 5810 R, Hamburg, Germany). Various dilutions of the ethanolic extract were prepared in a 1% (v/v) formic acid in water solution and filtered through a 0.45 µm syringe filter into HPLC injection vials. The HPLC conditions used for this analysis were as we previously described (Nain et al., 2021). The catechins were identified and quantified by comparing retention times and peak areas to those of a pure standard mixture of EGC, EC, ECGC, and ECG, prepared at different concentrations to obtain calibration curves. The analysis was repeated four times for each extract, and the results are expressed in mg/g of extract.

2.3. Oxidative Stability Test

The antioxidant effects of GTE1 and GTE2 were evaluated in a DHA-rich oil under accelerated ageing at 30°C during a storage period of 21 days. GTE1 was added to the oil at a concentration of 1000 ppm (250 ppm of major catechins), which was the most effective concentration in our previous study (Nain et al., 2021), and GTE2 was incorporated into the oil at a concentration of 450 ppm to ensure a total catechin content of 250 ppm as in GTE1-

supplemented samples. To evaluate the contribution of major catechins to the antioxidant effect of extracts, oil samples were also supplemented with reconstituted mixtures of catechins in the same proportions present in GTE1 (CatMix1) and GTE2 (CatMix2). The effect of individual catechins (EGC, EC, EGCG and ECG) were also evaluated at a concentration of 250 ppm. The GTEs and catechins were added to the oil as an ethanolic solution. Each GTE powder was precisely weighed in a centrifuge tube and solubilized in absolute ethanol by vortexing for 1 min and sonicating for 15 min. The quantity of ethanol used did not exceed 4% weight of the final oil mixture. As for the reconstituted catechins mixtures, EGC, EC, EGCG, and ECG powders were each dissolved in ethanol to obtain solutions with a concentration of 5 mg/mL. Combinations of these catechins solutions were then prepared to obtain catechins profiles similar to GTE1 and GTE2 (Table 1). The oil and antioxidant blends were mixed by stirring with a glass rod for 3 min and flushed with nitrogen for 10 min to evaporate the ethanol. Control samples were prepared in the same way as the treatments except without any added catechins. The oil samples were then distributed (10 g) in amber bottles (30 mL, Ø 2.7 cm), and the opened bottles were stored in an oven at 30°C for 21 days with a sampling interval of 7 days (day 0, 7, 14, and 21). All samples were prepared in triplicate. The oxidative status of oils was determined on the sampling day by measuring the PV and para-anisidine value (*p-AV*), and the remaining oil was flushed with argon and stored at −20°C for subsequent analyses of catechins and fatty acid contents.

Table 1. Mean content of epistructured catechins in an EGC-rich green tea extract (GTE1) and an EGCG-rich green tea extract (GTE2).

	GTE1		GTE2	
Catechins	**Absolute (mg/g Extract)**	**Relative (% Total Catechins)**	**Absolute (mg/g Extract)**	**Relative (% Total Catechins)**
Epigallocatechin (EGC)	147.2 ± 1.4	58.0	99.2 ± 4.1	17.7
Epicatechin (EC)	20.0 ± 0.9	7.9	54.7 ± 2.8	9.8
Epigallocatechin gallate (EGCG)	76.4 ± 7.9	30.1	338.8 ± 25.5	60.6
Epicatechin gallate (ECG)	10.0 ± 3.4	3.9	66.2 ± 5.2	11.8
Total	253.6 ± 11.2	100.0	558.9 ± 37.7	100.0

Results are expressed as mean ± standard deviation of four repeated analyses of each green tea extract.

2.4. Evaluation of Oxidative Status

The PV, which measures primary oxidation products, was determined by iodometric titration according to the AOAC official method 965.33 (AOAC, 2000). The *p-AV* was measured according to the AOCS official method Cd 18–19 (AOCS, 1998). *p-AV* is a measurement of secondary oxidation products generated during the decomposition of hydroperoxides, primarily 2-alkenals and 2,4-alkadienals.

2.5. Quantification of the Catechins Content in Oil Samples

Catechins were extracted from oil samples supplemented with GTE and quantified as previously described (Nain et al., 2021). To extract catechins, 0.5 g of oil sample was mixed with 1 mL of 90% ethanol in a 1.5 mL Eppendorf tube, vortexed for 1 min and centrifuged for 5 min at 20,000× *g*. The ethanolic phase (supernatant) was transferred into a 2 mL Eppendorf tube, and a second extraction of the remaining oil phase was carried out. The supernatants from the two extractions were combined and the ethanol was evaporated under vacuum in a SpeedVac concentrator Savant SPD121P (ThermoFisher Scientific, Asheville, NC, USA). The residue was solubilized in 1 mL or 2 mL of 1% (*v*/*v*) formic acid in water depending on the expected concentration of catechins and filtered through a 0.45 µL syringe filter into an HPLC vial. The samples were then analyzed using the reverse-phase HPLC conditions described earlier. The results are expressed in µg/g of oil.

2.6. Identification of Catechins Degradation Products

To identify catechins degradation products detected on the reverse-phase HPLC chromatograms of oil treatments, the catechins extracts from these treatments were analyzed by high-performance liquid chromatography-electrospray ionization-high resolution-mass spectrometry (HPLC-ESI-HR-MS). The HPLC-ESI-HR-MS system consisted of an HPLC equipped with a degasser, a

pump, an autosampler, and a PDA detector, and this was connected to a Thermo Instruments ESI-MS system (LTQ-Orbitrap-XL mass spectrometer). The HPLC column used was a Phenomenex Kinetex 2.6µm PFP 100A column (150 mm x 4.6 mm, and the HPLC conditions were the same as previously described (Nain et al., 2021). The MS was operated both in the positive ion mode in a mass range of 100–2000 m/z and in a negative ion mode. In positive ion mode, the MS conditions were the following: sheath gas flow rate: 10 au (arbitrary unit), auxiliary gas flow rate: 5 au, sweep gas flow rate: 5 au, capillary temperature: 265°C, capillary voltage: 15 V, tube lens: 90 V and I spray voltage: 3 kV. In negative ion mode, the MS conditions were the following: sheath gas flow rate: 10 au (arbitrary unit), auxiliary gas flow rate: 10 au, sweep gas flow rate: 10 au, capillary temperature: 275°C, capillary voltage: −35 V, tube lens: −110 V and I spray voltage: 5 kV.

2.7. Quantification of Fatty Acids

The fatty acid composition of the samples was analyzed at the start of the storage test (day 0) and the end of the test (day 21) using the method previously described by Mellery et al. (2016). The fatty acids in oil were first converted to fatty acid methyl esters (FAME) via a 2-step methylation with KOH and HCl with nonadecanoic acid (C19: 0, Sigma-Aldrich) used as an internal standard. The FAMEs were then extracted in hexane and separated by gas chromatography. Fatty acids in the samples were identified and quantified by comparing the retention times and peak areas with a standard mixture of 43 pure FAME standards (Larodan, Solna, Sweden). Chromatograms were processed using Chromeleon 7 (ThermoFisher Scientific, Waltham, MA, USA), and the results are expressed as mg/g of oil.

2.8. Statistical Analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation. The data for the oxidative status and catechins degradation were subjected to a one-way or

two-way analysis of variance (ANOVA), and means were compared between treatments using the Tukey–Kramer HSD (honestly significant difference) test. Differences in means between the fatty acid contents on day 0 and day 21 for each treatment was achieved by the Student's *t*-test. All statistical analyses were performed using JMP Pro version 16 (SAS institute inc., Cary, NC, USA), and the differences between the means were considered significant at $p \leq 0.05$.

3. Results

3.1. Catechins Content of Green Tea Extracts

The catechins contents of GTE1 and GTE2 analyzed by HPLC are shown in Table 1. The catechins of interest were the epistructured catechins, generally considered the major green tea catechins. The total content of these catechins in GTE1 represented 25.3% (w/w) of the extract, with the most abundant catechins being EGC accounting for 58% of the quantified catechins, followed by EGCG (30%), EC (7.9%), and ECG (3.9%). In contrast, GTE2 contained 55.8 % (w/w) of total catechins in the following order of abundance EGCG (60.6%) > EGC (17.7%) > ECG (11.8%) > EC (9.8%). Therefore, the total catechins content of GTE2 was 2.2 times higher than that of GTE1. Apart from the quantified catechins, other peaks were visible in the GTE chromatograms, identified as gallic acid (1), gallocatechin (2), caffeine (3), and gallocatechin gallate (4) (Figure 2).

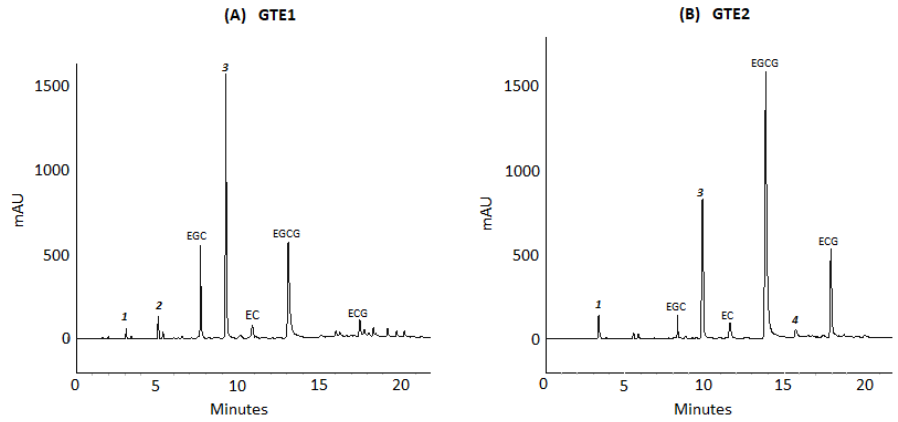

Figure 2. Chromatographic separation of catechins from two green tea extracts, GTE1 (**A**) and GTE2 (**B**). A binary solvent gradient system with solvent **A** (1% formic acid in water) and solvent **B** (1% formic acid in acetonitrile) was used. Identified and quantified compounds: EGC = epigallocatechin, EC = epicatechin, EGCG = epigallocatechin gallate, ECG = epicatechin gallate. Other compounds 1= gallic acid, 2 = gallocatechin, 3 = caffeine, 4 = gallocatechin gallate.

3.2. Oxidative Status of Oil during Storage

3.2.1. Peroxide Value

The PV of the DHA-rich oil was 3 meq O_2/kg at the start of the storage test, and the increase rate during the 21 days of storage at 30°C varied with respect to the treatment. As shown in Figure 3, incorporating GTE1 and GTE2 into the oil slowed the formation of hydroperoxides. The PV of the control sample increased by 95-fold in 21 days, which was significantly different ($p < 0.0001$) from the 9-fold and 12-fold increases observed for treatments with GTE1 and GTE2, respectively. No significant difference was detected between the PV of oils supplemented with GTE1 and GTE2 from day 0 to day 14 of storage, but by day 21, the GTE1-supplemented oil had a significantly lower ($p = 0.02$) PV than the oil supplemented with GTE2. This indicates a more persistent antioxidant activity for GTE1 than for GTE2.

To understand the contribution of the major catechins to the antioxidant activity of GTE, the effect of GTE1 and GTE2 on PV was compared to the reconstituted catechins mixtures (Figure 3). There was no significant difference between the PV of treatments with GTE1 and its reconstituted catechins mixture, CatMix1, throughout the storage period, although a slightly lower PV was recorded for GTE1-supplemented samples (PV of 25.5 ± 2.3 meq O_2/ kg oil) compared to CatMix1 (30.9 ± 2.3 meq O_2/kg) after 21 days of storage. On the other hand, the PV of GTE2-supplemented oil (37.5 ± 2.4 meq O_2/ kg oil) was significantly lower ($p < 0.0001$) than that of oil supplemented with CatMix2 (59.1 ± 2.4 meq O_2/ kg oil) after 21 days of storage, with no significant differences observed from day 0 to day 14.

The effect of individual catechins on the PV of the DHA-rich oil was also tested, and the results are also presented in Figure 3. The PVs of oils supplemented with individual catechins were significantly lower than the control. The PV increase was significantly higher for EC250 than for the other catechins-supplemented samples throughout the storage period, whereas EGC250 resulted in a significantly lower PV than the other treatments. EGC250 had, thus, a more potent effect on preventing PV increase than EGCG250, which is consistent with the observation that GTE1 rich in EGC (58% of major catechins) is also more effective than GTE2 rich in EGCG (60% of major catechins).

Comparing the change in PV among the samples rich in EGC and those rich in EGCG, we observed no significant differences among the PV of treatments with GTE1, CatMix1, and EGC250. Accordingly, there was no significant difference between the PV of oils supplemented with CatMix2 and EGCG250. Therefore, the most abundant individual catechins in each of these treatments appears to be responsible for the majority of the antioxidant activity.

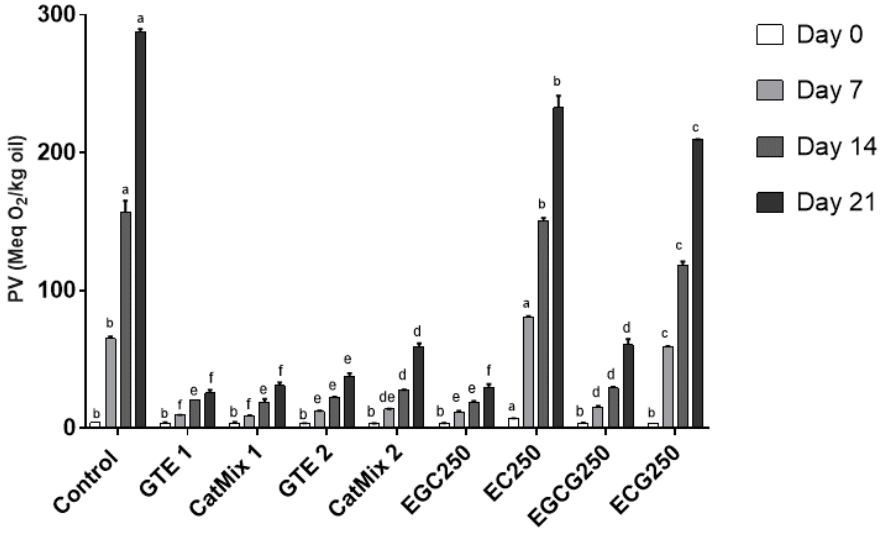

Figure 3. Effect of green tea extracts (GTEs) and catechins on the peroxide value (PV) of DHA-rich oil during a 21-day storage at 30°C. Results are expressed as mean ± standard deviation, n = 3. Control = DHA-rich oil, GTE1 = DHA-rich oil + 1000 ppm GTE1, CatMix1 = DHA-rich oil + reconstituted catechin mixture of GTE1, GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix2 = DHA-rich oil + reconstituted catechin mixture of GTE2, EGC250, EC250, EGCG250 and ECG250 = DHA-rich oil + 250 ppm of EGC or EC or EGCG or ECG, respectively. Significant differences ($p \leq 0.05$) are represented by different lower-case letters (a–f) among treatments at a specific storage time.

3.2.2. *p*-Anisidine Value

The *p-AV* is a measure of the accumulation of secondary oxidation products, such as aldehydes, that arise from the breakdown of hydroperoxides. As shown in Table 2, the *p-AV* of the control sample increased significantly from 2.8 ± 0.6 to 100.6 ± 8.2 over the 21-day storage period. The incorporation of the GTEs and catechins slowed the formation of secondary products. A decrease or no significant change was observed in the *p-AV* of samples containing GTE1, CatMix1, GTE2, CatMix2, EGC250, and EGCG250 during the storage, whereas samples supplemented with EC250 and ECG250 showed a significant increase in *p-AV*. The *p-AV* of the ECG250 condition remained lower than the control throughout the storage

period, whereas the difference was significant only on days 7 and 14 for the EC250 condition. These results are in accordance with the PV results, as samples that recorded higher PVs also revealed an increase in the *p-AV*.

The overall effect of treatments in improving the oxidative status of oil during storage was in the decreasing order GTE1 ≈ EGC250 ≈ CatMix1 > GTE2 > EGCG250 ≈ CatMix2 > ECG250 >EC250.

Table 2. Changes in the *p*-anisidine value (*p*-AV) in DHA-rich oil supplemented or not with green tea extracts or catechins during storage at 30°C for 21 days.

	***p*-AV**			
Treatment	**Day 0**	**Day 7**	**Day 14**	**Day 21**
Control	2.8 ± 0.6^{aD}	15.9 ± 2.8^{aC}	47.9 ± 1.5^{aB}	100.6 ± 8.2^{aA}
GTE1	0.0 ± 0.0^{bA}	0.0 ± 0.0^{cA}	0.3 ± 0.5^{deA}	0.32 ± 0.3^{cA}
CatMix1	0.0 ± 0.0^{bA}	0.0 ± 0.0^{cA}	0.0 ± 0.0^{eA}	0.0 ± 0.0^{cA}
GTE2	3.3 ± 0.5^{aA}	0.0 ± 0.0^{cC}	$1.2 \pm 0{,}8^{deB}$	0.0 ± 0.0^{cC}
CatMix2	2.9 ± 0.78^{aA}	1.1 ± 0.9^{cAB}	2.1 ± 1.0^{deA}	0.0 ± 0.0^{cB}
EGC250	3.2 ± 0.5^{aA}	1.3 ± 1.2^{cB}	$3.6 \pm 0{,}5^{dA}$	0.0 ± 0.0^{cC}
EC250	3.5 ± 0.1^{aD}	13.2 ± 3.9^{abC}	28.0 ± 1.8^{bB}	93.1 ± 6.4^{aA}
EGCG250	2.6 ± 0.4^{aA}	1.2 ± 1.1^{cA}	$3.2 \pm 2{,}2^{deA}$	0.0 ± 0.0^{cB}
ECG250	3.4 ± 0.3^{aC}	8.9 ± 1.7^{bC}	$15.3 \pm 1{,}1^{cB}$	69.6 ± 4.2^{bA}

Results are expressed as mean ± standard deviation, n = 3. Significant differences ($p \leq 0.05$) within a column are represented by different lower-case letters (a–e) and by different uppercase letters (A-D) within a row. Control = DHA-rich oil, GTE1 = DHA-rich oil + 1000 ppm GTE1, CatMix1 = DHA-rich oil + reconstituted catechin mixture of GTE1, GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix2 = DHA-rich oil + reconstituted catechin mixture of GTE2, EGC250, EC250, EGCG250 and ECG250 = DHA-rich oil + 250 ppm of EGC or EC or EGCG or ECG, respectively.

3.3. Changes in Fatty Acid Content of Oil during Storage

The DHA-rich oil used in this study had a PUFA content of 906.8 mg/g, with the major PUFAs being DHA (C22:6c4,c7,c10,c13,c16,c19) at 661.5 mg/g and EPA (C20:5c5,c8,c11,c14,c17) at 137.4 mg/g. The sum of monounsaturated fatty acids in the oil was 7.9 mg/g, while saturated fatty acids were 1.2 mg/g of oil.

Figure 4 shows the effect of the different catechins treatments on EPA, DHA, and total PUFA content of the DHA-rich oil after 21 days of storage. The control oil showed a significant decrease in EPA (p = 0.0023), DHA (p = 0.0019), and total PUFA (p = 0.0018), with a 12.4%, 13.6%, and 13.2% reduction, respectively. The supplementation of DHA-rich oil with GTE1, GTE2, and their respective catechins mixtures prevented any loss in fatty acids during the storage period, which was also true for EGC250 and EGCG250 treatments. A non-significant reduction in EPA, DHA, and total PUFA, of 4%, 4.9%, and 4.6%, respectively, was observed for the oil with ECG250, whereas the oil with EC250 recorded a significant decrease in these fatty acids, with a reduction of 5% for EPA (p = 0.0065), 5.4% for DHA (p = 0.0065), and 5.2% for total PUFA (p = 0.0063). These results are consistent with the changes observed in the *p-AV* of the oil samples.

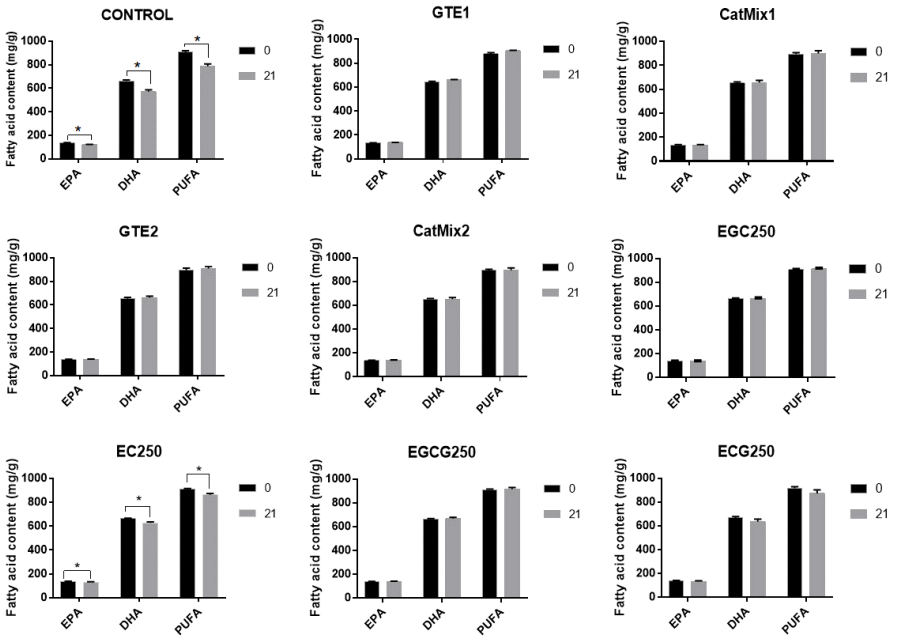

Figure 4. Effect of green tea extracts (GTE) and catechins on the eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total polyunsaturated fatty acid (PUFA) content of a DHA-rich oil after 21 days of storage at 30°C. Results are expressed as mean ± standard deviation, n = 3. A significant difference between pairs is indicated by a *. Control = DHA-rich oil, GTE1 = DHA-rich oil + 1000 ppm GTE1, GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix1 = DHA-rich oil + reconstituted catechins mixture of GTE1, CatMix2 = DHA-rich oil + reconstituted catechins mixture of GTE2, EGC250 = DHA-rich oil + 250 ppm EGC, EC250 = DHA-rich oil + 250 ppm EC, EGCG250 = DHA-rich oil + 250 ppm EGCG, ECG250 = DHA-rich oil + 250 ppm ECG.

3.4. Depletion of Catechins

All treatments were prepared to contain about 250 µg/g (250 ppm) of total catechins, equivalent to 1000 ppm of GTE1 and 450 ppm of GTE2. The activity of the catechins in the oil started immediately upon addition, as their depletion was already evident in extracts from day 0 oil samples (Table S1). The level of depletion of these catechins during the storage period varied with respect to treatment (Figure 5A–C). After 7 days of storage, the oil supplemented with GTE1 had the highest residual total catechins content of 186 µg/g (74.5 % of initial content). The residual total catechins in the other treatments was as follows: EGC250 with 150 µg/g (60.1% initial content), GTE2 with 147.8 µg/g (59.1% initial content), EC250 with 80.9 µg/g (32.3% initial content), EGCG250 with 74.5 µg/g (29.8% initial content), and ECG250 with 16.25 µg/g (6.5% initial content) (Figure 5A). By the end of the 21-day storage period, the catechins in EC250, EGCG250, and ECG250 treatments were almost completely depleted, while GTE1, EGC250, and GTE2 samples still had 27.2%, 14.1%, and 10.1% residual catechins, respectively. A slower loss in catechins was, therefore, observed for the GTE1 supplemented samples, while the fastest depletion was recorded for the ECG250 condition.

Figure 5B shows the residual catechins in oils supplemented with GTE and CatMix. The depletion rate of catechins was higher in CatMix samples compared to their corresponding GTE counterparts, suggesting an effect of the other polyphenolic compounds and caffeine present in the GTEs. After the first 7 days of storage, the catechins remaining in the CatMix1-supplemented oil were at 56.1% of the initial content, which was significantly different (p = 0.0053) from the 74.5% residual content in the GTE1 condition. After 21 days, a significantly lower value (p = 0.0016) was still evident for CatMix1. As for CatMix2 and GTE2 samples, total residual catechins contents of 50.4% and 59.1%, respectively, were recorded after 7 days of storage, and a significant difference

(p = 0.0265) was observed after 21 days of storage with a residual content of 6.4% and 10.1%, respectively.

The level of depletion of the individual catechins in oil samples supplemented with GTE1, CatMix1, GTE2, and CatMix2 are shown in Figure 5C. The least depleted catechins in these treatments was EC, with the remaining content after 21 days being 13.0 ± 0.6 μg/g (64.9% of initial content) for GTE1, 10.4 ± 0.5 μg/g (52.2% of initial content) for CatMix1, 10.1 ± 0.4 μg/g (42.1% of initial content) for GTE2, and 9.9 ± 0.3 μg/g (41% of initial content) for CatMix2. On the flip side, EGCG was the fastest depleted compound, with almost none left in GTE2 (1.5%) and CatMix2 (0.8%) after 21 days, while 16.6% and 5.7% of the initial content was left in treatments with GTE1 and CatMix1, respectively. The absolute concentrations of the catechins in the oil during storage are presented as supplementary data (Table S1). The depletion of the individual catechins in the GTE and CatMix samples was in the decreasing order of EGCG > EGC > ECG > EC, irrespective of the initial individual catechins concentrations in the samples.

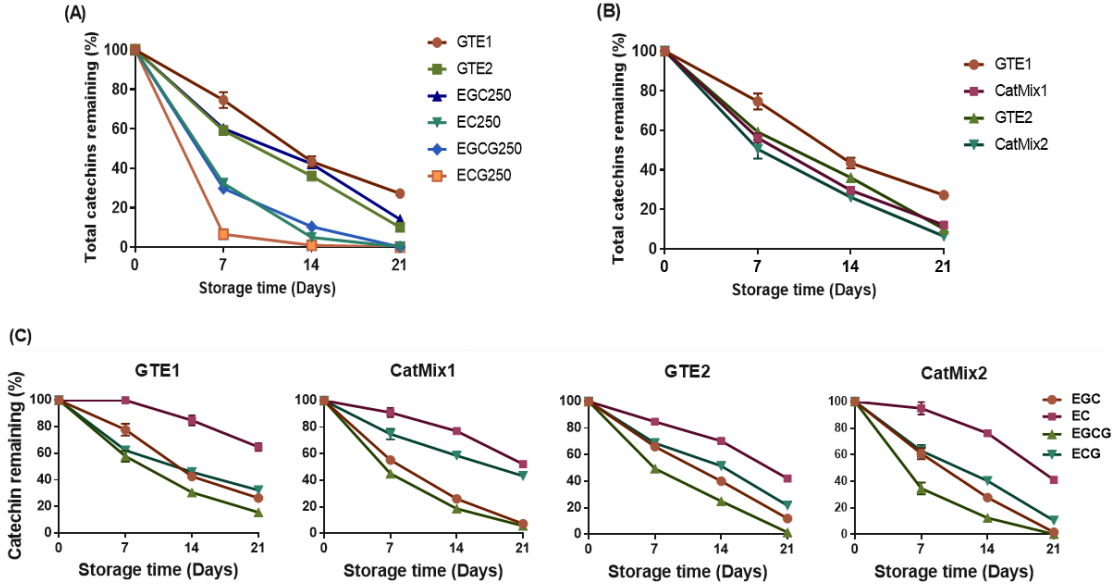

Figure 5. Kinetics of catechins depletion in DHA-rich oil supplemented with green tea extracts, catechins mixtures or individual catechins. (**A**) Depletion of total catechins from GTE-supplemented and catechins-supplemented oil samples; (**B**) comparing depletion of total catechins in GTE and CatMix-supplemented oil samples; (**C**) depletion of individual catechins present in oil supplemented with GTEs and their reconstituted mixtures. GTE1 = DHA-rich oil + 1000 ppm GTE1, GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix1 = DHA-rich oil + reconstituted catechins mixture of GTE1, CatMix2 = DHA-rich oil + reconstituted catechins mixture of GTE2, EGC250 = DHA-rich oil + 250 ppm EGC, EC250 = DHA-rich oil + 250 ppm EC, EGCG250 = DHA-rich oil + 250 ppm EGCG, ECG250 = DHA-rich oil + 250 ppm ECG.

3.5. Degradation Product of Epigallocatechin Gallate or an Intermediate Product in Its Antioxidant Activity?

The HPLC chromatograms of catechins extracted from the oil treatments supplemented with GTE2, CatMix2, and EGCG250 revealed the presence of an unexpected peak that elutes at a retention time of 7.65 min (Figure 6), but this peak was not detected in the other treatments (Figure S1). Since this peak was not present in the chromatogram of GTE2 (Figure 2B) nor the chromatograms of EGC250, EC250, and ECG250 oil samples, we postulated that this compound was a degradation product of EGCG, which resulted from its reaction in the oil. Thus, we quantified the new product as an EGCG equivalent (Table 3). This product was detected in small amounts in day 0 oil samples and increased after seven days at 30°C before showing a decreasing trend. The product was more abundant in the oil with EGCG250, which could be explained by the higher depletion level of EGCG in this treatment (Figure 5). The depletion of this new product was slower in oil samples supplemented with GTE2 compared to the EGCG250 and CatMix2 treatments.

Analysis by HPLC-ESI-HR-MS to identify this compound was futile as no signal was observed on MS both in the positive and negative electron ionization modes. The absence of a signal on MS could be due to the low concentration of this product in our samples or poor ionization characteristics.

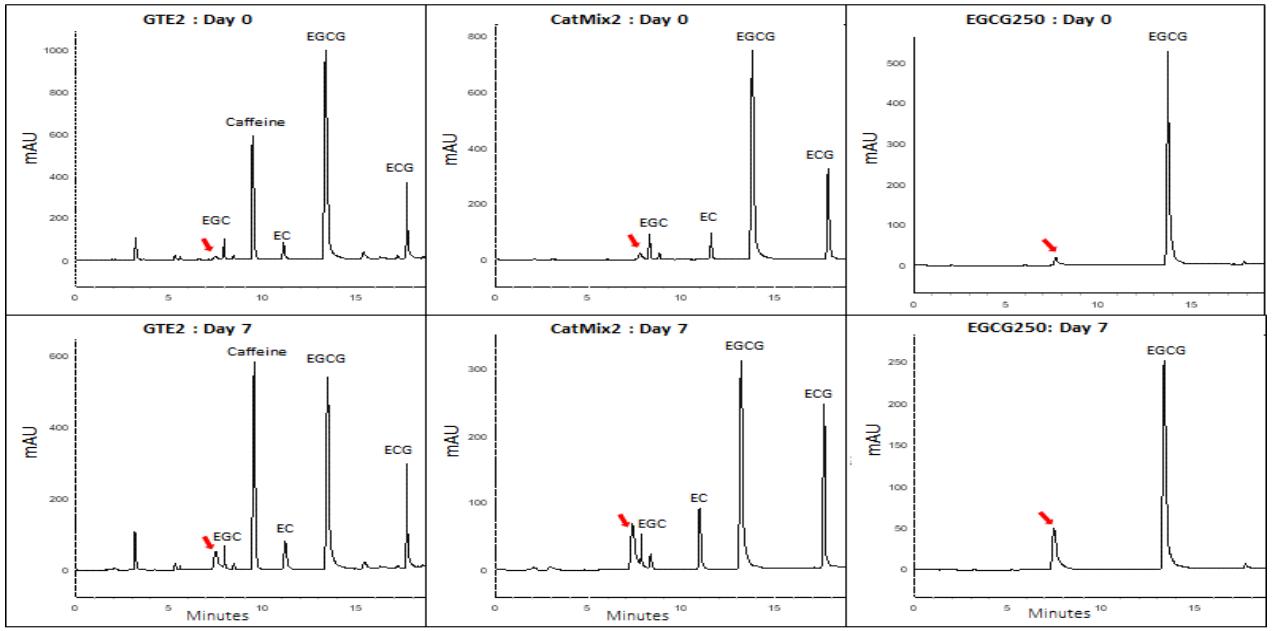

Figure 6. HPLC-UV chromatograms of a degradation product formed in catechins-supplemented DHA-rich oils during an accelerated storage test at 30°C. GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix2 = DHA-rich oil + reconstituted catechins mixture of GTE2, EGCG250 = DHA-rich oil + 250 ppm EGCG. The extracts from EGCG250-supplemented oil injected on HPLC were two times more diluted than the GTE2 and CatMix2 samples to avoid peak saturation. The degradation product is indicated by the arrow.

Table 3. Changes in the content of an EGCG degradation product during the storage of a DHA-rich oil supplemented with a green tea extract rich in EGCG (GTE2), a corresponding catechins mixture (CatMix2) or pure EGCG (EGCG250) at 30°C for 21 days.

Storage time (Days)	Concentration (µg EGCG Equivalent /g oil)		
	GTE2	**CatMix2**	**EGCG250**
0	3.2 ± 0.2^{c}	4.5 ± 0.7^{bc}	10.7 ± 0.6^{b} *
7	11.7 ± 0.8^{a}	13.1 ± 1.8^{a}	27.0 ± 3.1^{a} *
14	9.3 ± 1.0^{b}	7.2 ± 1.2^{b}	14.3 ± 3.7^{b} *
21	8.1 ± 1.0^{b} *	2.1 ± 0.3^{c}	2.1 ± 0.2^{c}

Values are mean ± SD (n = 3). Significant differences ($p \leq 0.05$) within columns are represented by different lower-case letters (a-c). * indicates a significant difference ($p \leq 0.05$) between treatments at a specific storage time (within a row). GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix2 = DHA-rich oil + reconstituted catechin mixture of GTE2, EGCG250 = DHA-rich oil + 250 ppm EGCG. The day 0 and 7 extracts from the EGCG250-supplemented oil injected on HPLC were two times more diluted than GTE2 and CatMix2.

4. Discussion

The antioxidant activity of catechins (flavan-3-ols) and GTEs has been demonstrated in various food matrices, including pastry, meat products, table spreads, food emulsions, and bulk oils (Boroski et al., 2020; Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009; Shen et al., 2021; Tian and Huang, 2019). The effectiveness of catechins as antioxidants is attributed to their ability to donate hydrogen atoms to free radicals, thus acting as free radical scavengers (Senanayake, 2013). Catechins also slow oxidation by acting as metal ion chelators, binding metal ions such as iron and copper, which otherwise initiate the oxidation process (Michalak, 2006). In our previous study, our results indicate that GTE was more effective in improving the oxidative stability of a DHA-rich oil than α-tocopherol (Nain et al., 2021). Investigating the activity of GTE1 and GTE2 in the present study confirmed the ability of these two GTEs to delay the oxidation of a DHA-rich oil. However, GTE1 resulted in a lower PV in the oil compared to GTE2

after 21 days at 30°C. Since the oil samples were prepared to contain a similar total catechins content, the difference in the protective effect of GTE1 and GTE2 could be attributed to the difference in their catechins profiles. In a related study, Toschi et al. (2000) evaluated the antioxidant ability of three GTEs in a refined peanut oil subjected to forced oxidation at 98°C and concluded that the two GTEs with higher levels of EGCG and ECG were more effective antioxidants than the less concentrated extract. However, the concentration of total catechins added in the peanut oil samples was not similar at the start of the experiment, thus suggesting that the differences observed may be explained by the difference in concentration of catechins rather than the catechins composition of the GTEs, as is the case in the present study.

The higher antioxidant activity observed for GTE1 could be associated with its high EGC content (58% of the total catechins), given that the EGC250 samples recorded the lowest PV compared to oils supplemented with the other individual catechins. Indeed, the ability of the individual catechins to slow oxidation was in the order EGC250 > EGCG250 > ECG250 > EC250. This result is similar to that reported by Chen and Chan (1996), who reported an antioxidant activity of catechins in the decreasing order EGC > EGCG > EC > ECG when incorporated at a concentration of 200 ppm in canola oil heated at 95°C. Moreover, in line with our results, Huang and Frankel (1997) investigated the ability of catechins to inhibit hydroperoxide formation in corn oil oxidized at 50°C and observed the effectiveness in the order EGC ≈ EGCG ≈ ECG > EC. Contrary to our results, some studies have reported a higher antioxidant activity for EGCG compared to EGC. For instance, the antioxidant activity of catechins was in the order EGCG ≈ ECG > EGC >> EC in ground mackerel cooked at 75°C and stored for 7 days at 4°C (He and Shahidi, 1997). Lee et al. (2014) investigated the antioxidant activity of pure catechins by their ability to scavenge 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and reported

antioxidant activity in the order EGCG ≥ GCG ≥ ECG > EGC ≥ GC ≥ EC ≥ C. The free radical scavenging ability of catechins is partly related to their reduction potential, which indicates the ease with which the catechins will donate a hydrogen atom to a free radical and the stability of the resulting antioxidant radical (Higdon and Frei, 2003). EGC and EGCG have the lowest reduction potentials (0.43V) compared to ECG (0.55V) and EC (0.57V), suggesting they are superior electron donors (Higdon and Frei, 2003). In addition, the antioxidant activity of catechins is also associated with the presence of the trihydroxyphenyl B-ring and/or a galloyl group on the C-ring, with the trihydroxyphenyl B-ring acting as the principal site of antioxidant reactions in EGCG and EGC (Valcic et al., 2000).

Comparing the effect of GTEs with their reconstituted catechins mixtures, we observed no significant difference in the ability to delay hydroperoxide formation between GTE1 and CatMix1. Therefore, the antioxidant activity of GTE1 could mostly be attributed to the epistructured catechins. On the other hand, GTE2 acted as a better antioxidant than CatMix2 after 21 days at 30°C, indicating that other compounds present in the extract (other polyphenols and caffeine) exhibited antioxidant activity or acted in synergy with the major catechins. Similarly, a previous study reported a higher antioxidant activity of a crude GTE than its reconstituted mixture of individual catechins in a β-carotene linoleate model system (Amarowicz and Shahidi, 1995). Nonetheless, the crude GTE with a total catechins content of 300 mg/g made up of 17.2% (EC), 39.2% (EGC), 7.6% (ECG), and 36% (EGCG) was different from the GTEs used in this study, which contained higher levels of either EGC (GTE1) or EGCG (GTE2). Gallocatechin gallate, present in GTE2, might contribute to its antioxidant effectiveness since it has been reported to possess a similar antioxidant activity to EGCG when subjected to an anti-2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay, whereas gallocatechin, was shown to be less potent as an antioxidant (Xu et al., 2004). In another study, the results of online DPPH radical-

scavenging activity tests of a mixed catechins standard showed that the antioxidant activity of catechins decreased in the order EGC > gallic acid > EGCG > EC > ECG > catechin, while caffeine had almost no DPPH-scavenging activity (Zhao et al., 2014). The presence of gallic acid in GTE2 could thus also contribute to its antioxidant activity and an enhanced protective effect compared to CatMix2. Caffeine though inert to DPPH radicals, is effective in scavenging hydroxyl radicals and has also been shown to inhibit lipid peroxidation (Devasagayam et al., 1996; León-Carmona and Galano, 2011). Thus, the presence of caffeine in the extracts might also enhance the antioxidant activity.

The incorporation of GTEs in the DHA-rich oil used in the current study prevented the degradation of fatty acids, hence no change in DHA and EPA content was observed in GTE-supplemented oils during the 21-day storage, whereas a 12 to 13% decrease was observed in the control sample. The fatty acid content of the other treatments remained relatively stable, apart from EC250 and ECG250 treatments, which recorded a decrease in EPA and DHA content. Yin et al. (2021)observed no change in the DHA content of a mixed antioxidant-supplemented microalgal DHA-rich oil (44.37 % DHA) subjected to accelerated oxidation at 60°C for 10 days, but a decrease of 4.35% was observed after 20 days of oxidation. In another recent study, the DHA content of purified algal oil stored for two days at 60°C significantly decreased from 35.3% to 31.6%, which equals a 10.5% decrease (Wen et al., 2022). In the present study, a decrease in fatty acid was only observed for treatments with PVs > 200 meq O_2/ kg oil and *p-AV*s > 50, which may imply that a change in fatty acid content is only evident when oxidation is at a very advanced stage.

As the catechins protected the oil from oxidation, they were depleted in the process. The rate of depletion of the total catechins varied among treatments in the decreasing order ECG250 > EC250 ≈ EGCG250 > GTE2 > EGC250 > GTE1. As free radical scavengers, catechins donate hydrogen from the hydroxyl group, thus breaking

the auto-oxidation chain. The resulting unpaired electron in the catechins is delocalized within the ring structure, resulting in a relatively unreactive free radical, which forms an o-quinone, followed by reactions such as oligomerization and intramolecular rearrangement (Tan et al., 2020). Catechins losses could be due to the combined effect of oxidation, degradation, epimerization, and polymerization during storage, with contributing factors being temperature and oxygen availability (Ananingsih et al., 2013). The depletion of the individual catechins in the GTE and CatMix-supplemented oils was observed in the order EGCG > EGC > ECG > EC. Catechins with higher antioxidant activity (EGCG and EGC) were depleted faster, and this could be linked to their lower reduction potential, which makes hydrogen abstraction easier. Wang and Zhou (2004) incorporated GTE in bread and observed losses in catechins during the breadmaking process in the order EGC > EGCG > ECG > EC. The catechins depletion rate was higher for the CatMix samples than for the GTE-supplemented samples, which might indicate that other polyphenolic compounds and caffeine present in the extracts play a protective role on the catechins. It is worth noting that the pattern of depletion of individual catechins, when added singly to the oil, differed from when added as a mixture.

Although all the catechins recorded losses during storage, a product of degradation was only detected for EGCG, which could indicate that the HPLC method used was not suitable for detecting the degradation products of the other catechins or the concentrations were too low to be detected. The depletion of EGCG resulted in a new peak that eluted at 7.65 min, just prior to the elution of EGC on the chromatogram. This unidentified product was more polar than EGCG, given that on reverse-phase HPLC, more polar compounds are eluted first. Furthermore, since this molecule eluted very close to EGC, it is reasonable to assume that it is similar in structure or polarity to EGC, possibly derived from an EGCG by loss of its gallic acid group. In a previous study, Dai et al. (2017)

observed a product ion of m/z 305 after thermal treatment at 100°C of a solution containing EGCG. They concluded that this product resulted from a loss of a gallic acyl group from EGCG. Kondo et al. (1999) suggested that the scavenging effect of EGCG on peroxyl radicals in liposomal and aqueous systems resulted in the conversion of EGCG to an anthocyanin-like compound with cleavage of the galloyl moiety. Contrarily, all three oxidation products of EGCG derived from its reaction with peroxyl radicals, which were identified and isolated by Valcic et al. (1999) resulted from changes in the B-ring of EGCG rather than the galloyl moiety. The EGCG product observed in the present study decreased over time, which may suggest that it is an intermediate breakdown product or a product that also possesses antioxidant activity.

5. Conclusion

This study confirmed the effect of GTEs and catechins as natural antioxidants that can retard the oxidation of highly unsaturated oils. The results of this study also indicate that the catechins profile of GTEs has an impact on their antioxidant effectiveness in DHA-rich oil. Both GTE1 and GTE2 enhanced the oxidative stability of the respective supplemented DHA-rich oil. However, GTE1, containing a higher proportion of EGC, was more effective in delaying oxidation than GTE2 rich in EGCG. The epistructured catechins (EGC, EC, EGCG, and ECG) were shown to be responsible for the antioxidant activity of GTE1, as no significant difference was observed between the antioxidant activity of GTE1 and CatMix1 during the storage period. In contrast, the effect of GTE2 was more prominent than that of CatMix2, suggesting that apart from the epistructured catechins, other compounds in GTE2 contributed to its antioxidant activity. Among individual catechins, EGC demonstrated a stronger antioxidant activity in the DHA-rich oil. Overall, the antioxidant activity of the GTEs and catechins were observed in the decreasing order GTE1≈ EGC250 > GTE2 > EGCG250 > ECG250 > EC250. A significant reduction in fatty acid content was observed for the control and EC250-supplemented

oils, and the catechins were most stable in GTE1-supplemented oil. These findings indicate that the EGC-rich GTE is a more potent antioxidant in DHA-rich oil than the EGCG-rich GTE.

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Supplementary materials

Table S1: Changes in the concentration (μg/g) of catechins in DHA-rich oil supplemented with green tea extracts, catechins mixtures or individual catechins during storage at 30°C for 21 days.

		The concentration of catechins (μg/g oil)					
Treatment		**Storage day**	**EGC**	**EC**	**EGCG**	**ECG**	**Total**
GTE1	**Added**		**147.2**	**20**	**76.4**	**10**	**253.6**
	Extracted	0	146.2 ± 7.6	20.5 ± 2.0	57.9 ± 4.2	6.6 ± 0.5	231.1 ± 13.4
		7	114.8 ± 6.1	20.6 ± 0.6	44.3 ± 3.1	6.3 ± 0.2	186.0 ± 9.4
		14	62.9 ± 4.1	17.0 ± 0.8	23.3 ± 1.4	4.6 ± 0.1	107.9 ± 6.0
		21	39.3 ± 1.1	13.0 ± 0.6	12.6 ± 0.6	3.2 ± 0.1	68.1 ± 2.4
CatMix1	**Added**		**145.8**	**20**	**75**	**10**	**250.8**
	Extracted	0	107.6 ± 6.2	17.7 ± 1.0	47.9 ± 2.2	7.6 ± 0.3	180.8 ± 9.6
		7	80.1 ± 3.5	18.2 ± 0.7	34.3 ± 1.4	7.5 ± 0.4	140.1± 5.6
		14	37.3 ± 1.4	15.4 ± 0.2	14.0 ± 0.5	5.9 ± 0.2	72.6 ± 1.8
		21	10.4 ± 2.8	10.4 ± 0.5	4.3 ± 0.9	4.3 ± 0.2	29.4 ± 4.4
GTE2	**Added**		**44.6**	**24.6**	**152.5**	**29.8**	**251.6**
	Extracted	0	38.3 ± 1.1	22.4 ± 0.3	132.2 ± 4.5	24.4 ± 0.9	217.3 ± 6.5
		7	31.1 ± 1.0	20.7 ± 0.8	75.9 ± 2.3	19.9 ± 0.3	147.6 ± 4.3

		14	17.6 ± 0.7	17.5 ± 0.6	37.9 ± 1.1	14.9 ± 0.2	88.0 ± 1.3
		21	6.3 ± 0.8	10.1 ± 0.4	2.2 ± 0.6	6.6 ± 0.3	25.2 ± 1.8
CatMix2	**Added**		**44.6**	**24.6**	**152.4**	**29.8**	**251.5**
	Extracted	0	35.6 ± 1.0	22.7 ± 0.6	101.8 ± 4.2	21.2 ±0.7	181.2 ± 6.4
		7	28.8 ± 1.8	24.0 ± 1.4	55.1 ± 5.7	18.2 ± 1.3	126.1 ± 10.2
		14	12.2 ± 0.2	18.3 ± 0.5	18.9 ± 1.8	11.7 ± 0.3	61.1 ±2.6
		21	0.7 ± 0.2	9.9 ± 0.3	1.3 ± 0.1	4.0 ± 0.1	15.9 ± 0.4
EGC250	**Added**		**250.0**				**250.0**
	Extracted	0	203.4 ± 2.1	-	-	-	203.4 ± 2.1
		7	150.3 ± 1.7		-	-	150.3 ± 1.7
		14	105.6 ± 3.4	-	-	-	105.6 ± 3.4
		21	35.1 ± 3.9	-	-	-	35.1 ± 3.9
EC250	**Added**		**-**	**250.0**	**-**	**-**	**250.0**
	Extracted	0	-	220.6 ± 1.1	-	-	220.6 ± 1.1
		7	-	80.9 ± 1.5	-	-	80.9 ± 1.5
		14	-	12.4 ± 2.0	-	-	12.4 ± 2.0
		21	-	0.4 ± 0.01	-	-	0.4 ± 0.01
EGCG250	**Added**		**-**	**-**	**250.0**	**-**	**250.0**
	Extracted	0	-	-	179.9 ± 3.4	-	179.9 ± 3.4
		7	-	-	74.6 ± 3.1	-	74.6 ± 3.1
		14	-	-	26.0 ± 1.9	-	26.0 ± 1.9
		21	-	-	0.3 ± 0.02	-	0.3 ± 0.02

ECG250	**Added**		**-**	**-**	**-**	**250.0**	**250.0**
	Extracted	0	-	-	-	175.3 ± 8	175.3 ± 8
		7	-	-	-	16.3 ± 1.0	16.3 ± 1.0
		14	-	-	-	2.4 ± 0.03	2.4 ± 0.03
		21	-	-	-	0.2 ± 0.1	0.2 ± 0.1

EGC = epigallocatechin, EC = epicatechin, EGCG = epigallocatechin gallate, ECG = epicatechin gallate. Control = DHA-rich oil, GTE1 = DHA-rich oil + 1000 ppm GTE1, GTE2 = DHA-rich oil + 450 ppm GTE2, CatMix1 = DHA-rich oil + reconstituted catechins mixture of GTE1, CatMix2 = DHA-rich oil + reconstituted catechins mixture of GTE2, EGC250 = DHA-rich oil + 250 ppm EGC, EC250 = DHA-rich oil + 250 ppm EC, EGCG250 = DHA-rich oil + 250 ppm EGCG, ECG250 = DHA-rich oil + 250 ppm ECG.

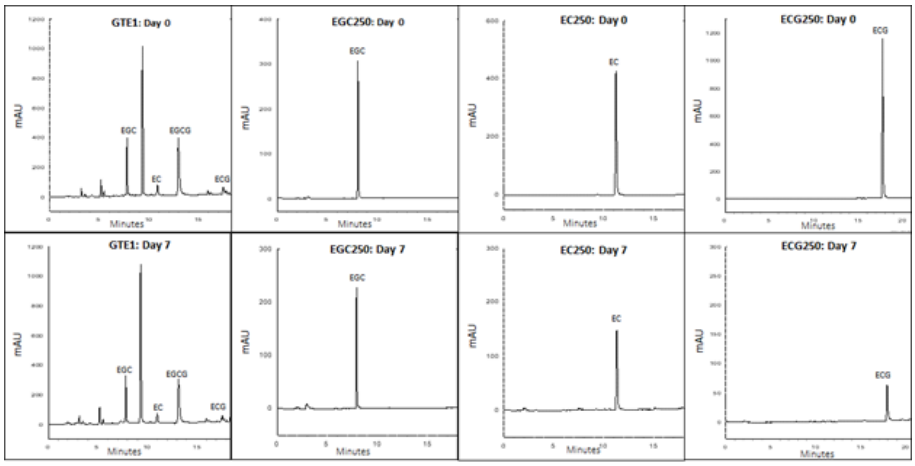

Figure S1. HPLC-UV chromatograms of catechins extracted from DHA-rich oils supplemented with GTE or catechins during accelerated ageing at 30°C. EGC = epigallocatechin, EC = epicatechin, EGCG = epigallocatechin gallate, ECG = epicatechin gallate. GTE1 = DHA-rich oil + 1000 ppm GTE1, EGC250 = DHA-rich oil + 250 ppm EGC, EC250 = DHA-rich oil + 250 ppm EC, ECG250 = DHA-rich oil + 250 ppm ECG. The degradation product at a retention time of 7.65 min was not detected in these samples.

Chapter 5. Effect of green tea extracts on the oxidative stability and organoleptic quality of omega-3 fatty acid enriched food products

Foreword

Lipid oxidation poses a major challenge in the use of omega-3 PUFAs in food product development as it gives rise to the formation of undesirable off-flavors, unhealthy compounds and may reduce the level of omega-3 fatty acids. The addition of antioxidants is, therefore, vital in PUFA-enriched food products. In this chapter, we investigated the effect of GTE supplementation in food products enriched with omega-3 PUFA-rich oils, such as salad dressings made with linseed oil, mayonnaise made with sacha inchi oil, and fruit and vegetable-based crackers enriched with DHA and α-linolenic acid. The results obtained in this chapter show the effect of processing or storage conditions on the oxidation level and demonstrate the antioxidant activity of green tea catechins in some complex food systems enriched with omega-3 PUFAs.

Chapter 5A. Oxidative stability and quality characteristics of linseed oil-based salad dressings supplemented with green tea extract.

Nain C.W., Fraipont L., Larondelle Y.

Abstract

This study investigated the effect of green tea extract (GTE) supplementation on the oxidative stability and sensory properties of linseed oil-based salad dressings. Two types of salad dressings were assessed, a stabilised or emulsified dressing and a two-phase dressing or vinaigrette. First, to determine the effectiveness of GTE in the dressings, samples were prepared with GTE at 0 ppm (control), 400 ppm, 1000 ppm and 2500 ppm, and an accelerated ageing test was carried out at 30°C in open bottles. After 4 weeks of storage, the best protection against oxidation was provided by GTE at 2500 ppm. A second experiment was then carried out at 20°C to evaluate the effect of GTE (2500 ppm) during long-term storage (49 weeks) in closed bottles. Thirdly, to imitate normal household use of dressings, bottles initially stored for 8 weeks at 20°C were used for a storage test under refrigerated conditions (4°C) with samples taken out from bottles every week for 3 weeks. The effect of GTE on sensory attributes of the stabilised dressing, was assessed through tests carried out with a consumer panel. The incorporation of GTE enhanced the oxidative stability of both dressings during storage at 30°C, with a more potent effect observed for the stabilised dressing. Meanwhile, no oxidation was observed for the dressings stored at 20°C or 4°C. GTE supplementation of the stabilised dressing resulted in a slight physical destabilisation of the emulsion and a less appreciated colour. Overall, our results suggest that incorporating GTE in these linseed oil-based salad dressings might not be necessary to enhance oxidative stability if an appropriate room or refrigeration temperature is used during storage

Keywords: Salad dressings, Linseed oil, green tea extract, peroxide value, sensory properties, storage

1. Introduction

Oils and fats are indispensable in the diet as a source of energy and essential fatty acids. In addition, they also provide liposoluble vitamins and other bioactive substances. Among the bioactive compounds are the omega-3 fatty acids: alpha-linolenic acid (ALA), eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid, whose consumption has been associated with a decrease in the risk of diseases such as cardiovascular diseases, inflammatory diseases, cancer, diabetes and neurological disorders (Jiang et al., 2022; Vázquez et al., 2019). ALA is an essential fatty acid because, although necessary for health, it cannot be synthesised by humans and must be obtained from dietary sources. Plants are the primary source of ALA, found in nuts and seeds, such as linseed (or flaxseed), chia seed, walnuts, echium seeds and canola (Shahidi and Ambigaipalan, 2018).

Linseed oil is one of the richest sources of ALA, which accounts for more than 50% of its fatty acids, making the total polyunsaturated fatty acid (PUFA) content of linseed oil >70% (Verghese et al., 2011). However, the high PUFA, especially high ALA content, makes linseed oil highly susceptible to oxidation, which results in sensory and nutritional quality deterioration and the production of toxic compounds (Ahmed et al., 2016). Being prone to oxidation, linseed oil is not recommended for cooking and frying applications since high temperatures act as a catalyst for oxidation (Choo et al., 2007). Food products with little or no heat treatments, such as salad dressings, are a better means to increase the consumption of linseed oil and, thus, omega-3 fatty acids in the diet.

The demand for various salad dressings and sauces has increased in recent years due to the rise in health awareness, which has led to an increase in the consumption of salads (Early et al., 2010). Traditionally, salad dressings are oil-in-water emulsions having a

continuous aqueous phase, such as vinegar or lemon juice, in which oil droplets are dispersed, and the dressings could be emulsified or not (Yang and Lai, 2003). According to the United States Food and Drug Administration (FDA), salad dressings should contain a minimum of 30% vegetable oil (Hill et al., 2020). Consuming salad dressings based on linseed oil will contribute to reducing the 15:1 omega-6/omega-3 ratio, which is reported for the western diet. The susceptibility of PUFAs to oxidation is one of the main causes of quality deterioration in emulsions. Lipid oxidation in emulsions differs from that in bulk oils since the aqueous phase may contain pro-oxidants, and the creation of an interfacial area between the oil and the aqueous phase promotes the contact between the unsaturated lipids and pro-oxidant compounds (Berton-Carabin et al., 2014). Other factors that can influence the lipid oxidation rate in emulsions include fatty acid composition, pH of the aqueous phase and ionic composition, oxygen concentration, lipid droplet characteristics such as size, concentration and physical state, and emulsion droplet interfacial properties (McClements and Decker, 2000). Therefore, incorporating antioxidants is necessary to protect salad dressings from oxidation. In a previous study, we showed that green tea extract (GTE) was effective in enhancing the oxidative stability of linseed oil (unpublished data), and other studies also demonstrated the antioxidant effectiveness of GTEs and catechins in food matrices such as pastries, meat products, table spreads, food emulsions and bulk oils (Boroski et al., 2020; Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009; Shen et al., 2021; Tian and Huang, 2019).

This study aimed at evaluating the oxidative stability of linseed oil-based salad dressings under different storage conditions and the impact of GTE supplementation on their oxidative stability. In addition, the effect of GTE on the taste and general appreciation of the dressings and any effect on physical stability were also assessed. This study was carried out in partnership with a Belgian company that specialises in the production of rapeseed oil and a

range of salad dressings based on this oil. With a desire to venture into linseed oil production, this study was an initial step in evaluating the feasibility of producing oxidatively stable salad dressings based on linseed oil. The company has two main types of salad dressings, a non-emulsified or 2-phase salad dressing and an emulsified or stabilised salad dressing, both being assessed in this study.

2. Materials and methods

2.1. Material

Linseed oil was provided by Alvenat (Achêne, Belgium). The oil was obtained by cold-pressing of seeds and had a total PUFA content of 73% wt. of total fatty acids, and the major fatty acids were ALA (57.5%), linoleic acid (15.6%), oleic acid (16.0%) and palmitic acid (5.6%). The other ingredients used in the production of the salad dressing (vinegar, mustard, water, egg-yolk powder, spices) were also provided by Alvenat. GTE (ED840370), a product of Naturex (Avignon, France), was a powdered extract of green tea leaves, standardised to a minimum content of 95% polyphenols, 60% catechins and 30% epigallocatechin gallate (EGCG). Based on our analysis, the major catechins in the GTE were EGCG (33.8%), epigallocatechin (EGC, 9.9%), epicatechin gallate (ECG, 6.6%), and epicatechin (EC, 5.5%). All other chemicals used in this study were either GC-grade, HPLC-grade or analytical-grade.

2.2. Sample preparation and oxidation conditions

Two types of salad dressings were evaluated in this study. First, a two-phase salad dressing (vinaigrette) was prepared by mixing linseed oil (55.5%), vinegar (25.1%), mustard (12.6%), water (5%), herbs and spices (1.2%) and salt (0.6%). A stabilised dressing was also prepared to contain linseed oil (40%), mustard (16.7%), vinegar (12%), water (22.5%), herbs and spices (3.8%), salt (3%) and egg-yolk powder (2%) as an emulsifier. Both

dressings were produced using a household mixer with an emulsification plate.

Three experiments were set up under different storage conditions to assess the antioxidant effectiveness of GTE in these salad dressings. First, the GTE was solubilised in vinegar, which was then used to produce the salad dressings. The preparation and bottling of the dressings were done at the Alvenat processing unit (Achêne, Belgium).

Experiment 1: Assessing the antioxidant effectiveness of different concentrations of GTE in salad dressings

This first experiment aimed at determining the GTE concentration that would best improve the oxidative stability of the two-phase and stabilised dressings. An accelerated oxidation test was carried out at 30°C for 8 weeks in open bottles. Four sample conditions for each salad dressing were prepared with concentrations of either 0 ppm (control), 400 ppm, 1000 ppm or 2500 ppm GTE. Forty grams of each sample were distributed in brown glass bottles (100 mL, 4 cm diameter) at production. All treatments were prepared in triplicate. The open bottles were then stored in an oven at 30°C in the dark for 0 days, 1 week, 2 weeks, 4 weeks, 6 weeks and 8 weeks to accelerate oxidation. The parameters measured were the peroxide value (PV) and the distribution of catechins between phases in the two-phase dressing.

Experiment 2: Effect of GTE on oxidation of salad dressings during long-term storage at 20°C

This experiment had as aim to evaluate the oxidative stability of dressings during shelf life like when in the warehouse or store. The two types of salad dressings with a GTE concentration of 2500 ppm and control without GTE were each prepared in triplicate. The concentration of either 0 ppm (control) or 2500 ppm was chosen based on the first experiment's results at 4 weeks of storage. For this long-term storage, the salad dressings were filled into brown

glass bottles of 250 mL, the standard bottles used for packaging dressings at the company. The bottles were tightly closed using a capping machine and stored in a 20°C oven for 0 days, 8 weeks, 16 weeks, 32 weeks and 49 weeks. The oxidative status of dressings was assessed by measurement of the PV. Samples stored for 20 weeks in this experiment were used to evaluate the effect of GTE on the sensory quality of the dressings.

Experiment 3: Oxidative stability of the salad dressings during refrigeration storage

In this third storage experiment, the conditions of regular household use of the salad dressings were imitated. The samples with a GTE concentration of 2500 ppm and the control samples with no added GTE were stored in a fridge at 4-6°C for 3 weeks in closed 250 ml bottles. The salad dressings used for this experiment were initially stored for 8 weeks at 20°C. To imitate household conditions at each sampling time, the bottles were taken out of the fridge, vigorously shaken and kept at room temperature for 15 minutes. Samples of 40 mL were then collected from the bottles and analysed for their oxidative status. The bottles were then closed and returned to the fridge. Analyses were done on day 0, week 1, week 2 and week 3. The samples were taken out of the same bottles each time, leading to an increase in the headspace volume at each sampling time.

2.3. Determining the oxidative status of salad dressings

2.3.1. Extraction of the lipid fraction and Peroxide value analysis

The PV analysis is classically performed on oil samples, making the separation of the oil from the salad dressings necessary. For the 2-phase dressings, centrifugation at 4480 x g for 10 minutes allowed a perfect separation of the oil phase. For the stabilised dressings, 40 mL of each were first centrifuged at 4480 x g for 10 min, resulting in a creamy layer on top with a mayonnaise-like

consistency separated from an aqueous lower phase. The creamy layer was then collected in a round bottom flask designed for rotary evaporation and mixed with 50 mL diethyl ether. After stirring the mixture for 10 minutes in the closed flask, the diethyl ether was evaporated on a rotary evaporator. The flask containing the resulting oil was then flushed with nitrogen for 1 minute to remove all traces of diethyl ether. The oily residue was then transferred to a 15 mL Falcon tube. The tube was centrifuged at 4480 x g for 10 minutes to separate the oil from any residues. The oil samples from both types of salad dressings were stored in the freezer at -20°C until further analysis.

The PV of the lipid fraction of the salad dressings was determined by iodometric titration according to the AOAC official method 965.33 (AOAC, 2000).

2.4. The physical stability of the stabilised salad dressing during storage

The stabilised dressings were visually evaluated in terms of phase separation during the storage tests at 30°C and 20°C.

2.5. Catechins distribution in the two-phase dressing

The catechins EGC, EC, EGCG and ECG were quantified in the oil and aqueous phases of the GTE2500 two-phase dressing by HPLC. The dressing was centrifuged (Figure 1), and catechins were extracted from the oil phase and quantified as we previously described (Nain et al., 2021).

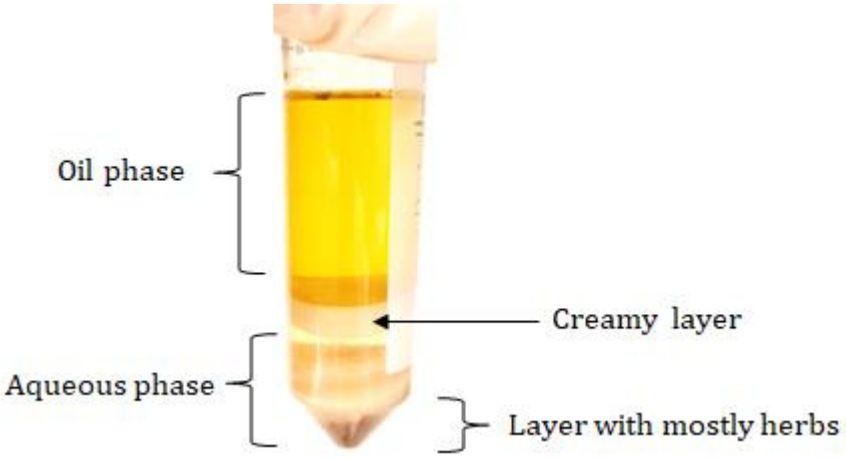

Figure 1. The separation of the two-phase dressing upon centrifugation

Briefly, 0.5 g of oil sample was mixed with 1 ml of 90% ethanol in a 1.5 ml Eppendorf tube, vortexed for 1 min and centrifuged for 5 min at 20000 x *g*. Next, the ethanolic phase (supernatant) was transferred into a 2 ml Eppendorf tube, and a second extraction of the remaining oil phase was carried out. The ethanolic phases from the two extractions were combined, and the ethanol evaporated under vacuum in a SpeedVac concentrator. The residue was then solubilised in 1 ml of 1% (v/v) formic acid in water and filtered through a 0.45 µl syringe filter into an HPLC vial.

For the aqueous phase, 50 µl were diluted with a 950 µl solution of 1% formic acid in water (v/v) and filtered through a 0.45 µL syringe filter into an HPLC vial before injection. The HPLC used was equipped with a Phenomenex kinetex 2.6 µm PFP 100A column (150 mm * 4.6 mm), an AS1000 autosampler (oven at 40°C and tray at 4°C), a P4000 pump (Thermo Separation Products) and a DAD UV 6000 LP diode array detector (DAD, Thermo Separation Products). The mobile phase consisted of the following two solvents: solvent A made of water with 1% formic acid (v/v) and solvent B made of acetonitrile with 1% formic acid (v/v), which were used according to the following elution protocol: 0 min, 3% B; 5 min, 9% B; 11 min, 10.5% B; 20 min, 25% B; 25 min, 100% B; 30

min, 100% B; 37 min, 3% B; 40 min, 3% B. The sample injection volume was 10 µL, and the flow rate was set at 1 mL/min. The catechins were identified and quantified by comparing retention times and peak areas to those of a pure standard mixture of EGC, EC, ECGC, and ECG (Extrasynthese, Genay, France) prepared at different concentrations to obtain calibration curves. Results are expressed in µg/g of oil or µg/ml of aqueous phase.

2.6. Sensory evaluation of the stabilised salad dressings

The sensory evaluation was performed on two stabilised dressings after storage at 20°C for 20 weeks, that is, the control and GTE2500 samples. The sensory tests were conducted at the Center of Investigation in Clinical Nutrition (Louvain-la-Neuve, Belgium) under controlled conditions. The microbiological safety of the salad dressings was ascertained before tasting.

Forty participants were recruited for the tasting. The salad dressing bottles were opened on the test day, and all samples for the day were prepared prior to the arrival of the participants: 5 g of salad dressings were poured into 30 mL cups, covered with a lid and stored in the fridge. After their arrival and installation, the assessors were given the instructions for the triangle and appreciation tests. The triangle test was conducted first, and participants were provided with the 3 samples for tasting, as well as water and neutral toast to cleanse their palate between samples. Two of the three samples served to the participants were identical and one different. Participants were asked to taste the samples and identify the odd one out of the three. An orange light was used for this test to minimise the effects of colour differences in the dressings on the participant's choice. Once the participants finished the first test, one of the samples provided in double was retrieved. The assessors were then asked to evaluate the colour, odour, taste and texture of the salad dressings using a nine-point hedonic scale and to rank the samples according to preference.

2.7. Statistical analysis

The samples were prepared in triplicate for all analyses, and the results are reported as mean ± standard deviation. Effects of the treatments with different GTE concentrations during the first storage experiment were assessed by subjecting data to an analysis of variance (ANOVA), and sample means were compared using a Tukey HSD (honestly significant difference). The effect of storage time on PV was also assessed for all experiments by one-way ANOVA. Differences between the means of the control and 2500 ppm GTE-supplemented samples at different storage times were achieved by the student's t-test. The data from the hedonic test were also subjected to a one-way ANOVA, whereas a reference table was used to determine significance in the triangle test. The data from the catechin analysis were also subjected to a t-test. All statistical analyses were performed using JMP Pro version 16 (SAS, USA), and the differences between the means were considered significant at $p \leq 0.05$.

3. Results

3.1. Oxidative stability of linseed-based salad dressing under accelerated storage at 30°C in open bottles

The antioxidant effectiveness of GTE (0 ppm (control), 400 ppm, 1000 ppm and 2500 ppm) was evaluated in the two-phase and the stabilised salad dressings stored in open bottles in an oven at 30°C for 8 weeks. The results obtained are presented in Figure 2.

The PVs of the two-phase dressings ranged from 4.5 to 5.5 meq O_2/kg oil at the start of the storage test, and no significant change was observed after the first week of storage (Figure 2A). After that, all conditions showed a significant increase in PV between each time point. The PV of the salad dressing without added antioxidants was significantly higher than all the other conditions from week 0 to week 6. Comparing the effect of the different treatments, it was noted that the PV of GTE2500-supplemented dressings was significantly lower than those of the other treatments after 4 weeks

of storage. After 6 weeks, the GTE2500 condition still had the lowest PV (13.7 ± 1.2 meq O_2/kg oil) but showed no significant differences with GTE1000 (15.4 ± 1.3 meq O_2/kg oil) and GTE400 (16.5 ± 0.6 meq O_2/kg oil) treatments. After 8 weeks of storage, the PV of the control samples (27.5 meq O_2/kg oil) showed no significant difference with the samples supplemented with GTE at 400 ppm (26.5 meq O_2/kg) but remained significantly higher than the 1000 ppm (21.6 meq O_2/kg) and 2500 ppm (22.5 meq O_2/kg) GTE-supplemented dressings. The PVs of the samples supplemented with GTE at 1000 ppm and 2500 ppm were 21.4% and 18.2% lower than the control at 8 weeks of storage.

The stabilised salad dressings showed no significant difference in PV (5.2 to 5.6 meq O_2/kg) at the start of the storage, and all the conditions also remained stable during the first week of storage (Figure 2B). The control then steadily increased, reaching a value of 25 ± 0.28 meq O_2/kg oil at week 8. The PV of the stabilised dressings supplemented with GTE either remained stable or showed a slight decrease after 4 weeks of storage, followed by a significant increase at week 6 and week 8. The PV of the control was significantly higher than that of the GTE-supplemented samples from week 4 onwards. While no significant differences were observed in the PV of the GTE-supplemented samples after 6 weeks of storage, the PV of GTE400 (13.9 ±0.8 meq O_2/kg oil) samples was significantly higher than that of GTE1000 (11.5 ± 0.6 meq O_2/kg oil). Still, both were not significantly different from the PV of the stabilised dressings supplemented with GTE2500 (12.9 ± 0.5 meq O_2/kg oil). The PV of stabilised dressings supplemented with GTE at 400 ppm, 1000 ppm and 2500 ppm was 45.2%, 54.7% and 48.9% lower than the control, respectively, after 8 weeks of storage at 30°C. The antioxidant effect of the GTE seemed more prominent in the stabilised dressings than in the two-phase dressings.

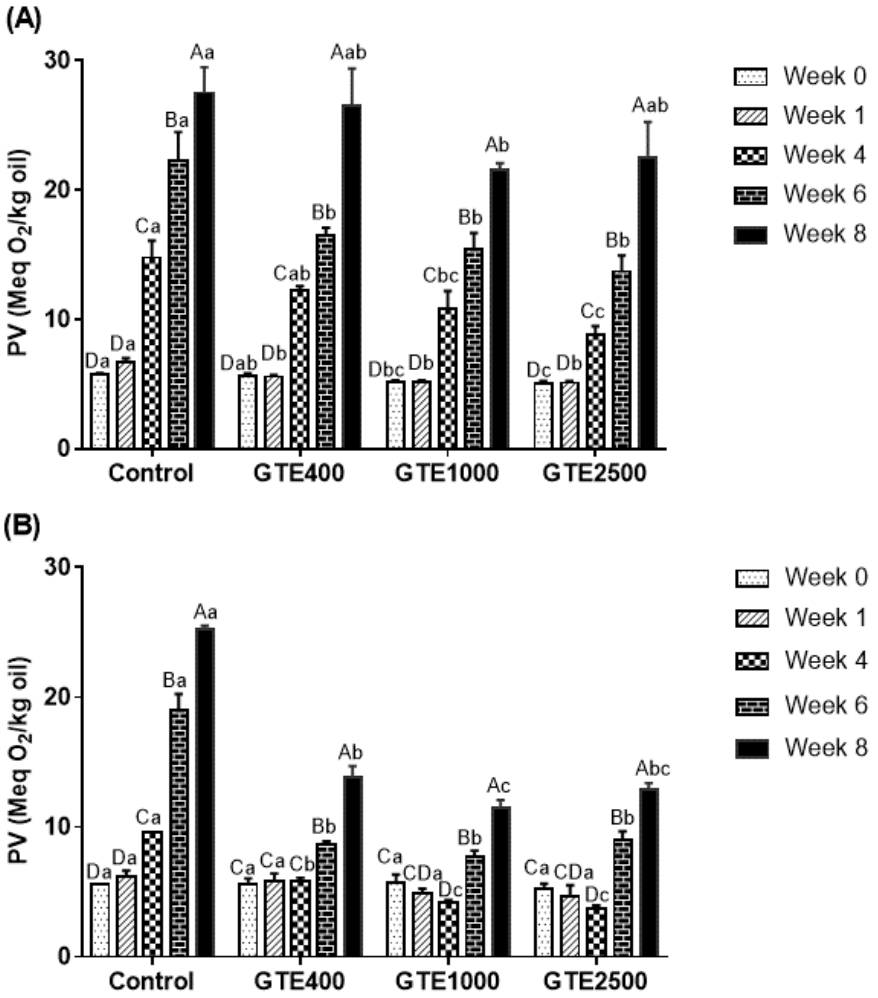

Figure 2. Evolution of peroxide values (PV) in linseed oil-based two-phase salad dressings (A) and stabilised salad dressings (B) during accelerated storage at 30°C in open bottles in the dark. Values are mean ± SD (n = 3). No common capital letter (A–D) indicates a significant difference ($p \leq 0.05$) during storage within a sample condition; no common lower-case letter (a–c) among samples indicates a significant difference ($p \leq 0.05$) at a specific storage time. Control = salad dressing without added antioxidants; GTE400, GTE1000 and GTE2500 = salad dressing supplemented with 400 ppm, 1000 ppm and 2500 ppm of GTE, respectively.

3.2. Oxidative stability of salad dressings during long-term storage at 20°C in closed bottles

Samples of the two-phase and the stabilised dressings were stored in capped 250 ml bottles at 20°C over 49 weeks to assess the effect of GTE (2500 ppm) incorporation on the oxidative stability of salad dressings when stored at room temperature.

The PVs of the freshly produced two-phase dressing without added antioxidant (control) and the dressing supplemented with GTE2500 were 6.5 ± 0.2 meq O_2/kg and 5.9 ± 0.1 meq O_2/kg, respectively. As shown in Figure 3A, the PV of the control slightly decreased during storage, while the GTE-supplemented samples showed no significant change in PV after 49 weeks of storage. On the other hand, the freshly produced stabilised dressings without added antioxidants (control) had a PV of 6.4 ± 0.4 meq O_2/kg, which was not significantly different from the PV of the corresponding GTE-supplemented dressing (5.9 ± 0.5 meq O_2/kg). The PVs of control and supplemented dressings showed a similar pattern during storage, with a slightly lower PV recorded after 49 weeks of storage (Figure 3B). There were no significant differences between the PV of the control samples and GTE-supplemented samples at the different sampling time points. Overall, the PVs of the control and GTE-supplemented salad dressings showed no increase during the 49 weeks of storage at 20°C.

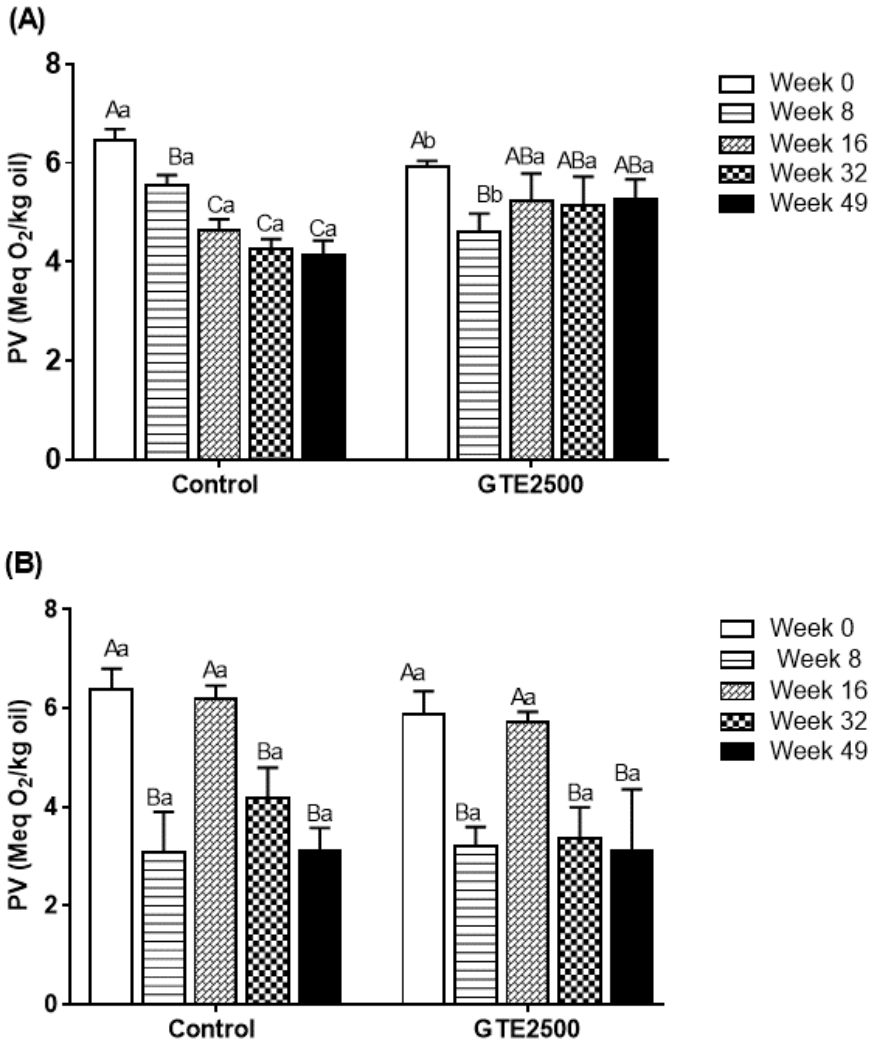

Figure 3. Changes in peroxide values (PV) in linseed oil-based two-phase salad dressings (A) and stabilised salad dressings (B) during long-term storage at 20°C in closed bottles in the dark. Values are mean ± SD (n = 3). No common capital letter (A and B) indicates significant differences ($p \leq 0.05$) during storage within a sample; a common lower-case letter (a) between samples at a specific storage time indicates no significant difference ($p > 0.05$). Control = salad dressing without added antioxidants; GTE2500 = salad dressing supplemented with 2500 ppm of green tea extract.

3.3. Oxidative stability of salad dressings under refrigeration storage at 4°C

The two-phase and stabilised dressings were stored at 4°C, with samples taken out of the same bottles weekly to mimic the household use of dressings. The PV of the dressings during the 3-week storage is shown in Figure 4. The PVs of the two-phase (Figure 4A) and stabilised dressings (Figure 4B) showed no significant change during storage, and there were no significant differences between the GTE-supplemented samples and the control samples throughout the storage period.

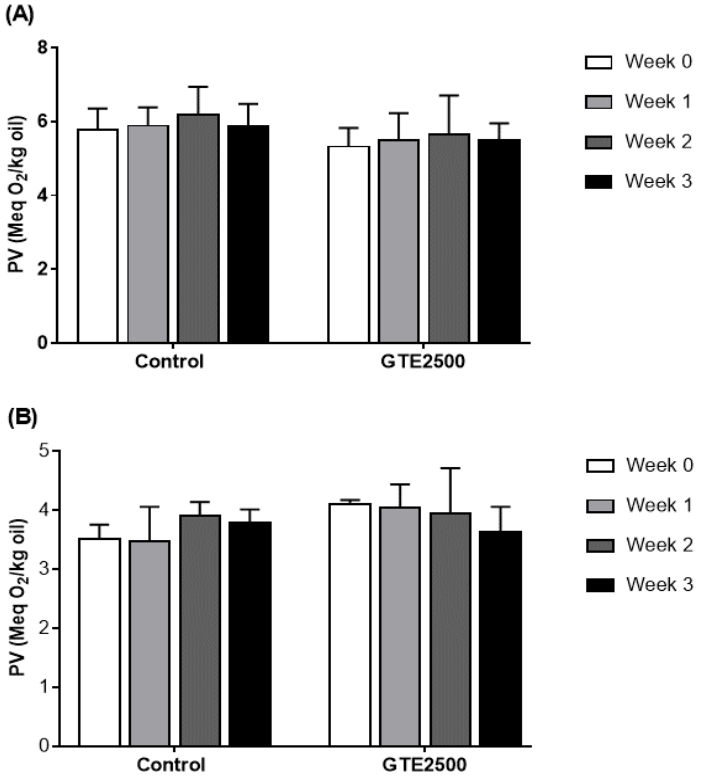

Figure 4. Peroxide values (PV) in linseed oil-based two-phase salad dressings (A) and stabilised salad dressings (B) during refrigeration at 4°C under household conditions. *Values are mean ± SD (n = 3). No significant differences (p > 0.05) observed. Control = salad dressing without added antioxidant; GTE2500 = salad dressing supplemented with 2500 ppm of green tea extract*.

3.4. Catechins distribution in the two-phase dressing

The distribution of catechins in the oil and aqueous phases of the two-phase dressing was assessed to get more insight into their antioxidant effect in this dressing. As shown in Table 1, the amount of catechins incorporated in the oil phase was minimal. This might explain the lesser antioxidant effect of GTE in this dressing since, with the separation of the phases, the oil phase had little protection from the catechins. Also, the amount of catechins quantified in the two phases (810.4 ± 70.4 μg/g) was less than the 1400 μg/g expected in the dressing with 2500 ppm of GTE, which could indicate that some of the catechins were incorporated in the other two layers observed after centrifugation of the salad dressings (Figure 1).

Table 1. Distribution of catechins between the aqueous phase and oil phase of a two-phase salad dressing supplemented with 2500 ppm green tea extract.

Catechins	Catechins content		LOQ (μg/ml)
	*Aqueous phase (μg/ml of vinegar)	*Oil phase (μg/g oil)	
EGC	262.9 ± 17.1	3.1 ± 1.9	0.70
EC	151.2 ± 6.9	3.5 ± 0.7	0.22
EGCG	326.1 ± 38.9	2.1 ± 1.2	0.81
ECG	59.0 ± 6.7	2.6 ± 0.4	0.86
Total	799.2 ± 69.5	11.2 ± 0.9	

*Values are mean ± SD (n = 3). EGC = epigallocatechin, EC = epicatechin, EGCG = epigallocatechin gallate, ECG = epicatechin gallate.
LOQ: limit of quantification based on signal-to-noise ratio of 10:1 in μg/ml of sample injected in HPLC.

3.5. Physical stability of stabilised salad dressing during storage

The samples of the stabilised salad dressing stored at 30°C for 8 weeks in open bottles and those held at 20°C in closed bottles for 49 weeks were checked for phase separation and oiling-off. Dressings stored at 30°C showed no oiling-off until week 6, when a tiny layer of free oil was observed in the salad dressings. However, centrifugation revealed evident signs of emulsion destabilisation in the GTE-supplemented dressing already after week 2 of storage, as a layer of oil was visible in the dressings supplemented with 1000 ppm and 2500 ppm GTE but not in the control and GTE400 samples after centrifugation. Over time, oil separation was evident in all samples, even the control group, with the volume of oil increasing with an increase in GTE concentration. This seems to indicate that supplementation with GTE increased the emulsion's destabilisation rate. As for the stabilised dressings stored at 20°C, oiling-off was evident only in one of the GTE-supplemented samples before centrifugation at week 8. However, upon centrifuging, a layer of oil was visible in all the GTE-supplemented samples, which was not the case for the control dressings. Nonetheless, the control samples showed some oiling-off at week 32 as well.

3.6. Sensory properties of the stabilised salad dressing

The sensory analysis was conducted only on the stabilised dressings as they showed better oxidative stability, especially during the accelerated storage. The dressings evaluated had been stored for 20 weeks at 20°C. A triangle test was carried out to determine whether there was a perceivable difference in the taste of the control and GTE-supplemented (GTE2500) salad dressings. A nine-point hedonic scale was used to score the different quality attributes of the dressings, followed by a preference test. Forty untrained assessors participated in the sensory evaluation, with

70% female and 30% male. Ninety per cent of the assessors were between the ages of 20 and 30 years.

A statistical reference table for determining significance in a triangle test (Roessler et al., 1978) was used to establish whether or not there was a significant taste difference ($p < 0.05$) between the control and GTE-supplemented salad dressings. Out of 40 assessors, a minimum of 19 persons must correctly identify the odd sample among the three tasted for a significant difference. In this case, 19 participants correctly identified the odd sample, while 21 participants did not pick out the odd sample. These results indicate a significant taste difference ($p < 0.05$) between GTE-supplemented and control salad dressings.

Regarding preference, 65 % of assessors preferred the control dressings, in which no GTE had been added, while the others (35%) liked the GTE-supplemented dressing. As for the quality attributes of the salad dressings, the colour of the control dressing had a significantly higher score than the GTE-supplemented dressing (Figure 5), with assessors indicating that the GTE-supplemented dressing had a darker colour compared to the control sample. Meanwhile, there were no significant differences between the odour, taste and texture scores between the two dressings.

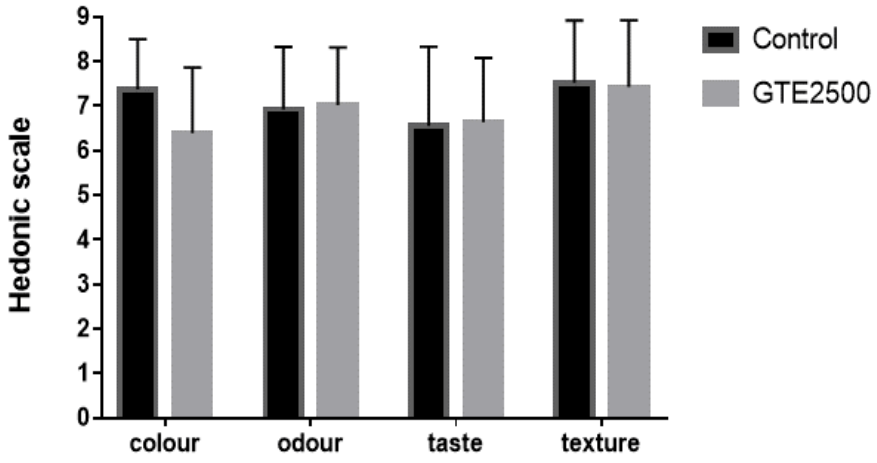

Figure 5. Sensory properties of linseed-salad dressings with and without incorporation of green tea extract (GTE) after 20 weeks of storage at 20°C. Values are mean ± SD (n = 40). For each attribute, different letters indicate significant differences at ($p \leq 0.05$). The sensory evaluation was performed based on nine-point hedonic scale where 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely.

4. Discussion

The oxidation pattern of the salad dressings varied with storage conditions. The PV of salad dressings stored at 30°C in open bottles increased during storage, whereas those held at 20°C for long-term storage in closed bottles or at 4°C under household conditions either slightly decreased or did not change during the storage period. The household conditions applied in this study were only approximate, given that some home refrigerators may work at temperatures up to 8°C, and how long the dressings are left out of the fridge during a meal may also vary. The dressings stored at 30°C were in open bottles with a considerable headspace volume, therefore more exposed to oxygen than dressings at 20°C and 4°C. A principal step in lipid oxidation is the reaction of oxygen with free radicals forming reactive oxygen species, which further accelerate

the oxidation process (Johnson and Decker, 2015). Krichene et al. (2010), assessing the oxidative stability of virgin olive oil in open and closed bottles under accelerated oxidation at 50°C, observed that the PV of the olive oil in open bottles was about 4 to 6 times higher than that of the oils in the closed bottles after 33 weeks of storage. In addition, the oxidation rate can be influenced by the storage temperature. Mohanan et al. (2018) observed that the rate of PV increase in LSO was more with an increase in storage temperature (25°C, 40°C, and 60°C) and showed a doubling of PV with an increase in storage temperature from 25°C to 60°C. The PV of an oil-in-water emulsion prepared with sunflower oil also showed a temperature-dependent increase when stored at temperatures of 5°C, 25°C and 40°C (Choulitoudi et al., 2021).

The salad dressings supplemented with GTE showed enhanced oxidative stability during storage at 30°C. The antioxidant activity of GTE has been ascribed to catechins, and the major catechins (EGC, EC, EGCG, ECG) are considered responsible for most of the positive effects. The antioxidant activity of green tea polyphenols is primarily attributed to the combination of aromatic rings and hydroxyl groups. Tea catechins scavenge free radicals by donating their hydrogen atoms, forming less reactive radicals and also act as antioxidants by chelating metal ions that will otherwise catalyse the oxidation process (Senanayake, 2013). Similarly, Roedig-Penman and Gordon (1997) had shown that a tea extract added to sunflower oil-in-water emulsion (30% sunflower oil) was effective in enhancing the oxidative stability of the emulsion during 40 days of storage at 30°C. The sunflower oil emulsion without added antioxidants recorded a PV about 2.5 times higher than that supplemented with the tea extract at the end of 40 days. Other studies have also explored the oxidative stabilisation of emulsions by adding natural compounds with antioxidant properties. Goyal et al. (2015) observed higher oxidative stability of linseed oil emulsions with an increase in the amount of whey protein

concentrate added to the emulsions, and they attributed this to the antioxidative properties of the protein and the ability to bind pro-oxidants such as metal ions. In another study, supplementation of salad dressings based on a 60% oil blend of rice bran oil and sunflower oil (4:1 ratio) with Spanish plum leaf extract resulted in decreased total oxidation (TOTOX value) with increased leaf extract concentration during storage at 4°C for 4 weeks (Kupongsak and Manomaiwajee, 2016). The major phenolic compounds reported in the Spanish plum leaf extract were gallic acid, EGC, and gallocatechin. Aksoy et al. (2021) investigated the potential of grape seed oil by-product powder and pomegranate seed oil by-product powder as natural antioxidants in a corn oil salad dressing by using the OXITEST method, and the supplemented salad dressings showed a higher induction period (IP) than the control sample. The major phenolic compounds in these powders were EC or EGC. In the studies mentioned above, the positive effect of supplementing salad dressings with some extracts was observable even at refrigeration temperatures. This could not be observed in our study, likely because the control condition did not show any increase in PV. The salad dressings produced in this study contained herbs and spices (e.g. chives, white pepper, parsley, ginger, fennel) as well as mustard, which are rich sources of vitamins (C and pro-A) and flavonoid antioxidants such as quercetin and kaempferol that may enhance the stability of even the control samples at low temperatures. For the salad dressings stored at 20°C, a decrease in PV was observed during storage, which may be due to the decomposition of hydroperoxides or their reaction with other compounds in the dressings. Hydroperoxides are unstable and break down to form secondary oxidation products starting with cleavage of O-O or O-H bond to form alkoxyl or peroxyl radicals, respectively. These reactions are catalysed by metal ions (Ahmed et al., 2016). Some of the ingredients used in the dressings, such as egg yolk and/or mustard, are known to contain trace elements like iron and copper, which act as catalysts in the

decomposition of hydroperoxides as shown in the following equations:

- $ROOH + Fe^{3+}(or\ Cu^{2+}) \rightarrow ROO^{\bullet} + Fe^{2+}\ (or\ Cu^{+}) \rightarrow + H^{+}$
- $ROOH + Fe^{2+}\ (or\ Cu^{+}) \rightarrow RO^{\bullet} + Fe^{3+}\ (or\ Cu^{2+}) \rightarrow\ + OH^{-}$

The salad dressings under this long-term storage at 20°C were in closed bottles with little headspace and, thus, limited exposure to oxygen. At low oxygen concentrations, oxidation rates are very low, which implies minimal or no additional hydroperoxides are formed.

The antioxidant activity of GTE was more potent in the stabilised dressing than in the two-phase dressing. This could be explained by the progressive separation of the two-phase dressing into the oil and aqueous phases during storage. Emulsions are thermodynamically unstable systems that tend to separate over time (McClements and Decker, 2000). Since the catechins are mostly hydrophilic, the separation of phases progressively led to lesser protection of the oil phase, which became the top layer. As shown by the results of catechins distribution in the salad dressing, only a minimal amount of catechins were detected in the oil phase. On the other hand, the stabilised dressings remained in the form of emulsions throughout the storage period. With the addition of an emulsifier (in our case, egg yolk) prior to homogenisation, a layer forms around the oil droplets during blending, preventing the droplets from aggregating (McClements and Jafari, 2018). Thus, the antioxidants had a higher protective effect on the oil droplets in the stabilised dressing.

Although the supplementation with GTE had a positive effect on the oxidative stability of the stabilised dressing, a faster physical destabilisation of the dressing was observed with an increase in the concentration of GTE. This may be due to reactions between catechins and proteins. Egg yolks used as an emulsifier in the stabilised dressings contain proteins. Proteins and polyphenols

have been shown to interact, forming protein-polyphenol complexes through covalent or non-covalent interactions (Ishii et al., 2008; Li et al., 2021). The catechins interact with proteins via non-covalent forces such as hydrogen bonding and hydrophobic interactions. Meanwhile, covalent interactions occur between quinones or semiquinones (formed from the oxidation of polyphenols) and nucleophilic groups of proteins, such as the ε-amino group of Lys and sulfhydryl (thiol) group of cysteine (Sun et al., 2022). These interactions may change the structure of proteins, thereby affecting their ability to keep the emulsion stabilised. Tian et al. (2021) investigated the effect of tea polyphenols on walnut oil-in-water emulsions stabilised with whey proteins and showed that a relatively low dose of polyphenols (0.01 or 0.02%) improved the physical stability of the emulsion by reducing the rate of droplets aggregation during incubation. In contrast, the incorporation of high levels of tea polyphenols (0.04%) led to small droplets being produced immediately after homogenisation but more susceptible to aggregation during storage, thus reducing the physical stability of the emulsion. The amount of emulsifier and the homogenisation pressure used in preparing an emulsion could also affect its physical stability (Goyal et al., 2015). An insufficient amount of emulsifier could lead to incomplete encapsulation of oil droplets formed, thus giving room for coalescence.

The colour of the stabilised dressing was affected by GTE supplementation. This was, however, expected because the GTE used in this study had a brownish colour. The triangle test also indicated a taste difference between the control and GTE-supplemented dressings, although this was not evident from the hedonic test responses. This might imply the taste difference was subtle. However, tea catechins are shown to be responsible for an astringent and bitter taste, especially EGCG (Scharbert and Hofmann, 2005), which was most abundant in the GTE used in this study. Wang et al. (2007) reported a decrease in the brightness and sweetness of bread supplemented with GTE and an increase in

astringency. The threshold level of GTE for its effect on the bread attributes to be significantly detectable by trained panellists was 5.0 g/kg flour (5000 ppm) for astringency and sweetness and 1.5 g/kg flour (1500 ppm) for brightness, which were correlated with instrumental analysis. The salad dressing evaluated for sensory attributes in this study contained 2500 ppm. Thus, a lower dose of GTE, such as 1000 ppm should be tested in further studies, as it might have a less negative impact on the sensory properties without affecting the antioxidant effectiveness.

5. Conclusion

The oxidative stability of linseed oil-based salad dressings assessed under accelerated ageing at 30°C, long-term storage at room temperature (20°C), and refrigerated storage (4°C) under household conditions showed different patterns for both the two-phase and stabilised dressings. Incorporating GTE in the dressings enhanced the oxidative stability of samples during accelerated ageing at 30°C, with a more potent effect in the stabilised dressing. The GTE concentrations of 2500 ppm and 1000 ppm showed a similar ability to delay the formation of hydroperoxides in the dressings, but the latter had a lesser destabilising effect on the emulsion. Thus, GTE at a concentration of 1000 ppm could be a better choice to delay oxidation in these products. However, samples stored at room temperature or under refrigeration showed no increase in PV for neither the control nor the supplemented samples, which was an indication that the PUFAs did not oxidise under these storage conditions. Overall, our results suggest that incorporating GTE in these linseed oil-based salad dressings might not be necessary to enhance oxidative stability if an appropriate room or refrigeration temperature is used during storage but becomes useful if the storage conditions are not met.

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Chapter 5B. Effect of natural antioxidants on the oxidative stability and sensory properties of mayonnaise enriched with sacha inchi oil.

Nain CW., Foncea P., Larondelle Y.

Abstract

Mayonnaise is one of the most widely consumed oil-based foods and could be a source of polyunsaturated fatty acids. The aim of this study was to: ***i)*** Formulate a mayonnaise rich in α-linolenic acid (ALA) through enrichment with sacha inchi oil (SIO), ***ii***) Investigate its oxidative stability and sensory parameters when supplemented with hydrophilic and oil-soluble green tea extracts (GTE and OSGT) and with Phytrox, an oil-soluble mixed antioxidant formulation. Two mayonnaises were prepared: One with 56% rapeseed oil (MRO) and another with 9% rapeseed oil replaced with SIO (MRSIO). Then, five SIO-based mayonnaises were prepared by supplementing with GTE, Phytrox and three doses of OSGT. The fatty acid profile of mayonnaise was determined by GC analysis, the oxidative stability by measuring peroxide value (PV) and p-anisidine value (*p*-AV) during storage at 30°C for 28 days, and sensory evaluation was carried out with an untrained panel. Incorporating SIO in mayonnaise led to a 48% increase in ALA from 42.3 mg/g of ALA in MRO to 81.3 mg/g in MRSIO, with a decrease in the ω-6/ω-3 ratio from 2.5 to 1.5. The PV and p-AV of MRSIO at the end of storage were, respectively, 42.4% and 30% higher than that of MRO. Supplementing MRSIO with antioxidants enhanced its oxidative stability in the decreasing order GTE ≥ OSGT (3000 ppm) > OSGT (2000 ppm) > Phytrox > OSGT (1000 ppm). The sensory evaluation showed that except for GTE-supplemented MRSIO, the taste of mayonnaises containing SIO was scored lower than MRO by the assessors. Overall, an acceptable mayonnaise enriched in ALA (ω-3 fatty acid) can be obtained by adding SIO, but antioxidants are necessary to delay oxidation.

Keywords: Sacha inchi oil, mayonnaise, oxidation, green tea extract, catechins, α-linolenic acid, sensory evaluation

1. Introduction

Mayonnaise is one of the most widely consumed oil-based food products. It is a low-pH oil-in-water emulsion obtained by emulsifying vegetable oils, egg yolk, vinegar (or lemon juice), sugar and spices (Alizadeh et al., 2019; Jacobsen, 2016). Conventionally, mayonnaise contains 70-80% of fat, but in recent years formulations have been developed with reduced oil content (Nikzade et al., 2012; Satriawan et al., 2022; Taslikh et al., 2022). Mayonnaise can be made from a single oil or a mixture of oils, and the oil characteristics determine the sensory, physicochemical and nutritional properties of the mayonnaise (Gaipova et al., 2022). The oils commonly used in mayonnaise include sunflower, soybean, rapeseed and corn oils. Meanwhile, oils with large quantities of saturated acids, such as palm oil, or oils that solidify at refrigeration temperatures, such as peanut and olive oils, are seldom used because they tend to break the emulsion at refrigeration temperatures (Hill et al., 2005). Meanwhile, some studies have investigated blending regular vegetable oils with more unsaturated ones, such as linseed and fish oils, to produce healthier mayonnaise (El-Waseif et al., 2022; Jacobsen et al., 2000; Raudsepp et al., 2014). Mayonnaise may be classified as semi-perishable because its low pH makes it relatively resistant to microbial spoilage and can be stored reasonably without refrigeration. However, like other high-fat foods, oxidation is one of the major causes of its deterioration.

In emulsified products like mayonnaise, oxidation is a reaction initiated at the interface between the two phases and influenced by both the presence of prooxidant and antioxidant compounds and the interactions between the different components in the system (McClements and Decker, 2000). In addition to the fact that a vast area of the oil is exposed to an aqueous phase, which may contain dissolved oxygen, air bubbles are introduced into the emulsion through the blending process, which can further fuel lipid oxidation (Depree and Savage, 2001). Lipid oxidation leads to the

degradation of flavour, aroma, colour, texture, and loss of beneficial polyunsaturated lipids and the formation of secondary and potentially toxic compounds (Ahmed et al., 2016). The most common strategy to avoid or retard lipid oxidation is to add antioxidants. The effectiveness of antioxidants in emulsions is associated with their partitioning in the emulsion. Antioxidants that partition into the interfacial region protect the oil droplets more than those soluble in the oil or aqueous phases (McClements and Decker, 2000). However, the polarity of the antioxidant could be influenced by the pH of the aqueous phase (Schwarz et al., 1996). Other factors affecting the efficiency of antioxidants include the nature and type of emulsifier and the chemical composition of the aqueous and oil phases (Di Mattia et al., 2009). Hence, the effectiveness of an antioxidant in a specific emulsion cannot be directly predicted from its effectiveness in bulk oil or another emulsified system.

Synthetic antioxidants like EDTA, TBHQ and propyl gallate are effective in mayonnaise (Ghorbani Gorji et al., 2016), but due to health concerns relative to the use of these antioxidants, a number of studies investigated potential natural antioxidants in mayonnaise and among those that seem to enhance oxidative stability are rosemary (Alizadeh et al., 2019; Sørensen et al., 2010) and green tea (Ramsaha et al., 2015).

The objective of the present study was to formulate an α-linolenic acid-rich mayonnaise and investigate its oxidative stability, as well as its sensory properties when supplemented with natural antioxidants. The mayonnaise was prepared by partially substituting rapeseed oil (RO) with sacha inchi oil (SIO). The antioxidant activities of GTE, Phytrox and OSGT (oil-soluble green tea extract), which we had investigated in sacha inchi oil, were also evaluated in the mayonnaise during storage at 30°C for 28 days. In addition, the sensory attributes of the different mayonnaise samples were assessed using a 9-point hedonic scale.

2. Materials and Methods

2.1. Materials

***Mayonnaise ingredients*:** The oils used in the mayonnaise were SIO and rapeseed oil (RO). RO is one of the oils commonly used in the production of mayonnaises. Due to its low saturated fatty acid content it does not solidify at refrigeration temperature. In addition, the oil also contains ALA. SIO was extracted from sacha inchi seeds from Peru, provided for this study by Prellencia (Gembloux, Belgium). Cold-pressed RO, and the other ingredients (eggs, mustard, lemon, salt, and white pepper) were purchased from a local store. The eggs were pasteurised in water at 60°C for 5 minutes and cold down in ice water. This was followed by the careful separation of the yolks from the white.

Natural antioxidants: This consisted of three products. A water-soluble green tea leaf extract (GTE) was a product of Naturex (Avignon, France) and contained 25% of major green tea catechins: EGCG (76.4 mg/g), EC (20.0 mg/g), and ECG (10.0 mg/g). An oil-soluble green tea extract (OSGT) containing 14% of active antioxidant molecules (derivatives of epigallocatechin) was kindly provided EGC (147.2 mg/g), by Kemin industries (Herentals, Belgium). The third antioxidant was Phytrox LTR15-IP MB (Phytrox), a product of Jan Dekker (Amsterdam, The Netherlands). Phytrox is a liposoluble liquid formulation containing a mixture of vegetable-derived tocopherols, ascorbyl palmitate and rosemary extract blended in lecithin and vegetable oil.

2.2. Mayonnaise preparation

Mayonnaises were made from either RO, one of the oils commonly used in mayonnaise production, or from a blend of RO and SIO using the ingredient list in Table 1. This mayonnaise recipe and the proportion of RO and SIO were fixed on the basis of preliminary trials, which took into consideration the effect of SIO on taste.

Table 1. ingredients used in the preparation of mayonnaise

Ingredients	Weight (g)	
	MRO	MRSIO
Rapeseed oil (RO)	56	47
Sacha inchi oil (SIO)	-	9
Mustard	24	24
Egg yolk	12	12
Lemon	7	7
Black pepper	0.3	0.3
Salt	0.7	0.7

MRO= RO-based mayonnaise, MRSIO= mayonnaise with a blend of RO and SIO

Mayonnaise containing 56% of plant-derived oils was prepared in batches as follows. First, the mustard, egg yolk, salt, and pepper were mixed using an electric hand blender (Bosch, ErgoMixx, 750 W, Germany) to obtain a smooth paste. Next, oil was added gradually to the paste while mixing continuously with the electric hand blender for five minutes. Once the emulsion was obtained, lemon juice was added while mixing for one additional minute. The mayonnaise with RO was prepared with no added antioxidants (MRO), while the mayonnaise with a blend of RO and SIO was prepared with different concentrations of antioxidants as follows: no antioxidant (MRSIO), GTE at 1000 ppm (MRSIO-GTE), Phytrox at 1000 ppm (MRSIO-Phytrox) and with three doses of OSGT 1000, 2000 and 3000 ppm (MRSIO-OSGT1, MRSIO-OSGT2 and MRSIO-OSGT3). The oil-soluble antioxidants (OSGT and Phytrox) were solubilised in the oil, while GTE was solubilised in lemon juice.

2.3. Fatty acid content of mayonnaise

The oil phase of the mayonnaises was extracted by freezing samples at -22°C for 24 hours, followed by thawing for 1 hour at room temperature to break the emulsion. They were then centrifuged at 3200*g for five minutes to separate the phases.

The oils extracted from MRO and MRSIO were converted to fatty acid methyl esters (FAMEs) via a two-step methylation with KOH and HCl. The FAME was extracted in hexane, and the fatty acids were identified and quantified on a gas chromatograph equipped with a flame ionisation detector. Results are presented in mg/g.

2.4. Determination of the oxidative stability of mayonnaise

The oxidation rate of the different mayonnaises was assessed during an accelerated ageing test at 30°C for 28 days. Samples (25g) of the different mayonnaises were stored in open 50 ml Falcon tubes in an incubator at 30°C. Samples were analysed on days 0, 7, 14, 21 and 28 of the storage period. The oxidation state of oil extracted from the mayonnaise samples was determined by measuring the peroxide value (PV) according to the AOAC official method 965.33 (AOAC, 2000) and *p*-anisidine value (*p*-AV) according to the AOCS official method Cd 18–19 (AOCS, 1998).

2.5. Sensory evaluation of mayonnaise

The sensory evaluation was carried out in the tasting room of the Clinical Investigation Centre in Nutrition (CICN, Belgium). The mayonnaises tasted were MRO, MRSIO, MRSIO-GTE and MRSIO-Phytrox. The sensory analysis was conducted with a panel of 65 untrained assessors aged 18 to 70 years (median 24 years) and composed of 30 women, 34 men and one non-binary person. Five grams of the mayonnaise samples were presented to each assessor in a coded transparent plastic cup. The sensory attributes evaluated were overall acceptability, taste, smell, colour, and texture. The appreciation of each attribute was scored on a 9-point

hedonic scale, with 1 being extremely disliked and 9 being extremely liked. The samples were presented in a randomised order. Assessors were provided with water and bread to rinse their mouths between samples.

2.6. Statistical analysis

Statistical analyses were performed using the R studio software version 4.1.3 and JMP Pro version 16 (SAS institute inc., Cary, NC, USA) and differences between the means were considered significant at $p \leq 0.05$. The fatty acid profiles of MRO and MRSIO were compared by performing a t-test. The PV and *p*-AV were subjected to an analysis of variance, and sample means were compared by Turkey's HSD (honestly significant difference) test. The acceptability and appreciation scores of samples were analysed using a Friedman test and a Conover's post hoc test, with Bonferroni correction, then performed for all pairwise comparisons.

3. Results

3.1. The fatty acid profile of mayonnaise

The fatty acid profile of the lipid fraction of mayonnaises showed that MRO contained oleic acid (58.6%), linoleic acid (19.2 %wt.) and ALA (7.6 wt.%) as its major fatty acids (Table 2). The blending of RO with SIO (84:16) resulted in a significant decrease in saturated fatty acids ($p = 0.04$) and monosaturated fatty acids ($p < 0.0003$) and an increase in the total PUFA ($p < 0.0001$). The ALA content of MRSIO was 48% higher than in MRO, while the total PUFA content increased by 25%. The ω-6/ω-3 ratio of the mayonnaise was also improved from 2.5 to 1.5. Taking into consideration the oil content of the mayonnaises, MRO and MRSIO were estimated to contain, respectively, 42.3 mg/g and 81.3 mg/g of ALA.

Table 2. Fatty acid composition of the lipid fraction of mayonnaises made with rapeseed oil (MRO) or a blend of rapeseed (86% wt.) and sacha inchi (16%) oils (MRSIO).

Fatty acid	**MRO** (mg/g of oil)	**MRSIO** (mg/g of oil)
C16:0	50.2 ± 0.1[a]	44.0 ± 1.0[b]
C18:0	18.4 ± 0.1[a]	16.2 ± 0.7[b]
C18:1cis9	585.8 ± 1.8[a]	533.8 ± 5.3[b]
C18:2c9c12	191.7 ± 0.4[b]	211.5 ± 0.9[a]
C18:3c9c12c15	75.5 ± 0.7[b]	145.1 ± 1.7[a]
Total SFA	83.2 ± 5.4[a]	70.1 ± 1.8[b]
Total MUFA	633.1 ± 2.1[a]	576.7 ± 4.2[b]
Total PUFA	267.9 ± 1[b]	357.3 ± 1,2[a]
ω-6/ω-3	2.5	1.5

Values are mean ± SD (n=3). Different superscript letters (a and b) indicate a significant difference ($p \leq 0.05$) within a row.

3.2. The oxidative stability of mayonnaise

3.2. 1. Effect of Sacha inchi oil on oxidative stability

The PV and *p*-AV of MRO and MRSIO were measured every 7 days during the storage at 30°C for 28 days to assess the effect of incorporating SIO on the oxidative stability of the mayonnaise (Figure 1). The PV increased in both mayonnaises during storage, but a faster increase was observed for MRSIO (Figure 1A). After 28 days of storage, the PV of MRSIO (12.5 meq O_2/Kg oil) was 42.4% higher than that of MRO (7.2 5 meq O_2/Kg oil). As for the *p*-AV, both mayonnaises had a similar *p*-AV on day 0, but the rate of increase was higher for MRSIO during most of the storage period (Figure

1B). At the end of storage, the *p*-AV of MRSIO (10.01) was 30% higher than that of MRO (7.02). This indicates that SIO is more susceptible to oxidation than RO.

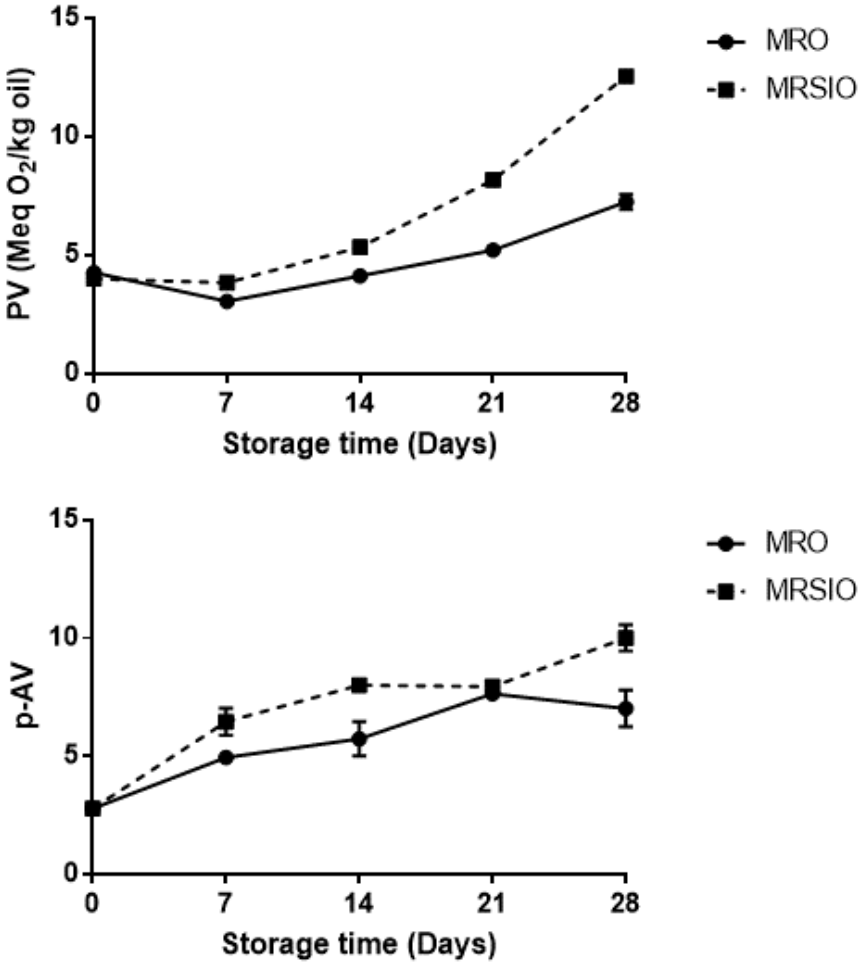

Figure 1. Effect of sacha inchi oil incorporation on the oxidative stability of mayonnaise: (A) peroxide value and (B) *p*-anisidine value. *Values are mean ± SD (n = 3). MRO = mayonnaise containing rapeseed oil (56%); MRSIO = mayonnaise containing 47% rapeseed oil and 9% sacha inchi oil without antioxidants*

3.2.2. Effect of antioxidants on oxidative stability of mayonnaise containing SIO

To improve the oxidative stability of MRSIO, different natural antioxidants were added during its preparation, and the PV and *p*-AV were also monitored during storage at 30°C (Figure 2).

The PV of the samples on day 0 ranged from 3.5 meq O_2/kg oil to 4.9 meq O_2/kg oil. It then changed at different rates depending on the treatment (Figure 2A). The PV of the mayonnaise without added antioxidant (MRSIO) was stable during the first 7 days of storage, whereas a significant decrease was observed for all other mayonnaises. From day 14, a significant increase in PV was observed for MRSIO and MRSIO-OSGT1. For the other mayonnaise samples an increase was observed from day 21. After 28 days of storage, the mayonnaise with the highest PV was MRSIO (12.6 meq O_2/kg oil) and that with the lowest was MRSIO-GTE (4.7 meq O_2/kg oil). The addition of GTE (1000 ppm), Phytrox (1000 ppm), OSGT (2000 ppm), and OSGT (3000 ppm) in the mayonnaise significantly lowered the PV by 62.4%, 25.6%, 51.1%, and 56.4%, respectively. Adding antioxidants in the mayonnaise reduced the rate of hydroperoxide formation except for OSGT at 1000 ppm, which did not slow down oxidation but seemed to show a prooxidant effect until day 21 of storage. The PV of MRSIO-GTE (4.7 meq O_2/kg oil), MRSIO-OSGT2 (6.1 meq O_2/kg oil) and MRSIO-OSGT3 (5.5 meq O_2/kg oil) were also lower than the PV of MRO (7.2 5 meq O_2/Kg oil), after 28 days at 30°C.

The *p*-AV of the mayonnaise samples also changed during storage (Figure 2B). On day 0, the *p*-AV ranged from 2.1 to 3.5, and all increased significantly during storage. Mayonnaises with the highest *p*-AV at the end of storage were MRSIO (10.0 ± 0.5) and MRSIO-OSGT1 (10.1 ± 0.6), with *p*-AV significantly higher than in the other samples. The *p*-AV of MRSIO-GTE, MRSIO-Phytrox, MRSIO-OSGT2 and MRSIO-OSGT3 were not significantly different on day 28 but were lower than that of MRSIO by 28.1, 23.4%, 22.1 and 31.1%, respectively.

Overall, the effect of the antioxidants in enhancing the oxidative stability of mayonnaise was in the decreasing order GTE ≥ OSGT (3000 ppm) > OSGT (2000 ppm) > Phytrox > OSGT (1000 ppm)

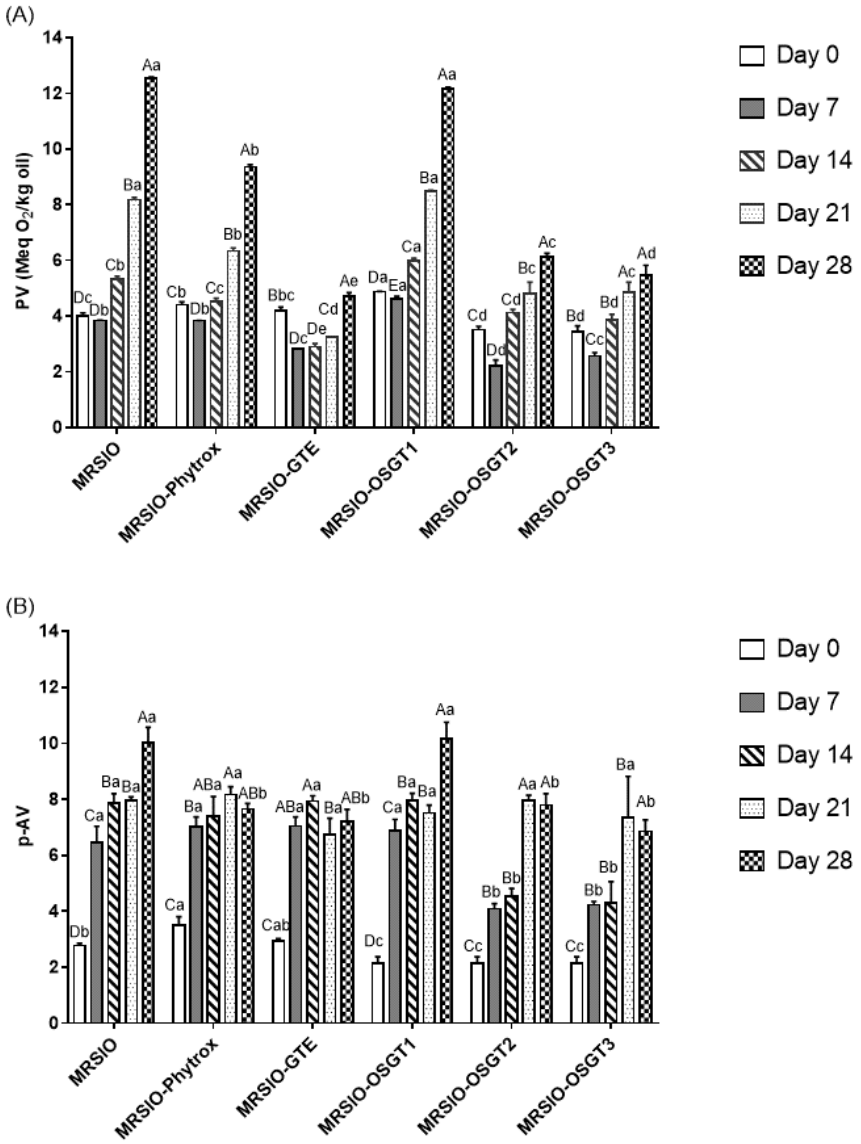

Figure 2. Change in peroxide value (PV)(A) and p-anisidine value (*p*-AV) (B) of mayonnaise containing a blend of rapeseed oil and sacha inchi oil (84:16) during storage at 30°C in open tubes*. Values are mean ± SD (n = 3). No common uppercase letter (A–E) indicates significant differences (p ≤ 0.05) during storage within mayonnaise treatment; no common lower-case*

letter (a-e) indicates a significant difference ($p \leq 0.05$) at a specific storage time between mayonnaise treatments. MRSIO = mayonnaise containing rapeseed (47%) and sacha inchi oils (9%) without antioxidants; MRSIO-Phytrox and MRSIO-GTE = MRSIO supplemented with Phytrox or green tea extract powder (GTE at 1000 ppm concentration. MRSIO-OSGT1, 2 and 3 = MRSIO supplemented with oil-soluble green tea extract 1000 ppm, 2000 ppm and 3000 ppm.

3.3. Sensory properties of mayonnaise

During the sensory analysis, panelists rated the mayonnaises on the basis of their appreciation of the taste, smell, colour, texture and overall acceptability. The sensory attributes were graded on a nine-point hedonic scale where 9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much, 1 = dislike extremely. The average scores for each mayonnaise are shown in Table 3.

The mayonnaise with rapeseed oil (MRO) had a significantly higher score ($p \leq 0.05$) in terms of taste and overall acceptability compared to MRSIO and MRSIO-Phytrox but was not significantly different ($p > 0.05$) from MRSIO-GTE. No significant difference was observed in the level of appreciation of the smell, colour and texture between the samples. The partial replacement of RO with SIO in the mayonnaise negatively impacted the taste, resulting in a lower score and incorporating GTE seemed to improve the taste of this mayonnaise.

Table 3. Sensory scores for mayonnaises enriched or not with sacha inchi oil and supplemented with natural antioxidants

Sensory attributes	MRO	MRSIO	MRSIO-GTE	MRSIO-Phytrox
Taste	7.0 ± 1.4^{a}	5.1 ± 2.0^{c}	6.0 ± 2.0^{ab}	5.3 ± 2.0^{bc}
Odour	6.8 ± 1.3^{a}	6.8 ± 1.4^{a}	6.7 ± 1.5^{a}	6.6 ± 1.6^{a}
Colour	7.3 ± 1.2^{a}	7.2 ± 1.2^{a}	7.1 ± 1.4^{a}	7.2 ± 1.1^{a}
Texture	7.4 ± 1.2^{a}	7.2 ± 1.2^{a}	7.0 ± 1.4^{a}	7.2 ± 1.3^{a}
Overall acceptability	7.0 ± 1.4^{a}	5.7 ± 2.0^{b}	6.3 ± 1.8^{ab}	5.8 ± 2.0^{b}

Values are mean ± SD *(n = 65). No common superscript letter (a-c) indicates a significant difference (p ≤0.05) between mayonnaises for each attribute. MRO = mayonnaise containing rapeseed oil (56%); MRSIO = mayonnaise containing rapeseed (47%) and sacha inchi oils (9%) without antioxidants; MRSIO-GTE and MRSIO-Phytrox = MRSIO supplemented with green tea extract powder (GTE) or Phytrox at 1000 ppm concentration.*

4. Discussion

The oils commonly used for the production of mayonnaise include soybean, rapeseed, sunflower, and corn oils. In this study, mayonnaises were prepared with RO (MRO) or a blend of RO and SIO (MRSIO). As expected, partially substituting RO with SIO resulted in an increase in the PUFA content of the mayonnaise, with ALA increasing by 48%. The Belgian Superior Health Council recommends a daily intake of ALA of 1% of the total energy intake, which implies 2.2 and 2.8 g/day for adult women and men, respectively, based on daily energy requirements of 2000 kcal for women and 2500 kcal for men (SHC, 2016). However, according to the 2014 food consumption survey of the Belgian population, intake of ω-3 fatty acids represents, on average, 0.7% of the total energy (De Ridder et al., 2016). Enriching mayonnaise in ALA is, therefore, a means of increasing the consumption of this fatty acid, considering that Belgians eat, on average, 27 g of sauce per day (De Ridder et al., 2016). Consuming 27 g of MRSIO implies consuming 2.2 g of ALA, corresponding to 100% and 78.6% of the recommended daily intake for women and men, respectively.

As expected, the rate of oxidation was higher in MRSIO than in MRO. Edible oils with higher contents of PUFA are more susceptible to oxidation (Syed, 2016). Medina-Marroquín et al. (2018) observed an increase in PV from 2.91 to 14.36 meq O_2/kg of mayonnaise made from SIO after 31 days of storage at 32°C in sealed aluminium/polyethylene bags. Golmakani et al. (2020) observed a 45% decrease in rapeseed oil's oxidative stability index when blended with 34% linseed oil, also rich in ALA. Torri et al. (2019) also reported a decrease in the oxidative stability index of extra virgin olive oil from 38.9 hours to 9 hours when blended with 25% v/v perilla seed oil containing 60% of ALA.

To improve the oxidative stability of MRSIO, mayonnaise samples were prepared with added antioxidants, i.e. GTE, Phytrox and OSGT. The supplementation with antioxidants reduced the oxidation rate of the mayonnaise, with GTE and OSGT (3000 ppm) being the most potent. Green tea catechins prevent oxidation by

acting as radical scavengers and metal chelators. Ramsaha et al. (2015) assessed the ability of green tea infusate (0.1% w/w.), *Piper betle* (0.2% w/w.) and *Ocimum tenuiflorum* (0.2% w/w) extracts to retard lipid oxidation in mayonnaise containing 74.7% w/w sunflower oil during storage at 40°C in closed containers. They reported that *P. betle* and green tea were more effective than BHT in both food systems, while *O. tenuiflorum* though not as potent as *P. betle* and green tea infusate, also delayed lipid oxidation. Contrarily, Ghorbani Gorji et al. (2019) reported a prooxidant activity of GTE (0.05%) in a mayonnaise made with 80% wt. sunflower oil and stored in closed containers at 38°C for 60 days, with an increased hydroperoxide formation recorded for GTE-supplemented mayonnaise. Zhou and Elias (2013) had previously shown that EGCG could act as an antioxidant or prooxidant in oil-in-water emulsions depending on the concentration used and the pH of the emulsion. According to the polar paradox, lipophilic antioxidants such as tocopherols and ascorbyl palmitate are more active in polar systems like oil-in-water emulsions, given that the non-polar, lipid-soluble antioxidants will be located in the oil phase where oxidation propagates (Jacobsen et al., 2008). However, this was not the case in this study because although Phytrox contained a mixture of tocopherols, ascorbyl palmitate and rosemary extract, its antioxidant effect was less potent than that of the green tea catechins. Di Mattia et al. (2009) noted that the efficiency of antioxidants in an emulsion depends not only on the polarity but also on the antioxidants' capacity and rapidity in donating a hydrogen atom. The oxidation of lipids in mayonnaise is influenced by its low pH and the presence of iron. The iron content of egg yolk is high, and at low pH, iron dissociates from phosvitin, thereby becoming more available to catalyse oxidation (Jacobsen, 2016). The antioxidant effect of GTE and OSGT in mayonnaise may, thus, be associated with the metal-chelating activity of catechins.

The taste of mayonnaises containing SIO was less appreciated than that of mayonnaise containing only RO. SIO is characterised by an intense taste of dried fruit and seed, a slightly

nutty taste and herbal or green odour notes associated with volatile compounds in the oil (Gutiérrez et al., 2019; Ramos-Escudero et al., 2021). The incorporation of SIO in mayonnaise imparted these flavours, which were perceivable to the panellists. Among the mayonnaises with SIO, MRSIO-GTE had the highest score for taste, suggesting that GTE had a partial masking effect on the SIO flavours.

5. Conclusion

The effect of partially replacing RO with SIO in mayonnaise was assessed in this study, as well as the antioxidant effect of some natural extracts on the developed mayonnaise. The incorporation of SIO resulted in an increase in the PUFA content of the mayonnaise, with a decrease in the ω-6/ω-3 ratio from 2.5 to 1.5. However, it negatively affected the oxidative stability, taste and overall acceptability of the mayonnaise. Meanwhile, supplementing the mayonnaise with antioxidants enhanced the oxidative stability in the decreasing order GTE ≥ OSGT (3000 ppm) > OSGT (2000 ppm) > Phytrox > OSGT (1000 ppm). The results of this study indicate that SIO can be used to enrich mayonnaise in ALA (ω-3 fatty acid), but the addition of antioxidants is necessary to delay oxidation. Further studies are needed to improve the mayonnaise recipe and assess the effect of long-term storage on physical and oxidative stability.

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Chapter 5C. Oxidative stability of Omega-3 fatty acid-enriched fruits and vegetable-based crackers

Nain, C.W.; Berdal, G.; Page, M.; Larondelle, Y.

Abstract

This study investigated the effect of green tea extract (GTE) on crackers enriched with omega-3 fatty acids from linseed and DHA-rich oils during storage at 30°C for nine weeks. Three crackers treatments were produced with oil containing GTE at a concentration of 0%, 0.1% and 1%. The oxidative stability of crackers was evaluated by measuring the peroxide and *p*-anisidine values during processing and storage. The control crackers (no GTE added) showed a high oxidation rate, with the peroxide value of the lipid fraction going beyond recommended limits after one week of storage. Supplementing crackers with GTE either showed no significant effect or increased the formation of primary and secondary oxidation products, thus exhibiting a pro-oxidant effect. In addition, the PUFA content of GTE-supplemented crackers decreased significantly during storage.

Keywords: Omega-3 fatty acids, green tea extract, oxidation, crackers.

1. Introduction

The enrichment of foods in omega-3 fatty acids is of interest due to their beneficial effects on health and the lower amounts in our diet compared to omega-6 fatty acids. Humans evolved on a diet whose ratio of omega-6 to omega-3 fatty acids was close to 1, but with modern western diets and food processing, the intake of omega-6 fatty acids has increased, leading to a ratio of 16:1 (Simopoulos and DiNicolantonio, 2016; Vázquez et al., 2019). The omega-3 fatty acids include α-linolenic acids (ALA, C18:3), the simplest omega-3 fatty acid, obtained primarily from plant sources, such as linseed oil. Meanwhile, the long-chain omega-3 fatty acids, eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) are obtained from marine organisms, with oily fish being the most common source in the human diet (Rubio-Rodríguez et al., 2010).

Considering the importance of these fatty acids in the human diet, the Belgian Superior Health Council (SHC, 2016) recommends an ALA intake of 2.2 and 2.8 g/day for adult women and men, respectively. On the other hand, a consumption of 250-500 mg/day is recommended for EPA + DHA. Various food products enriched with omega-3 fatty acids have been developed to encourage the consumption of these fatty acids, including dairy products, beverages and baked goods (Jacobsen et al., 2013). However, one of the main challenges involved with the enrichment of foods with omega-3 fatty acids is their high sensitivity to oxidation, which is particularly an issue in baked goods, given that the products are subjected to high temperatures and exposed to air during processing (Hernandez, 2013). EPA and DHA can rapidly react with oxygen in a free radical reaction process in which hydroperoxides are formed. These hydroperoxides then rapidly degrade to a range of volatile ketones and aldehydes with very low sensory threshold values resulting in undesirable off-flavours, harmful products, as

well as a reduction in the level of the omega-3 fatty acids (Barrow et al., 2013; Choe and Min, 2006).

Various strategies are implemented to prevent or delay lipid oxidation, including minimizing the exposure of the omega-3 fatty acids to air, light, metal ions, and high temperatures in both oils and enriched foods (Taneja and Singh, 2012). In addition, microencapsulation of omega-3 fatty acid-rich oils (Barrow et al., 2013) and active packaging technologies (Tian et al., 2013) have been implemented. However, the use of antioxidants remains a common method to delay lipid oxidation. In our previous study, incorporating green tea extract (GTE) in DHA-rich oil reduced the formation of primary and secondary oxidation products (Nain et al., 2021).

This study investigated the effect of green tea extract (GTE) on the oxidative stability of omega-3 fatty acid-enriched crackers. In a previous study, Birchall et al. (2021) formulated crackers based on apples and root vegetables (carrot, beetroot, parsnip or Jerusalem artichoke) with linseed oil as the fat source. The level of oxidation in the crackers remained within acceptable limits during 2 to 3 months of storage at 30°C. In the present study, DHA-rich oil was added to the crackers recipe used by Birchall et al. (2021) to obtain at least 250 mg of DHA per serving of crackers (50 g). The effect of GTE (0.1% and 1% relative to the amount of oil) was assessed in the crackers during cooking and storage.

2. Materials and methods

2.1. Materials

DHA-rich oil (DHAO) made from deep-sea fish oil concentrate was kindly provided by Atrium Innovations Inc. (Antwerpen, Belgium and linseed oil (LSO), cold pressed and unrefined, was supplied by Alvenat (Achêne, Belgium). The fatty acid composition and oxidative status of oils are shown in Table 1.

The green tea extract (GTE), a powdered extract standardised to a minimum content of 50% polyphenols, 20% catechins, and 7% EGCG, was a product of Naturex (Avignon, France).

Carrots were obtained from the UCLouvain-Lauzelle farm (Louvain-la-Neuve, Belgium), and all other ingredients (apples, white quinoa, flaxseeds, and eggs) for the crackers were purchased from a local organic produce store.

Table 1: Fatty acid composition and oxidative status of DHA-rich oil (DHAO) and linseed oil (LSO) used for production of crackers

	Characteristics	DHAO	LSO
Fatty acids (mg/g)	C16:1cis9	2.0 ± 0.1	53.8 ± 0.3
	C18:0	-	40.9 ± 0.2
	C18:1cis9	1.4 ± 0.1	162.5 ± 0.4
	C18:2c9,c12	-	149.4 ± 0.8
	C18:3c9,c12,c15	-	554.7 ± 2.6
	C20:4c5,c8,c11,c14	6.1 ± 0.2	-
	C20:5c5,c8,c11,c14,c17 (EPA)	149.3 ± 4.4	-
	C22:5c4,c7,c10,c13,c16	15.4 ± 0.5	-
	C22:5c7,c10,c13,c16,c19	54.7 ± 1.3	-
	C22:6c4,c7,c10,c13,c16,c19 (DHA)	626.7 ± 16.1	-
	c24:5c9,c12,c15,c18,c21	1.5 ± 0.1	-
	c24:6c6,c9,c12,c15,c18,c21	11.1 ± 0.3	-
	Total SFA	3.4 ± 0.8	100.3 ± 0.5
	Total MUFA	4.9 ± 0.6	170.9 ± 0.5
	Total PUFA	873 ± 22.8	705.8 ± 3.2
Oxidative status	Peroxide value (meq O_2/kg)	2.0 ± 0.0	1.6 ± 0.1
	p-anisidine value	5.2 ± 0.3	-

Values are means of three measurements ± SD. SFA= Saturated fatty acids, MUFA= Monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

2.2. Preparation of Crackers

The crackers were prepared according to a recipe developed within the context of a master's thesis (Birchall et al., 2021). The crackers

were initially prepared with LSO as the fat source, but in the present study, both LSO and DHAO were used. The formulation was such that 565 g of the raw ingredients resulted in 100-110 g crackers (Table 2). Three different treatments were produced: crackers without GTE (control), crackers supplemented with GTE at 1000 ppm (GTE 0.1%), and crackers supplemented with GTE at 10,000 ppm (GTE 1%). The concentration of GTE added was relative to the oil fraction added in the crackers. The concentrations used were based on results obtained in previous investigations in baked products (biscuits and sponge cake), whereby 1% of GTE exhibited the best protective effect (Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009), and our investigations in oils with 0.1% (1000 ppm) GTE being effective.

Table 2. Basic cracker formula

Ingredients	Control (g)	GTE 0.1% (g)	GTE 1% (g)
Apple	150	150	150
Carrot	150	150	150
White quinoa, cooked	160	160	160
Egg white	90	90	90
Flaxseed, ground	10	10	10
Linseed oil (LSO)	4	4	4
DHA-rich oil (DHAO)	1	1	1
Green tea extract (GTE)*	-	0.005	0.05

Control = crackers without added antioxidant; GTE 0.1%, GTE 1% = crackers supplemented with 1000 or 10,000 ppm of GTE relative to the oil content. *GTE was added to the oils prior to the production of crackers.

The apples and carrots were sliced, weighed and pre-cooked together for about five minutes on low heat while stirring regularly to ensure uniform cooking. The flaxseeds were ground with the aid of a laboratory mill (Retsch ZM 200, Haan, Germany). The white quinoa was cooked in three times its volume of water until all the water was absorbed. The egg whites were carefully separated from the yolks. Once all the ingredients were prepared, they were weighed and mixed for three minutes in a blender to obtain a

homogeneous paste without lumps. The oils were then added, and the mixture was thoroughly homogenised. The resulting paste was then spread on a baking sheet to a thickness of about 0.5 mm, and the baking trays were placed in an oven (Memmert, Gena, UK) at 85°C for 18 to 10 hours until crisp and dry. The resulting crackers were then sealed in plastic bags of 55 g portions. The crackers were analysed for protein, total lipid and moisture contents, and an accelerated storage test was carried out to evaluate oxidative stability.

2.3. Proximate analysis

The moisture content was determined according to the official AOAC 967.03 and 930.15 methods by drying samples in an oven at 105°C for 16 h (AOAC, 1995). The total lipid content was determined by the Soxhlet analysis following the AOAC Official method 920.39. The protein content was determined by measuring the total nitrogen content of the crackers using the Dumas combustion method, and the total nitrogen was converted to protein by multiplying with a conversion factor of 6.25 (Simonne et al., 1997). The carbohydrate (including fibres) content was calculated by difference. The results are reported as g/100 g of crackers.

2.4. Oxidative stability study

An accelerated oven storage test was carried out at 30°C for up to 9 weeks. During the storage tests, the oxidative status of crackers was determined on Weeks 0, 1, 2, 3, 6, and 9 by measuring the peroxide and p-anisidine values. The oxidative status of cracker mixtures before heat treatment was also measured to determine the effect of cooking on oxidation.

Each cracker sample (50-55 g) was ground using the Retsch ZM 200 grinder (Haan, Germany) and transferred into a 250 ml Erlenmeyer flask. Next, hexane (150 ml) was added and stirred on a magnetic stirrer plate for 20 min, followed by decantation of the lipid extract into a clean flask. A second extraction was then continued with a

fresh portion of hexane. The lipid extracts were filtered, and hexane was evaporated in a rotary evaporator at 30°C (Rotavapor R-3000, Buchi, Switzerland). The lipid obtained was flushed with nitrogen for one minute to remove any traces of hexane.

The degree of oxidation of the extracted lipid fraction was determined by measuring the peroxide value (PV) according to the AOAC official method 965.33 (AOAC, 2000) and *p*-anisidine value (*p*-AV) according to the AOCS official method Cd 18–19 (AOCS, 1998).

2.5. Determination of the fatty acid composition of crackers

The fatty acid composition was analysed for freshly prepared crackers and crackers after 9 weeks of storage at 30°C.

The Folch method (Folch et al., 1957) was used to extract lipids from the crackers, with nonadecanoic acid (C19:0, Sigma-Aldrich) added as an internal standard to evaluate the efficiency of the extraction. The fatty acids were methylated, as earlier described by Mellery et al. (2016). The fatty acid methyl esters (FAME) were extracted with hexane, followed by the separation and quantification of the FAMEs by gas chromatography (GC).

The GC was equipped with an RT2560 capillary column (100 m × 0.25 mm, 0.2 µm film thickness, Restek), an automatic injector, and a flame ionisation detector (FID) set at 255°C. The carrier gas used was hydrogen at a constant pressure of 200 kPa. The oven temperature program was as follows: the initial temperature of the column was set at 80°C, increased at 25°C/min up to 175°C and held for 25 min; With a second increase at 10°C/min, the column temperature was raised to 200°C, which was held for 20 min and was followed by an increase at 10°C/min to 220°C, held for 5 min, followed by an increase at 10°C/min up to 235°C, with a holding time of 15 min and then a decrease at 20°C/min to the initial temperature of 80°C. The fatty acid peaks were identified and quantified by comparison of retention times and peak areas with those of pure FAME calibration standards

(Larodan, Solna, Sweden). Chromatograms were processed using Chromeleon 7 software (ThermoFisher Scientific, Waltham, MA, USA). The results are expressed in mg/g of oil.

2.6. Statistical analysis

The crackers were prepared in triplicate batches, and results are expressed as mean ± standard deviation. One-way analyses of variance (one-way ANOVA) were performed using GraphPad Prism 9.1.2 statistical software to assess the effect of treatments during oven storage and differences between treatments. The differences in protein, total lipid, and dry matter content of treatments were also achieved by ANOVA. Sample means were compared using Tukey's multiple comparison test, with statistically significant differences at $p \leq 0.05$. Also, a One-way ANOVA and Tukey's multiple comparisons were performed to assess differences in fatty acid profile during storage using JMP pro 16 software.

3. Results

3.1. Characteristics of crackers

The crackers had an average dry matter content of 95% wt. The total fat content of the crackers ranged from 9.7-10.8% wt., while the protein content was 16.5-17% for all samples. No significant difference was observed in the composition between different crackers treatments (See Table 3).

Table 3. The proximate composition of crackers (g/100 g)

Parameter	Control	GTE 0.1%	GTE 1%
Moisture	5.01 ± 1.10[a]	5.2 ± 3.19[a]	3.79 ± 0.43[a]
Total fat	10.72 ± 0.14[a]	10.42 ± 0.05[a]	9.84 ± 0.18[a]
Protein	17.30 ± 0.63[a]	17.23 ± 0.55[a]	16.93 ± 0.26[a]
Carbohydrates	66.97	67.15	69.47

Control = crackers without added antioxidant; GTE 0.1%, GTE 1% = crackers supplemented with 1000 or 10,000 ppm of GTE relative to the oil content. Carbohydrates = 100- (moisture content + Total fat + protein content).

3.2. Effect of cooking on the oxidative stability of crackers

The PV and *p*-AV of the lipid fraction of cracker mixtures before and after cooking at 85°C for 10 hrs are shown in Figure 1. Before cooking, the PVs of the lipid fractions were in the range of 4.2-4.6 meq O_2/kg oil, with no significant difference observed among treatments. The PV of all treatments significantly increased after the cooking process (Figure 1A). However, the PV of treatment with GTE 0.1% (19.2 ± 5.2 meq O_2/kg oil) was significantly higher than that of the control (11.2 ± 0.4 meq O_2/kg oil), and GTE 1% supplemented samples (10.6 ± 0.6 meq O_2/kg oil). There is no set PV limit for food products. However, considering the recommended PV limits of 15 meq O_2/kg for virgin oils (Codex, 1999) and 5 meq O_2/kg for fish and DHA-rich oils (Codex, 2017), the crackers supplemented with GTE 0.1% exceeded both limits after the cooking process.

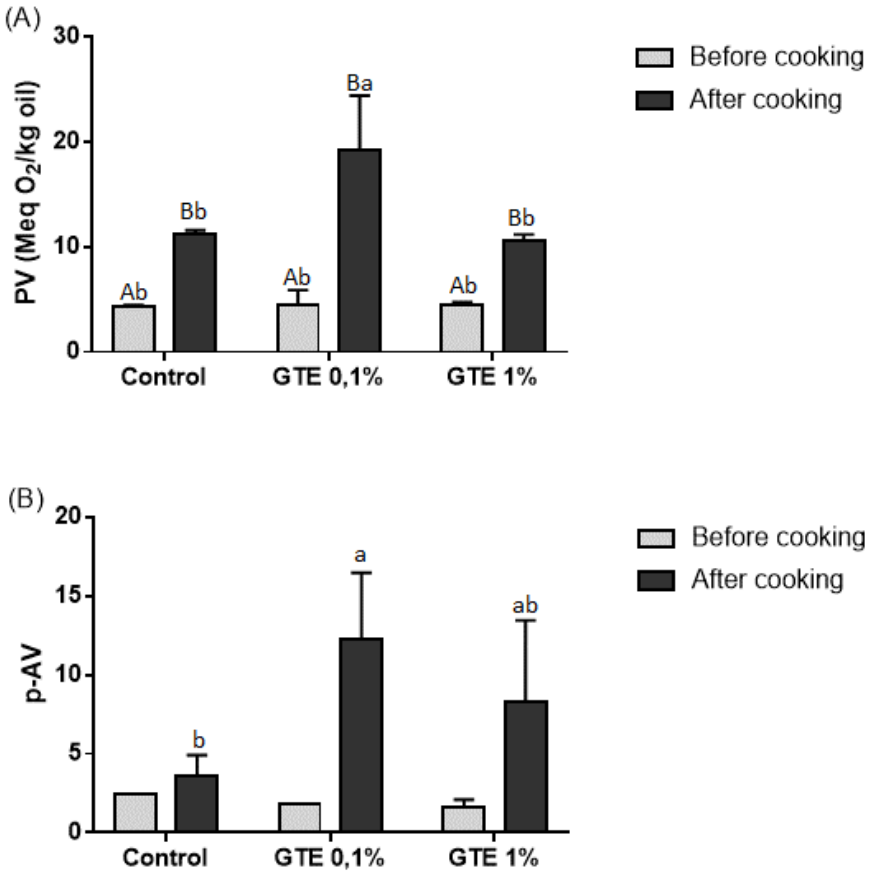

Figure 1. Effect of cooking (85°C, 10 hours) on the peroxide value (PV) (A) and p-anisidine value (p-AV) (B) of the lipid fraction of crackers supplemented or not with green tea extract (GTE). *Values are mean ± SD (n = 3) or means for p-AV control and GTE 0.1% before cooking. Different capital letters (A or B) indicate a significant difference ($p \leq 0.05$) before and after cooking within a sample; no common lower-case letters (a or b) among samples indicate a significant difference ($p \leq 0.05$) at a specific cooking time. Control = crackers without added antioxidant; GTE 0.1%, GTE 1% = crackers supplemented with 1000 or 10,000 ppm of GTE relative to the oil content.*

Regarding the *p*-AV, the values were between 1.5 and 2.5 before cooking and increased in all treatments after the cooking process (Figure 1B). Again, the crackers supplemented with GTE 0.1% had a higher *p*-AV (12.2 ± 4.2), which was 70.9% and 31.9% higher than the p-AV of the control and GTE 1% supplemented samples,

respectively. There is no p-AV limit for food products, but rancidity is noticeable for *p*-AVs above 20, which is also the recommended limit for *p*-AV in fish oils (Codex, 2017). This limit was not exceeded for any of the samples after the cooking period.

3.3. Oxidative stability of crackers during storage

The changes in PV and p-AV of crackers stored at 30°C for over 9 weeks are presented in Figure 2. The PV of all treatments increased during the storage period (Figure 2A). However, during the first 2 weeks of storage, the PV increase was not significant in any of the treatments. A significant increase in PV was observed for GTE 0.1% and GTE 1% supplemented samples after 3 weeks of storage and remained relatively stable up to 9 weeks. On the other hand, the PV of the control significantly increased after 6 weeks of storage, followed by a slight decrease on week 9. Comparing the PV among treatments, we observed that the PV of crackers supplemented with GTE 0.1% was higher than the others during the first week of storage, thereafter, no significant differences were detected among the treatments up to week 9.

The *p*-AV of all treatments also increased significantly during the storage period (Figure 2B), with a similar trend to that observed for PV. No significant difference was observed in the p-AV of any treatment during the first 2 weeks of storage. At week 3, a significant increase in *p*-AV was observed in all samples, which became even more prominent on week 6, except for the GTE 0.1% supplemented sample, which did not significantly change between weeks 3 and 6. Among the treatments, the p-AV of samples supplemented with GTE 0.1% was higher throughout the storage period, although no significant difference was observed with other treatments after week 6.

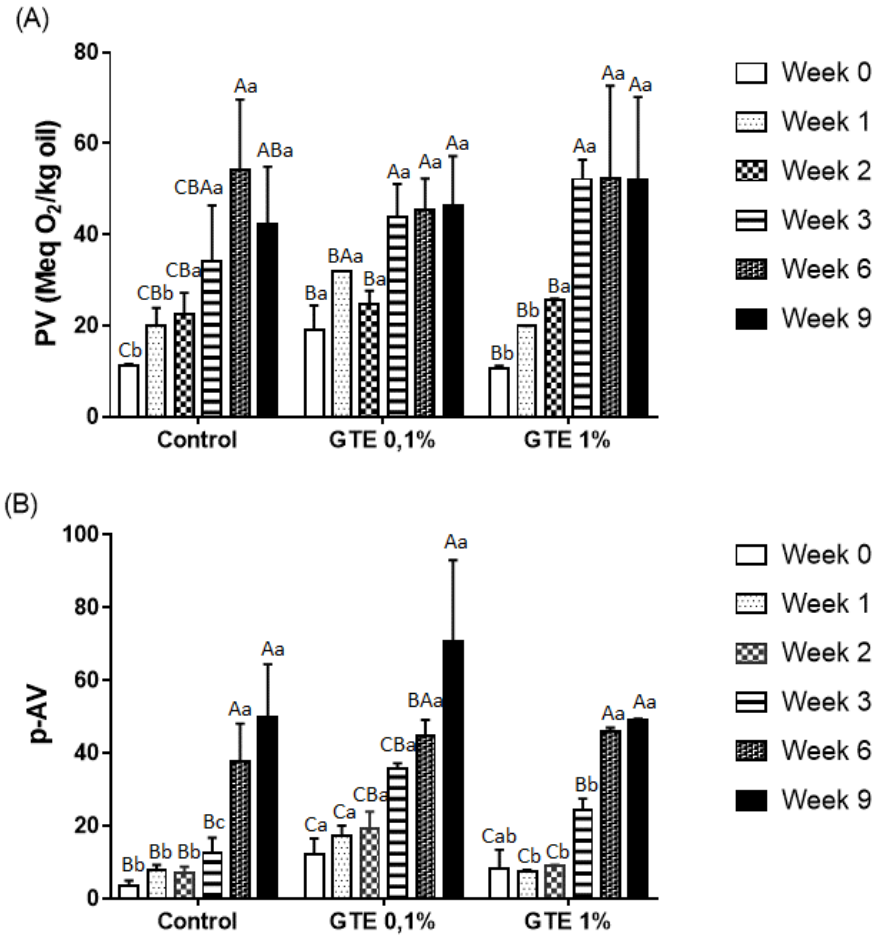

Figure 2. Changes in the peroxide value (PV) (A) and *p*-anisidine value (p-AV) (B) of the lipid fraction of DHA-enriched crackers supplemented or not with green tea extract (GTE) during an oven storage test at 30°C. *Values are mean ± SD (n = 3). No common capital letters (A-C) indicate significant differences ($p \leq 0.05$) during storage within a sample. No common lower-case letters (a-c) among samples indicate a significant difference ($p \leq 0.05$) at a specific storage time. Control = crackers without added antioxidant; GTE 0.1%, GTE 1% = crackers supplemented with 1000 or 10,000 ppm of GTE relative to the oil content.*

3.4. Fatty acid composition of crackers during storage

The fatty acid content of crackers was determined after production (week 0) and after 9 weeks of storage at 30°C. The changes in the content of major polyunsaturated fatty acids (PUFA) during storage are presented in Table 4.

The main PUFAs in the crackers were ALA, linoleic acid, DHA and EPA in decreasing order of concentration. On week 0, the fatty acid content of the control crackers was significantly lower than that of GTE 0.1%, and GTE 1% supplemented samples. For reference, 1 g of DHA-rich oil (626 mg of DHA and 149 mg of EPA) was used in a recipe that resulted in 100 g of crackers. Therefore, crackers are expected to have a DHA and EPA content of about 6.2 mg/g and 1.4 mg/g, respectively, which was the case for GTE 0.1% and GTE 1% supplemented samples. The content of DHA and EPA in the control crackers was less than expected by about 18% on week 0.

After 9 weeks of storage at 30°C, no significant change in fatty acid content was observed for the control, which was not the case for the GTE-supplemented samples. For the GTE 0.1% supplemented crackers, the content of linoleic acid, ALA, EPA, DHA and total PUFAs decreased after the 9-week storage by 17.6%, 22.7%, 40%, 40.9% and 23%, respectively, with no significant change observed in the saturated (SFA) and monounsaturated fatty acid (MUFA) contents. A significant decrease in fatty acid content was also observed for the GTE 1% supplemented crackers, with a loss of 23.6%, 25.7%, 35.7%, 38.7% and 26.4% for linoleic acid, ALA, EPA, DHA and total PUFAs, respectively. In addition, a decrease in MUFA content of 12.5% and 19.1% was recorded for the GTE 0.1%, and GTE 1% supplemented samples, respectively. The saturated fatty acids in the GTE-supplemented samples also slightly decreased, which was surprising given that saturated fatty acids were expected to be stable.

Table 4. Change in fatty acid content (mg/g) of omega-3 fatty acid-enriched crackers supplemented with green tea extract (GTE) during 9-week storage at 30°C.

	Control		**GTE 0.1%**		**GTE 1%**	
	Week 0	Week 9	Week 0	Week 9	Week 0	Week 9
Linoleic acid	19.2 ± 1.4^{Ac}	20.4 ± 1.1^{Aa}	24.03 ± 1.5^{Aa}	19.8 ± 1.8^{Ba}	25.0 ± 0.3^{Aa}	19.1 ± 1.4^{Ba}
ALA	41.0 ± 2.6^{Ab}	43.4 ± 4.4^{Aa}	51.2 ± 3.6^{Aa}	39.6 ± 6.9^{Ba}	51.7 ± 1.46^{Aa}	38.4 ± 4.61^{Ba}
EPA	1.1 ± 0.1^{Ab}	1.1 ± 0.2^{Aa}	1.5 ± 0.1^{Aa}	0.9 ± 0.3^{Ba}	1.4 ± 0.0^{Aa}	0.9 ± 0.3^{Ba}
DHA	5.1 ± 0.3^{Ab}	4.7 ± 0.7^{Aa}	6.6 ± 0.3^{Aa}	3.9 ± 1.3^{Ba}	6.2 ± 0.1^{Aa}	3.8 ± 1.1^{Ba}
∑ SFA	9.1 ± 0.7^{Ab}	9.9 ± 0.7^{Aa}	11.2 ± 0.7^{Aa}	9.8 ± 0.7^{Aa}	11.5 ± 0.3^{Aa}	9.3 ± 0.4^{Ba}
∑ MUFA	16.9 ± 1.1^{Ab}	18.4 ± 1.3^{Aa}	21.1 ± 1.3^{Aa}	18.0 ± 1.6^{Aa}	21.3 ± 0.4^{Aa}	16.7 ± 0.9^{Ba}
∑ PUFA	67.2 ± 4.1^{Ab}	70.2 ± 5.7^{Aa}	84.3 ± 5.3^{Aa}	64.9 ± 10.2^{Ba}	85.2 ± 1.60^{Aa}	62.7 ± 7.5^{Ba}
Total fatty acids	93.2 ± 5.9^{Ab}	98.5 ± 7.7^{Aa}	116.6 ± 7.3^{Aa}	92.7± 12.5^{Ba}	118.0 ± 2.3^{Aa}	88.7 ± 8.8^{Ba}

Values are mean ± SD (n = 3). Different capital letters (A or B) within a sample indicate a significant difference ($p < 0.05$) between week 0 and week 9 for a specific fatty acid; Different lower-case letters (a-c) within a row indicate significant differences ($p < 0.05$) among samples at a specific storage time for a specific fatty acid. *Control = crackers without added antioxidant; GTE 0.1%, GTE 1% = crackers supplemented with 1000 or 10,000 ppm of GTE relative to the oil content.* ALA=alpha-linolenic acid, DHA= docosahexaenoic acid, EPA= eicosapentaenoic acid, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

4. Discussion

The incorporation of GTE in the crackers either showed no significant effect on oxidation or resulted in a pro-oxidant effect. Indeed, higher levels of hydroperoxides and secondary oxidation products were detected in the GTE 0.1% treatment, whereas the accumulation of oxidation products in the GTE 1% treatment showed no significant difference from the control. This suggests that the concentration of GTE added to the crackers might have influenced the oxidation process. Logically, a higher concentration of GTE is more "protective" against oxidation, as it contains more catechins. Mildner-Szkudlarz et al. (2009) investigated the antioxidant effect of GTE (0.02%, 0.1% and 1%) in biscuits stored in foil packs at 60°C for 20 days and reported that GTE slowed down the formation of hydroperoxide, with the 1% concentration giving the best results. In a similar study, sponge cakes supplemented with GTE (0.02%, 0.2% and 1%) and stored in plastic bags at 63°C for 28 days showed far lower increases in PV and *p*-AV compared to the samples with no added antioxidants and GTE at a concentration of 1% again exhibited greater resistance to oxidation (Kozłowska et al., 2019). However, the lipid fraction in the studies on biscuits and sponge cake was provided by margarine, with a low PUFA content in contrast to the high PUFA oils used in the present study.

Linseed oil and DHA-rich oil used in the crackers are prone to oxidation due to their high degree of unsaturation. For example, Cosgrove et al. (1987) reported that the oxidizability of DHA (22:6) was five times greater than that of linoleic acid (18:2) based on the rate of oxygen uptake and in another study, α-linolenic acid was found to be 2.4 times more reactive than linoleic acid (Frankel, 2012). Therefore, incorporating DHA-rich oil in the crackers is undoubtedly a key factor in the oxidative instability of the product. In a previous study on similar crackers containing as lipid fraction only linseed oil supplemented with 0.1% GTE, Birchall et al. (2021) obtained a PV of 16.1 meq O2/kg oil after three months of storage

at 30°C. Meanwhile, the crackers in the present study already exceeded that PV after cooking or after 1 week of storage 30°C. Therefore, the difference in the oxidation pattern is largely due to the addition of DHA-rich oil in the current preparations.

The pro-oxidant effect of GTE at 0.1% in the crackers contrasts with the antioxidant activity of GTE observed in the DHA-rich oil (Nain et al., 2021). However, antioxidant efficacy is influenced by many factors, such as oil quality, concentration of antioxidants, temperature, pH of the food, availability of oxygen, and presence of metal ions (Ananingsih et al., 2013). Moreover, the reactivity of an antioxidant in a food system can increase or decrease due to interactions with other compounds present in the food system (Taneja and Singh, 2012). For example, in a previous study, Huang and Frankel (1997) found that catechins showed an antioxidant effect in corn oil oxidised at 50°C but acted as pro-oxidants in corresponding corn oil-in-water emulsions. The authors suggested that in the emulsion system, traces of metals present in the corn oil may be reduced (e.g. $Fe^{3+} \rightarrow Fe^{2+}$) more effectively by catechins, which may then become pro-oxidants. In like manner, the pro-oxidant effect of GTE observed in the present study may suggest an interaction of catechins with other compounds. Linseeds and quinoa used as ingredients in the crackers contain considerable amounts of iron, 59.9 mg/kg and 81 mg/kg, respectively (Ruales and Nair, 1993; Szyczewski et al., 2016), which might have been reduced by catechins. The reduced forms of transition metals (Fe^{2+}, Cu^{+}) accelerate lipid oxidation by catalysing the decomposition of hydroperoxides to lipid alkoxyl radicals (Ahmed et al., 2016; Lambert and Elias, 2010). In addition, catechins can form covalent or non-covalent bonds with proteins, forming protein-polyphenol complexes (Ishii et al., 2008; Li et al., 2021). These complexes may reduce or increase lipid and protein oxidation depending on the concentration of polyphenols and environmental conditions (Tian et al., 2021). The crackers in the present study contained about 17% wt. of proteins, which implies the formation of catechins-

protein complexes was possible. Hence, the antioxidant efficacy of catechins in GTE could be diminished by their interaction with other active compounds in the crackers. Apart from the possible interactions mentioned above, the ingredients used in the crackers also contained other antioxidants that could influence the catechins' activity. The crackers were made of carrots, which are rich in antioxidants such α- carotene and β-carotene, and apples, which contain various antioxidants including quercetin, catechin, phloridzin and chlorogenic acid (Stahl, 2016; Boyer and Liu, 2004). The presence of successive conjugated double bonds in carotenoid molecules may impart a prooxidant character and make them very susceptible to attack by free radical species. This explains why β-carotene has been reported to act as a pro-oxidant in certain circumstances, including high oxygen tensions, high carotenoid concentrations and environments that are poor in other antioxidants, while combination with other antioxidants offer a better overall protection (Palozza et al., 2011). With the different antioxidants present in the crackers, various interactions could have been possible. However, we did not quantify nor analyse the changes in the concentration of these antioxidants during the preparation and storage of the crackers.

Also, exposure of PUFAs to high temperatures increases the lipid autoxidation rate (Dridi, 2016). The crackers in this study were cooked at 85°C for 10 hours. The cooking conditions and the high surface area of crackers probably acted as catalysts for the oxidation reactions. García-Moreno et al. (2013) investigated the effect of temperature on the stability of cod liver oil using the Rancimat method, and they observed that at an airflow of 25 L/h, the oxidative stability index (induction time) of the oil decreased significantly with temperature from 17.46 hours at 60°C to 1.52 hours at 90°C. Higher temperatures, therefore, accelerate the oxidation process. In addition, the catechins in GTE can undergo degradation, oxidation, epimerisation and polymerisation reactions during food processing, resulting in a decrease in the total

catechins content and, thus, a reduction in its antioxidant activity (Ananingsih et al., 2013).

The crackers were formulated to contain, per 100 g, about 5 g of ALA and 600 mg of DHA. The lower level of fatty acids in the control crackers might suggest that less oil was incorporated during the preparation phase or might indicate a loss in fatty acids in this sample during cooking. A decrease (≈25%) in the PUFA content of the GTE-supplemented crackers was detected during the storage period. In a previous study, we observed an 8% decrease in the EPA and DHA content of DHA-rich oil after 9-week storage at 30°C, with the fatty acid content of GTE-supplemented samples remaining relatively stable (Nain et al., 2021). Meanwhile, in similar crackers containing only linseed oil as lipid fraction, Birchall et al. (2021) noted no significant change in ALA content after 3 months of storage at 30°C.

5. Conclusion

This study investigated the effect of GTE on crackers enriched with omega-3 fatty acids by including linseed and DHA-rich oils in the recipe. The crackers showed a high oxidation rate, with PV of the lipid fraction going beyond recommended limits after 1 week of storage at 30°C. Supplementing crackers with GTE either showed no significant effect or increased the formation of primary and secondary oxidation products, thus exhibiting a pro-oxidant effect. In addition, the PUFA content of GTE-supplemented crackers decreased significantly during storage.

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Chapter 6. General discussion and conclusion

General discussion

The importance of PUFAs in the human diet cannot be overemphasized. The consumption of these fatty acids provides various health benefits and is essential for the proper functioning of the human system. Despite dietary recommendations promoting the intake of PUFAs, especially omega-3 fatty acids, the level of consumption in the western diet remains low. The oils rich in PUFAs are very susceptible to oxidation, posing a major challenge in handling and thus limiting their incorporation in frequently consumed products. Oxidation of the unsaturated fatty acids results in off-flavours, loss of nutrients and the formation of toxic compounds. Therefore, healthy PUFAs can easily become unhealthy if care is not taken. Various strategies can be used to prevent or delay oxidation in these oils, including incorporating antioxidants.

The main objective of this thesis was to investigate the impact of green tea catechins on the oxidative stability of oils with a high PUFA content. To achieve this objective, the experiments carried out in this thesis allowed us to investigate:

1. the effect of GTE in different oils rich in PUFAs, such as DHA-rich oil, linseed oil, and sacha inchi oil,
2. the role of major catechins and catechins profile on the antioxidant activity of GTE,
3. the effect of GTE on PUFAs when incorporated in more complex food matrices such as salad dressings, mayonnaise and crackers.

The major outcomes of the investigations are discussed in the paragraphs below.

Supplementing PUFA-rich oils with green tea extract improved oxidative stability.

As mentioned above, there is great interest in improving the oxidative stability of PUFA-rich oils. We investigated the effect of incorporating GTE in DHA-rich oil (62% DHA and 14.9% EPA), linseed oil (55.4% ALA) and sacha inchi oil (48% ALA).

Lipid oxidation leads to the formation of hydroperoxides in the initial stages of oxidation, which increases until it reaches a maximum and then declines at the later stages of oxidation because of decomposition to secondary products such as aldehydes and ketones (Choe and Min, 2006). The early stage of oxidation is characterised by a decrease in antioxidants and a slow increase in oxidation products (induction phase). The time needed to reach the maximum level of hydroperoxides depends on the degree of unsaturation of fatty acids, the amount of antioxidants initially present in the matrix, the presence of pro-oxidants, the availability of oxygen and the temperature to which the lipid is exposed (Dagmey, 2020; Semb, 2012). It happens fast in highly unsaturated lipids because their hydroperoxides decompose more quickly. At the later stage, the hydroperoxides' decomposition rate surpasses the formation rate. This implies that the primary oxidation products will dominate in the early stage, and secondary oxidation products will be more in the later stages of oxidation. This also means oils might have the same PVs but are in different stages of oxidation. Therefore, to get a clear picture of the level of oxidation of oils in this study, we measured both primary and secondary oxidation products by monitoring the PV and p-AV, respectively.

The experiments presented in Chapters 3A, 3B, 3C and 4 have shown that GTE improved the oxidative stability of all three oils. The antioxidant activity of GTE was shown to be dose-dependent in DHA-rich oil, with the 1000 ppm concentration being the most effective (Chapter 3A). Incorporating this dose of GTE in linseed and sacha inchi oils also greatly reduced the formation of primary

and secondary oxidation products. These results corroborate previous studies that reported the antioxidant effectiveness of GTE in canola oil, marine oils, high linoleic sunflower oil and refined bleached and deodorized palm olein (Chen and Chan, 1996; Wanasundara and Shahidi, 1998; Womeni et al., 2016; Yin et al., 2012). The delay in oxidation observed in the different oils supplemented with GTE can be explained by a radical scavenging mechanism via hydrogen transfer (Banjarnahor and Artanti, 2015). This mechanism is characteristic of flavonoids, particularly catechins in this case. Based on the rules of redox reactions, any compound whose reduction potential is lower than a free radical can donate a hydrogen atom to this radical unless the reaction is kinetically unfavourable. The reduction potentials of the major catechins in GTE, that is, EC, EGC, ECG and EGCG, are 570, 430, 550 and 430 mV, respectively. On the other hand, peroxyl and alkoxyl radicals have reduction potentials of 600, 1000, and 1600 mV, respectively (Choe and Min, 2006, 2009). These catechins, therefore, easily donate hydrogen to these radicals formed during lipid oxidation, thus acting as free radical scavengers (Figure 1). In addition to the ability to transfer its hydrogen or electron, the effectiveness of an antioxidant is also determined by its reaction rate with free radicals in a system (scavenging rate constant) and the stability of the resulting antioxidant radical (Higdon and Frei, 2003). Catechins scavenge lipid radicals and become more stable phenoxy radicals, well known for their resonance stabilization and go on to form phenolic quinones.

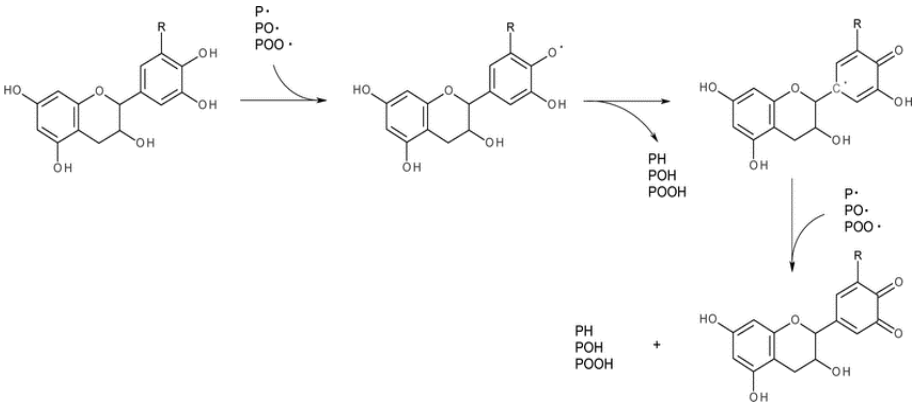

Figure 1. Mechanism of radical scavenging activity of catechins via hydrogen transfer (Agustin-Salazar et al., 2017)

Comparing the effect of GTE to that of α-tocopherol in the PUFA-rich oils, we observed that GTE acted as an antioxidant, whereas α-tocopherol was pro-oxidant in both DHA-rich oil and linseed oil. In previous studies, the antioxidant activity of GTE was shown to be more potent than that of α-tocopherol in marine oils (Wanasundara and Shahidi, 1998), as well as in cooked beef and chicken meat (Tang et al., 2001). Similar to our results, Mohanan et al. (2018) reported a pro-oxidant effect of α-tocopherol (50, 100, 200 and 400 ppm) in linseed oil samples stored at 25, 40 and 60°C for over 30 days. Also, supplementation of table spreads with α-tocopherol (250 ppm) resulted in pro-oxidant activity, with more hydroperoxides formed as compared to the control samples (Dwyer et al., 2012). The difference in the activity of GTE and α-tocopherol may be linked to their solubility/lipophilicity and relative portioning to the interfacial zone. The catechins in GTE are hydrophilic, whereas α-tocopherol is lipophilic. Based on a theory pioneered by Porter (1993), polar or hydrophilic antioxidants work better in non-polar matrices, and non-polar or lipophilic antioxidants work better in polar matrices. Frankel et al. (1994) postulated that this polar paradox was due to an interfacial phenomenon. Accordingly, in bulk oils, the increased effectiveness of hydrophilic antioxidants was attributed to their ability to migrate and concentrate at the air-oil interface where oxidation is

prevalent, whereas lipophilic antioxidants were solubilized in the oily phase, leading to less efficacy (Figure 2). In contrast, in an oil-in-water emulsion, non-polar antioxidants would concentrate in the oil-water interface (assumed to be the site where oxidation occurs) and inhibit oxidation more efficiently than polar antioxidants that partition into the water phase (Laguerre et al., 2015). However, it is difficult to predict the activity of an antioxidant on the sole basis of the polar paradox because the effectiveness of an antioxidant is also influenced by its concentration, presence of metals, metal binding constants, redox potential, antioxidant radical stability, and solubility (Laguerre et al., 2015; Zhong and Shahidi, 2012). For example, free-radical scavengers, like α-tocopherol, can inhibit lipid oxidation by producing low-energy oxidative species, but can also promote lipid oxidation when their redox potential is high enough to reduce metals into more pro-oxidative forms (Laguerre et al., 2015). In addition, tocopherol radicals at high concentration sometimes abstract hydrogen from lipids having a low concentration of peroxyl radicals and produce tocopherol and lipid radicals. The resulting lipid radicals can increase oxidation by reacting with triplet oxygen. So, in that case, tocopherol acts as a pro-oxidant instead of an antioxidant (Choe and Min, 2009; Kamal-Eldin, 2006).

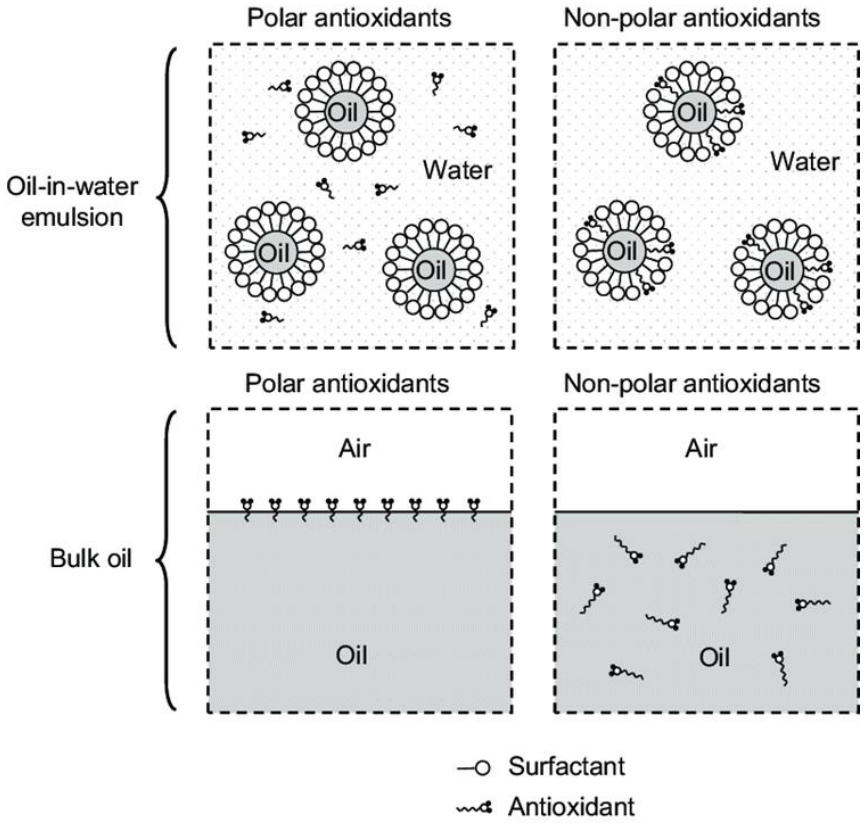

Figure 2. The effectiveness of antioxidants in different environments (Laguerre et al., 2015).

As expected, the rate of formation of the oxidation products was higher in DHA-rich oil than in linseed oil. For oils stored at 30°C, the hydroperoxides in non-supplemented linseed oil remained stable during the first 32-48 days of storage and secondary oxidation products were only detected after 96 days (≈13.5 weeks). Meanwhile, no induction period was observed for DHA-rich oil, with high peroxide values and fast accumulation of secondary products noted in the non-supplemented oil during the 9-week storage. On the other hand, the accumulation of oxidation products in sacha inchi oil was similar to that of linseed oil during accelerated storage for six days at 60°C. These results agree with previous studies indicating that the rate of oxidation in oils increases with the degree of unsaturation (Cosgrove et al., 1987;

Holman and Elmer, 1947; Syed, 2016). The oils under investigation in the present work are characterized by a high PUFA content, and a large percentage of fatty acids with at least three double bonds. DHA-rich oil contains long-chain omega-3 fatty acids, DHA and EPA, with six and five double bonds, respectively. On the other hand, the major fatty acid in linseed and sacha inchi oils is ALA, with three double bonds. The energy to extract hydrogen from the fatty acid depends on the position of the hydrogen in the molecule (Choe and Min, 2006). The bisallylic carbons, between two double-bonded carbon atoms, have a low activation energy for hydrogen loss and free radical formation. Thus, the ease by which fatty acid radicals are formed increases with an increased degree of unsaturation because the more double bonds there are, around a hydrogen atom, the less energy for its extraction (Holman and Elmer, 1947; Wang et al., 2017). DHA and EPA have more vulnerable bisallylic carbons (EPA : 4, DHA : 5) than ALA (2), explaining the higher susceptibility of DHA-rich oil to oxidation.

Supplementing omega-3 PUFA-rich oils with GTE, as shown in Chapters 3A and 3C, prevented or reduced losses in tocopherols present in the oils. The tocopherols in supplemented sacha inchi oil were stable during the 12 days of accelerated storage at 60°C. accordingly, the degradation of tocopherols was delayed in supplemented DHA-rich oil, and the level of depletion at the end of the storage period was lower than in the oil without GTE. A similar observation was made by Prabsangob and Benjakul (2018), who reported a delay in the frying-induced degradation of tocopherols in a soybean-tea seed oil blend supplemented with EGCG or EC. The effect of GTE supplementation on α-tocopherol might be linked to the nature of their interaction. A strong synergistic effect has been reported for the combination of α-tocopherol and GTE in an oil-in-water emulsion of methyl linoleate and pure bulk oil (Yin et al., 2012). Synergism was also observed in a ternary antioxidant system, including green tea polyphenols, α-tocopherol and ascorbic acid. Kinetic and mechanistic studies of the antioxidant process

revealed that this synergism was due to the regeneration of the α-tocopherol by green tea polyphenols and the latter by ascorbic acid (Dai et al., 2008). Green tea polyphenols regenerate α-tocopherol by reducing the α-tocopheroxyl radical (Choe and Min, 2009). The major catechins in the GTE used in this study are EGC and EGCG, each with a reduction potential of 430 mV. Both can thus easily donate hydrogen to regenerate α-tocopherol, which has a higher reduction potential of 480 mV. In addition, the hydrophilic catechins in GTE have a better affinity to the interface where oxidation takes place in bulk oils (Laguerre et al., 2015). Thus, they will be more active in scavenging free radicals and inhibiting the oxidation of fatty acids as well as lipophilic compounds such as α-tocopherol in the oil.

The antioxidant activity of GTE in PUFA-rich oils is influenced by its catechins profile and rate of degradation.

The antioxidant activity of GTE is attributed to its catechins content, with the major catechins in green tea being EGC, EC, EGCG, and ECG. Thus, to better understand the positive effect of GTE in PUFA-rich oils, it was important to investigate the role of the catechins.

The experiments in Chapter 4 compared the antioxidant activity of two GTEs, one rich in EGC and the other rich in EGCG, on the oxidative stability of a DHA-rich oil. The effect of the individual catechins was also shown. We observed that both GTEs enhanced the oxidative stability of DHA-rich oil. However, the EGC-rich extract showed a more potent effect, which is in line with the observation that EGC was the most effective in protecting the oil from oxidation among the individual catechins. The antioxidant activity of the individual catechins was shown to be in the order EGC250 > EGCG250 > ECG250 > EC250. Our results are similar to previous results in canola oil and corn oil (Chen and Chan, 1996; Huang and Frankel, 1997) but different from other studies that found EGCG or ECG to be the most potent catechins in oil or oil-

containing foods (Amarowicz and Shahidi, 1995; He and Shahidi, 1997; Lee et al., 2014; Wanasundara and Shahidi, 1996). The better antioxidant activity observed for EGC and EGC-rich GTE over EGCG might be related to their hydrophilicity. As indicated before, both EGC and EGCG have a reduction potential of 430 mV (Higdon and Frei, 2003), which implies they require a similar amount of energy for hydrogen transfer. Although catechins are hydrophilic, EGC is more hydrophilic than EGCG, evidenced by the faster elution of EGC on reversed-phase HPLC and a lower log P value of 1.46 for EGC compared to 3.08 for EGCG as predicted with computer software (Marczyński et al., 2018). Based on the polar paradox, EGC, which is more hydrophilic than EGCG, may tend to have better antioxidant activity in bulk oils.

The catechins content in the oil decreased during storage, as shown in Chapters 3A and 4. As the catechins depleted, the GTE's antioxidant activity decreased, which could be seen by an increase in the PV and p-AV of the oil. It was also observed that EGCG and EGC were more susceptible to degradation than EC and ECG. The decrease in catechins has been reported in various studies and attributed to degradation, epimerization, oxidation and/or polymerization (Ananingsih et al., 2013; Li et al., 2011; Wang and Zhou, 2004). As free radical scavengers, catechins donate hydrogen from the hydroxyl group, thus breaking the auto-oxidation chain. The resulting unpaired electron in the catechins is delocalized within the ring structure, resulting in a relatively unreactive free radical, which forms an o-quinone, followed by reactions such as oligomerization and intramolecular rearrangement (Tan et al., 2020). The catechins losses could be due to the combined effect of different reactions listed above (oxidation, degradation, epimerization, polymerization), with contributing factors being temperature and oxygen availability (Ananingsih et al., 2013). EGC and EGCG have the lowest reduction potentials (0.43V) compared to ECG (0.55V) and EC (0.57V), suggesting they are superior electron donors (Higdon and Frei, 2003). Being more effective

antioxidants may also imply a faster depletion rate of EGC and EGCG.

Incorporating green tea extract in complex food matrices enriched with omega-3 fatty acids can affect oxidation differently.

Various food matrices can be used to deliver omega-3 fatty acids to humans. Since GTE effectively protected the oils from oxidation, the experiments in Chapters 5A, 5B, and 5C investigated its effect on more complex food products such as salad dressings prepared with linseed oil, mayonnaise enriched with sacha inchi oil and crackers containing linseed and DHA-rich oils.

The incorporation of GTE enhanced the oxidative stability of the salad dressings and mayonnaise but showed a pro-oxidant effect in the crackers. Apart from differences in ingredients, the key difference between the processing method of crackers and emulsified products is the cooking step. Heat is known to catalyze oxidation, and catechins are also sensitive to high temperatures, which leads to an increase in the rate of epimerization and oxidation (Ananingsih et al., 2013). In contrast, catechins show good stability at a pH below 4 (Senanayake, 2013; Wang et al., 2017). This suggests that the catechins might have been more stable in the salad dressings and mayonnaise, which generally have low pH. In addition, the crackers contained ALA and DHA, which makes them more prone to oxidation. Antioxidants also react with compounds in the food system to which they are added, leading to an increase or decrease in their antioxidant activity (Taneja and Singh, 2012). Indeed, catechins can form complexes with proteins (Ishii et al., 2008; Li et al., 2021) and can interact with transition metals forming reduced metals, which catalyze decomposition of hydroperoxides into free radicals, such as peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$) radicals. The pro-oxidant effect observed in the crackers might be prevented by improving the recipe and adjusting the cooking temperature of the crackers.

Observations that require further investigation

Further studies are needed to understand better the activity of green tea catechins in PUFA-enriched foods, as well as to identify the degradation products of catechins.

Identifying the EGCG degradation product detected in DHA-rich oil supplemented with EGCG-rich GTE (GTE2) or EGCG (Chapter 4) might give a clue to the mechanism that led to its formation. Initial analysis by HPLC-ESI-HR-MS recorded no signals on MS, which we associated with the low concentration of the substrate. Furthermore, concentrating the catechins extract from these oil samples gave a cloudy viscous solution, which posed a problem for analysis by HPLC-MS. Therefore, a solid-phase extraction method should be put in place to purify the concentrated samples before proceeding with HPLC-MS analysis.

The crackers produced in the context of this thesis were cooked at 85°C for 10 hours, with the addition of GTE resulting in a pro-oxidant effect. In previous studies, the antioxidant activity of GTE was reported in biscuits baked at 205°C for 11 minutes and in sponge cake baked at 160°C for 45 minutes (Kozłowska et al., 2019; Mildner-Szkudlarz et al., 2009). Although the lipid fraction in the studies with biscuits and sponge cake was margarine with less PUFA, cooking at higher temperatures for a shorter period might have had less effect on the activity of GTE. Thus, instead of cooking the crackers at 85°C for a long period, a study should be carried out with cooking temperatures between 160 and 200°C with the assessment of the effect on oxidation, catechins and fatty acids.

We also observed that incorporating GTE (EGCG-rich) in salad dressings had a slightly destabilizing effect on the emulsion in a dose-dependent manner (Chapter 5A). Tian et al. (2021) observed a similar effect of tea polyphenols on the physical stability of Walnut oil-in-water emulsions. This effect is probably due to the interaction of catechins and proteins because proteins and

polyphenols have been shown to form complexes, which affect the emulsifying properties of proteins (Ishii et al., 2008; Li et al., 2021). In addition, some studies have reported a stronger affinity of gallated catechins (EGCG and ECG) to proteins, as compared to the non-gallated ones (EGC and EC), e.g. the affinity of EGCG towards human serum albumin was shown to be about 100 times stronger than the affinity of EGC (Minoda et al., 2010; Saeki et al., 2018). Therefore, incorporating the EGC-rich GTE in the salad dressing might have a lesser effect on the physical stability. Thus, further studies should be carried out to investigate the effect of the EGC-rich GTE on the physical stability of the salad dressing during storage. In addition to observing the emulsion separation, the changes in oil droplet size could also be assessed during the storage period.

Combining the addition of GTE with microencapsulation of oils may improve the oxidative stability of omega-3 PUFA-rich oils in complex food systems. Previous studies have shown that the form in which omega-3 PUFAs are added to foods, either as neat oil, microencapsulated oil or pre-emulsified oil, can affect product stability (Jacobsen, 2010; Let et al., 2007). Evaluating the oxidative stability of fish oil-enriched fish paté, Nielsen and Jacobsen (2013) showed that fish pâté with microencapsulated fish oil did not oxidize, whereas oxidation was slower in fish pâté with pre-emulsified oil compared to fish pâté with neat oil. Additional studies could be carried out to investigate the oxidative stability of foods enriched with microencapsulated GTE-supplemented PUFA-rich oils. However, the major disadvantage of using encapsulated oil will be the increase in production cost, as the encapsulation process can double the cost of the omega-3 ingredient (Hernandez, 2013).

Contribution and significance of our study

The studies carried out in this thesis demonstrated the effectiveness of GTE in protecting omega-3 PUFA-rich oils from

oxidation. Therefore, supplementing oils rich in omega-3 fatty acids with GTE could be a good strategy for the oil industry to reduce oxidation associated with handling such oils and increase shelf life. Furthermore, this could encourage the enrichment of more foods with omega-3 fatty acids, thus increasing the daily consumption to meet recommended values.

The DHA-rich oil used in our study is usually stabilized with a mixture of tocopherols, but as our results show, green tea catechins provide better protection to this oil than tocopherols. Thus, GTE could be a better choice of antioxidant for similar oils.

The oxidation of PUFAs leads to compounds with potential toxic effects, such as malondialdehyde, acrolein crotonaldehyde, 4-hydroxy-2-trans-nonenal, and 4-hydroxy-2-trans-hexenal. These compounds can react with proteins, phospholipids, and DNA, forming conjugates that may create abnormalities in protein functioning or damage the DNA (Ayala et al., 2014; Esterbauer et al., 1991; Long and Picklo, 2010). Although the compounds mentioned were not quantified in our study, we showed that supplementing PUFA-rich oils with green tea catechins reduces the formation of secondary oxidation products, which could therefore make the oils safer for consumers.

Besides the antioxidant activity of GTE, its antimicrobial effect has been reported in previous studies. For example, green tea catechins have demonstrated antimicrobial activities against foodborne pathogens such as *Enterococcus faecalis, Staphylococcus aureus, Escherichia coli and Salmonella typhimurium* (Liu et al., 2022; Mohebalian and Alizadeh, 2016). Therefore, supplementing PUFA-enriched foods with GTE will not only reduce oxidation but might also prevent microbial spoilage.

Adopting GTE as an antioxidant for PUFA-rich oils could also help reduce the waste generated during tea production. During the production process of tea, only the bud and the youngest leaves (2-3) are harvested. Thereafter, the plantations are pruned, and the

mature leaves are left on the ground as fertilizer. In addition, leaves with poor appearance during green tea processing are separated and sold as low-quality tea products or just discarded as tea waste (Athirojthanakij, 2020). These tea wastes contain significant amounts of catechins and thus could be better valorized through the production of GTEs (Chen et al., 2003; Debnath et al., 2021).

General conclusion

This thesis investigated the effect of green tea catechins on the oxidative stability of omega-3 PUFA-rich oils. Based on the results obtained, the following conclusions can be made.

Supplementing PUFA-rich oils, such as sacha inchi, linseed and DHA-rich oils, with green tea catechins enhances the oxidative stability of the oils by limiting the formation of primary and secondary oxidation products, limiting the degradation of fatty acids and inhibiting or reducing the losses in tocopherols. Furthermore, our finding that the oil-soluble green tea extract (OSGT) is also effective in these sensitive oils eliminates the need for solvents when supplementing PUFA-rich oils with green tea catechins.

The GTEs are effective antioxidants in complex food systems enriched with ALA, such as salad dressings and mayonnaise. The effect of GTE on the sensory properties of the products was minimal, with only the colour and physical stability of salad dressings slightly affected. However, a pro-oxidant effect of GTE was observed for crackers enriched with ALA and DHA, most likely due to the cooking process and interaction of catechins with other compounds in the matrix. Therefore, it is necessary to consider the cooking temperature and duration when incorporating GTE in products containing very sensitive PUFAs.

The catechins profile of GTE affects its antioxidant activity in oils. As observed in this study, the EGC-rich GTE was more effective in

DHA-rich oil than an EGCG-rich GTE, which was consistent with the effect of the individual catechins. Thus, this should be considered when choosing or preparing an extract for antioxidant application in PUFA-rich oils. In addition, the extracts showed a dose-dependent antioxidant activity, which means the concentration of catechins in the extract and quantity of extract added to oil or food product is vital to achieving a good antioxidant activity.

The results obtained in this thesis open new avenues for incorporating oil-soluble and water-soluble GTEs in oils and food products with high PUFA content.

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