UNIVERSITY OF SÃO PAULO

Faculty of Pharmaceutical Sciences

Graduate Program in Food Science

Area of Experimental Nutrition

Oxidative stability of *Echium plantagineum* oil

Gabriela Grassmann Roschel

Dissertation presented for the degree of
MASTER OF SCIENCE

Advisor: Prof. Dr. Inar Castro Erger

São Paulo

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“We pass through this world but once. Few tragedies can be more extensive than the stunting of life, few injustices deeper than the denial of an opportunity to strive or even to hope, by a limit imposed from without, but falsely identified as lying within.”
Stephen Jay Gould

“So do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand.”
Isaiah 41:10

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RESUMO

ROSCHEL, G. G. **Estabilidade Oxidativa do óleo de *echium plantagineum***. 2020. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2020.

As evidências do efeito cardioprotetor dos ácidos graxos ômega 3 (AG n-3), principalmente do ácido eicosapentenoico (EPA) e docosahexaenoico (DHA), tem aumentado o consumo desses ácidos graxos. *Echium plantagineum* é uma planta da família *Boragenacea*, conhecida como uma fonte potencial AG n-3 de origem não marinha. As sementes de Echium apresentam 12-16% de ácido estearidônico (SDA), que pode ser convertido em EPA e DHA a uma maior taxa que a obtida através do consumo do ácido alfa linolênico (ALA), presente em diversos óleos vegetais. Porém, o óleo de echium é extremamente suscetível à oxidação, por ter um alto teor de ácidos graxos poli-insaturados. Portanto, o objetivo desse estudo foi combinar três estratégias naturais para inibir a oxidação no óleo de echium. Na primeira parte do estudo, misturas contendo antioxidantes hidrofílicos (HM: ácido sinápico + ácido ascórbico + ácido cítrico) ou lipofílicos (LM: alfa-tocoferol + palmitado de ascobila + ácido cítrico) foram aplicados no óleo de linhaça, e mantidos a 40ºC por 15 dias. Os marcadores de oxidação foram comparados com óleo de linhaça no qual foram adicionados compostos artificiais: TBHQ (120 ppm) e EDTA (75 ppm). Os resultados mostraram que LM e HM apresentaram uma proteção antioxidante similar ao efeito apresentado pelos compostos artificiais, e que a mistura HM promoveu uma melhor proteção antioxidante que a mistura LM. A partir desse resultado, a mistura HM foi selecionada como estratégia a ser aplicada na etapa seguinte. Assim, na segunda parte do estudo, o óleo de echium foi obtido por dois processos de extração: prensagem mecânica continua (PRESS) e extração usando hexano (SOLV). A mistura HM e o óleo de girassol alto oleico foram selecionados como estratégias antioxidantes, além da redução de temperatura de estocagem. Duas condições foram analisadas: 6 meses em frascos fechados e 30 dias em frascos abertos. A oxidação foi quantificada através da determinação das concentrações de hidroperóxido, malonaldeído, tocoferol e compostos voláteis. No geral, os resultados mostraram que a redução de temperatura foi suficiente para manter a estabilidade do óleo durante o estoque. Portanto, objetivou-se combinar estratégias para aumentar a estabilidade oxidativa das amostras expostas ao oxigênio. Neste contexto, os melhores resultados foram obtidos quando 20% de óleo de girassol alto oleico foi combinado com a mistura hidrofílica de antioxidantes naturais (500 ppm de ácido sinápico, 250 ppm de ácido ascórbico e 150 ppm de ácido cítrico). Nessa condição, foi observada uma redução de 37-41% nos valores de hidroperóxidos e 40-75% na concentração de malonaldeído, quando comparado com a condição padrão.

Palavras-chave: Ômega 3, echium, oxidação, estearidônico, fenólicos, alto oleico

ABSTRACT

ROSCHEL, G. G. **Oxidative stability of *Echium plantagineum* oil**. 2020. Dissertation (Master) – Faculty of Pharmaceutical Science, University of São Paulo, São Paulo, 2020.

The evidences about the cardioprotective effects of omega-3 fatty acids (n-3 FA), especially EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), have increased the consumption of these fatty acids. *Echium plantagineum* is a plant from *Boragenacea* family, known as potential source of non-marine omega-3 fatty acids (n-3 FA). Echium seeds presents 12-16% of stearidonic acid (SDA), that can be converted to EPA and DHA at a more elevated rate than the conversion obtained from α-linolenic acid (ALA), present in several other vegetable oils. However, echium oil is highly susceptible to oxidation because it has a high proportion of polyunsaturated fatty acids. Thus, the objective of this study was to combine three natural strategies to inhibit the oxidative damage in echium oil. In the first step, a mixture containing hydrophilic (HM: synaptic + ascorbic + citric acids) or lipophilic (LM: α-tocopherol + ascorbyl palmitate + citric acid) antioxidants was applied in the flaxseed oil, kept at 40ºC/ 15 days. The oxidative markers were compared with the oil added of TBHQ (120 ppm) and EDTA (75 ppm), both artificial compounds. The results showed that LM and HM had an oxidative protection similar to the artificial antioxidants and that, HM promoted a better protection than LM. Based on this result, HM was selected as a strategy to be applied in the next step. In the second part of this study, Echium oil was obtained by two process: continuous screew pressing (PRESS) and extraction using hexane (SOLV). Both samples were added of HM combined with a high oleic sunflower oil and kept at different temperatures during storage. Two conditions were analyzed: 6 months into sealed flasks and 30 days into opened flasks. Oxidation reaction was followed by measuring the concentration of hydroperoxide, malondialdehyde, tocopherol and volatile compounds. In general, results showed that temperature reduction was enough to keep the oils stability during storage. Thus, the focus of the strategy's combination was directed toward samples after exposition to oxygen. In this context, better results were obtained by blending 20% of high oleic sunflower oil and the hydrophilic antioxidant mixture (500 ppm of synaptic acid, 250 ppm of ascorbic acid and 150 ppm of citric acid). In this condition it was observed 37-41% reduction in the hydroperoxide values and 40-75% in the malondialdehyde concentration in the samples prepared according to the optimized condition, when compared with the standard conditions by which the oil is currently extracted and processed.

Key-words: Omega 3, echium, oxidation, stearidonic, phenolics, high oleic

Abbreviations

$^{1}O_{2}$ (Singlet oxygen)

^{1}Sen* (Activated sensitive molecules)

$^{\bullet}$OH (Hydroxyl radical)

ALA (α-linolenic acid)

COX (Cicloxigenase)

CVD (Cardiovascular diseases)

DHA (Docosahexanoic acid)

EPA (Eicosapentanoic acid)

ESO (Echium oil)

FDA (Food and Drug Administration)

GLA (Gamma linolenic acid)

GMO (Genetic modified organisms)

L$^{\bullet}$ (Unsaturated lipid)

LO$^{\bullet}$ (alkoxyl radical)

LDL (Low denstity lipoprotein)

LNA (Linoleic acid)

LOX (Lipoxigenase)

LOO$^{\bullet}$ (Peroxyl radical)

LOOH (Hydroperoxides)

MCT (Medium chain tryglicerides)

MDA (Malondialdehyde)

MS (Mass spectrum)

n-3 FA (Omega 3 fatty acids)

n-6 FA (Omega 6 fatty acids)

NIST (National Institute of Standards and Technology)

PUFA (polyunsaturated fatty acids)

PV (Peroxide value)

SDA (Stearidonic acid)

Sen (Photosensitive molecules)

ROS (reactive oxygen species)

TEP (1,1,3,3-tetraethoxypropano)

TG (Triglycerides)

UV (Ultraviolet)

UV-DAD (Ultraviolet diode array)

R$^{\bullet}$ (Free radicals)

VLDL (Very low-density lipoprotein)

1. INTRODUCTION

Omega 3 fatty acids (n-3 FA) are polyunsaturated fatty acids (PUFA) that present well-established health effects in humans. These essential fatty acids take part in neural development, immune and inflammatory responses and can modulate several pathological conditions, including inflammatory bowel disease, arthritis and cardiovascular diseases (Siscovick et al., 2017).

The main commercial source of n-3 FA is fish oil (Tocher, et al., 2019). However, global demand for n-3 FA have significantly increased over the past few decades due to their health effects, which has raised concerns about the sustainability of sourcing these compounds from wild fish (Tocher et al., 2019). These concerns lead to a 'fish-oil arms race' between biotech companies seeking for alternative sources of n-3 FA, as transgenic oils, in order to protect fish species and the oceans' ecosystems (Adarme-Vega, Thomas-Hall and Schenk, 2014; Tocher et al., 2019).

Currently explored alternatives include plant oils with high n-3 FA content, algae oils and fed farmed fishes with microalgal biomasses (Ruiz et al., 2017; Tocher et al., 2019). Plants that are high in n-3 FA, such as flaxseed, primrose and hempseed, contain α – linolenic acid (ALA). However ALA needs to be *in vivo* converted in an active form of eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), to exert the health effects (Lenihan-Geels, Bishop and Ferguson, 2013; Ruiz et al., 2017). Algae-derived oils are vegetarian-friendly, easy to grow on a large scale due to their small size and lower contamination level (Shen et al., 2020). But the algae oil is still limited by cost, extraction, purification and stabilization methods (Lenihan-Geels, Bishop and Ferguson, 2013; Shen et al., 2020).

Echium plantagineum oil is n-3 FA naturally rich in, effective at providing long-chain n-3 FA to body tissues because it contains stearidonic acid (SDA), that can be converted 14-16% in EPA wile ALA can be converted only 5-6% (Prasad, et al., 2020). However, the fatty acids profile of echium oil makes it extremely unstable (Comunian et al., 2019), being necessary to provide strategies to prevent its degradation.

2. LITERATURE REVIEW

2.1. Omega 3 fatty acids

Cardiovascular diseases (CVD), usually manifested as coronary disease, angina, acute myocardial infarction and stroke, are the major cause of worldwide deaths, with atherosclerosis being the main process involved in the onset of these events (Benjamin, et al.,

2017). According to data from WHO (2016), about 15.6 million people die each year from cardiovascular diseases, with a projection of 23.6 million to 2030 (Lozano et al., 2012). The main cause of atherosclerosis is dyslipidemia, which is characterized by the increase of plasma total cholesterol and triglyceride levels and decrease of high density lipoprotein cholesterol, leading to the accumulation of fatty plaques in the arteries (Benjamin et al., 2017; Khan et al., 2020). This accumulation of fatty plaques increases the expression of pro-inflammatory molecules, undergoing more complex atheromas (Frostegart, 2013; Moore and Tabas, 2011; Weber & Noels, 2011; Khan et al., 2020).

N-3 FA are biologically active lipids with cardio protective effects, leading to the production of anti-inflammatory eicosanoids (Guertin, et al. 2018; Shabani et al., 2019). N-3 FA, such as ALA is an essential compound, not synthesized by humans and must be obtained from the diet. Most health effects of n-3 FA are mainly attributed to longer-chain n-3 FA, as EPA (20:5 n-3) and DHA (22:6 n-3), which are found in fish and krill oil (Rundblad, et al., 2018). The American Heart Association has recently published an updated meta-analysis showing that n-3 FA supplementation can reduce CVD risk and death, and the reduction risk is associated with increasing dose (Hu et al., 2019). However, as reported by Tocher et al., (2019), the traditional sources to supply EPA and DHA are wild fish or farmed fish, linking a gap between demand and supply. If the world's population follows fish intake recommendation, marine fish would become extinct due to the intensive fishing. Thus, new sources of n-3 FA must be found to answer the growing demand n-3 FA market and to meet the vegetarian and vegan population that has been growing in recent years (Tocher et al., 2019).

Sources of plant-derived n-3 FA include flaxseed (approximately 50% ALA, C18:3 n3), soybean (10% ALA), canola seeds and some nuts (Beker, et al., 2016). However, human synthesis of EPA and DHA from ALA is low (about 5%), because the conversion of ALA to EPA and DHA depends on the action of Δ-6 desaturase enzyme (Sayanova & Napier, 2011), as shown in **Figure 1.**

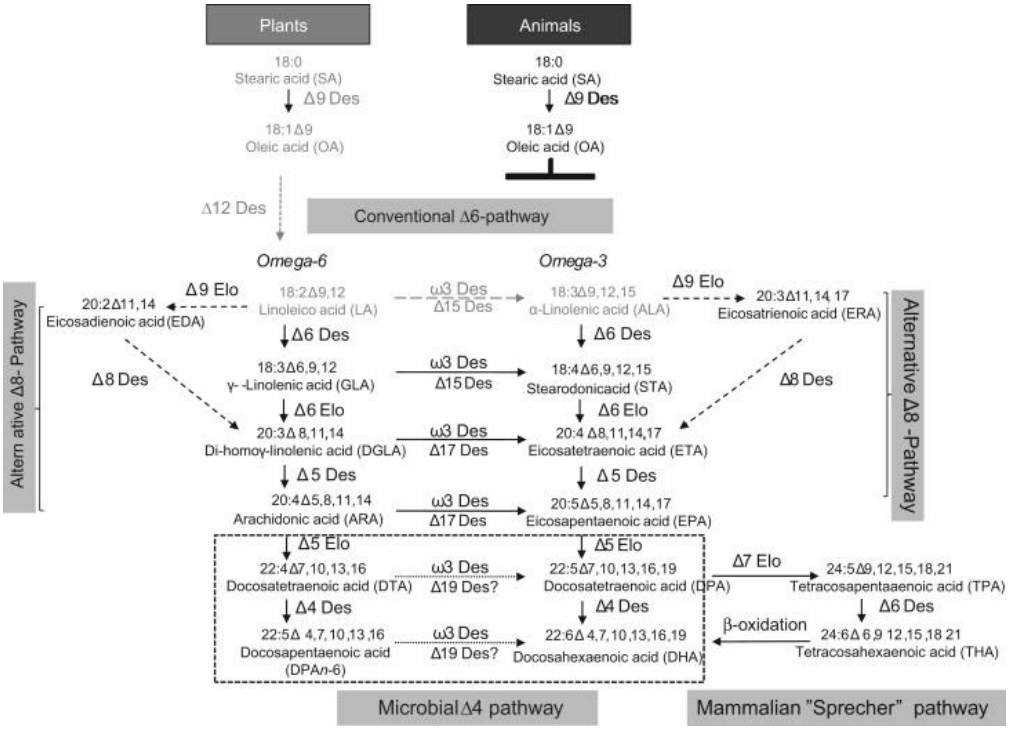

Figure 1: Simplified metabolism of polyunsaturated n-6 FA and n-3 FA (Adapted from Sayanova & Napier, 2011).

To overcome this limitation, the oil industry has started the production of transgenic plants that provide EPA and DHA enriched oils, such as Camelina oil (Petrie et al., 2014), by the addition of transgenic Δ-6 desaturase pathway, improving in 12% DHA content. Also, Monsanto Company developed an enriched soybean oil with stearidonic acid (SDA, C18:4 n3) (Hammond et al., 2008). Studies have shown that supplementation with these transgenic oils provide the right amount of EPA to the liver, in a similar concentration as obtained from fish oil intake (Hammond et al., 2008; Petrie et al., 2014). However, the production of genetic modified organisms (GMO) in some counties is still forbidden, allowing only to import from allowed countries, such as United Stated of America, Brazil, Argentina, Canada and India (GMO FAQ, 2020). In European Union is only allowed the production in Spain and Portugal. In Russia and some countries of Latin America and Africa both production and importation have still been forbidden (Rostoks et al., 2019; GMO FAQ, 2020), highlighting the importance of seeking other alternatives.

2.2. Echium seed oil

Echium *(Echium Plantagineum)*, non – GMO plant origin, is an herbaceous plant from the *Boraginaceae* family that produces a number of small seeds (Castejón, Luna, and Señoráns, 2018), that contains about 26-30% of oil, with a unique fatty acids profile, when compared with others n-3 FA vegetable oils (**Table 1**).

Table 1: n-6 and n-3 FA proximate composition of echium, flaxseed and hemp oils (USDA, 2018)

Fatty acids	Echium oil (%)	Flaxseed oil (%)	Hemp oil (%)
LNA (n6)	13	14	54
GLA (n6)	10	-	3
ALA (n3)	32	53	17
SDA (n3)	15	-	2

Echium oil can be considered an excellent source of n-3 FA, because SDA does not need the action of Δ-6 desaturase enzyme to be converted in EPA (**Figure 1**). For example, flaxseed has high concentration of ALA and LNA, and it does not present none of SDA or GLA, while borage oil has only omega 6 fatty acid (n-6 FA) (Bert et al., 2007; Mir, 2008; Comunian et al., 2019). Echium oil has already been used in the pharmaceutical industry, mainly in several dermatological treatments (Berti et al., 2007), and in food industry, in some countries in Europe countries (Castejón, Luna and Señoráns, 2018). In the last ten years, several animal and human studies have shown the beneficial effects of Echium oil on health, especially cardiovascular health (Botelho, et al., 2013; Forrest et al., 2013; Kuhnt, et al., 2014; Kuhnt, et al., 2016). Commercialization of Echium oil is approved by Food and Drug Administration (FDA) and European Commision, and has been classified as a novel food ingredient (Castejón, Luna and Señoráns, 2018).

Animal studies showed that the consumption of Echium oil or isolated SDA promotes a concentration of EPA in blood higher than the consumption of ALA (Kuhnt et al., 2016). Echium oil intake also showed decrease of plasma triglycerides (TG), low density lipoprotein (LDL) cholesterol, and Very Low Lipoprotein Cholesterol (VLDL) (Kuhnt et al., 2014). In a study conducted by our group, similar results were observed. Supplementation of Echium oil was compared with fish and algae oils, using a LDLr knockout mice fed with a high fat diet. It was

observed a reduction of VLDL, total cholesterol, and hepatic steatosis and an increase of EPA concentration in the liver of animals supplemented with Echium oil (Botelho et al., 2013). In another study, it was observed a decreased TG and VLDL by intracellular lipolysis in mice supplemented with Echium oil, when compared with mice supplemented with fish and palm oils (Forrest et al., 2013).

In human studies, similar results can be observed. In a study reported by Surette (2013), consumption of oils rich in SDA, such as Echium oil, promote high concentration of EPA in tissues and plasma. The conclusion of this study was that oils containing SDA, like Echium oil, can be classified with cardio protect effects. Another study (Miles, Banerjee and Calder, 2004) showed that the consumption of Echium oil decreased plasma TG in patients with hypertriglyceridemia, as the same rate as fish oil.

However, Echium oil is extremely susceptible to oxidation, because of the high level of unsaturation of the fatty acids present in its composition, resulting in the formation of primary and secondary oxidation products (Comunian, et al., 2019).

2.3. Oxidative stability of vegetable oils.

PUFA, as those found in Echium oil, are liquid at room temperature and present very low melting point (Dresler, et al. 2017). These fatty acids are extremely susceptible to oxidation due to their high index of unsaturation (Barden and Decker, 2013). Oils with high proportion of PUFA can undergo different oxidative processes known as autooxidation, photooxidation and oxidation mediated by enzymes (Jackson and Penumetcha, 2019).

2.3.1. Autoxidation.

Autoxidation occurs at temperatures below 100ºC degrees, in the presence of oxygen and radicals, as summarized in **Figure 2**.

Initiation:

$$LH + O_2 \rightarrow L^{\cdot} + OH^{\cdot}$$

Propagation:

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot}$$

Termination

$$L^{\cdot} + L^{\cdot} \rightarrow LL$$

$$L^{\cdot} + LOO^{\cdot} \rightarrow LOOL$$

$$LOO^{\cdot} + LOO^{\cdot} \rightarrow LOOL + O_2$$

Figure. 2: Summary of lipid oxidation steps (Adapted from Frankel, 2005).

During the initiation, radicals such as hydroxyl radical (•OH) can readily abstract a hydrogen from unsaturated fatty acids containing an allylic center (-CH=CH-CH_2-), by a radical displacement reaction (Frankel, 2005). In the propagation, the alkyl radical of unsaturated lipids (L•) reacts very rapidly with molecular oxygen to form peroxyl radicals (LOO•). After, there is a hydrogen transfer to form hydroperxides (LOOH) (Frankel, 2005). In the termination reaction, lipidic radical form secondary compounds potentially toxic, such as aldehydes, ketones, alcohols, lactones, hydrocarbons (Frankel, 2005; Samdani, McClements and Decker, 2018). Some of these secondary products cause unpleasant odor and reduce the nutritional quality of oils, becoming improper for consume or even toxic potential (Gray, 1978; Samdani, McClements and Decker, 2018). Aldehydes are the most formed secondary compounds in the oxidation. Some of these aldehydes have been associated with diseases, such as chronic inflammation, atherogenesis, neurodegenerative diseases and diabetes (Yuan, Shoeman and Csallany, 2018; Liu et al., 2020). Malondialdehyde, a three carbon dialdehyde, is a compound formed in the oxidation of PUFA containing more than two double bounds, being indicator of lipid oxidation (Ma and Liu, 2017; Xia and Budge, 2017; Liu et al., 2020).

2.3.2. Photooxidation

Photosensitive molecules (Sen) absorb visible or UV light becoming activated (^{1}Sen*). When return to their original state, in a process known as “intersystem crossing”, they can rapidly react with oxygen, forming singlet oxygen ($^1O_2$), or react with unsaturated fatty acids. There are two types of “Sen” molecules: Type I (ex: riboflavin) and Type II (ex: chlorophyll and erytrosin). In type I, the formation of LOOH occurs with free radicals (R•) while in type II, $^1O_2$ reacts with the double bonds in the fatty acids, forming different LOOH from those formed by autooxidation (**Figure 3**). The reaction of $^1O_2$ with fatty acids is about 1500 times faster than the triplet oxygen (O_2), the oxygen form in the autooxidation (Shahidi and Zhong, 2010; Li et al., 2019).

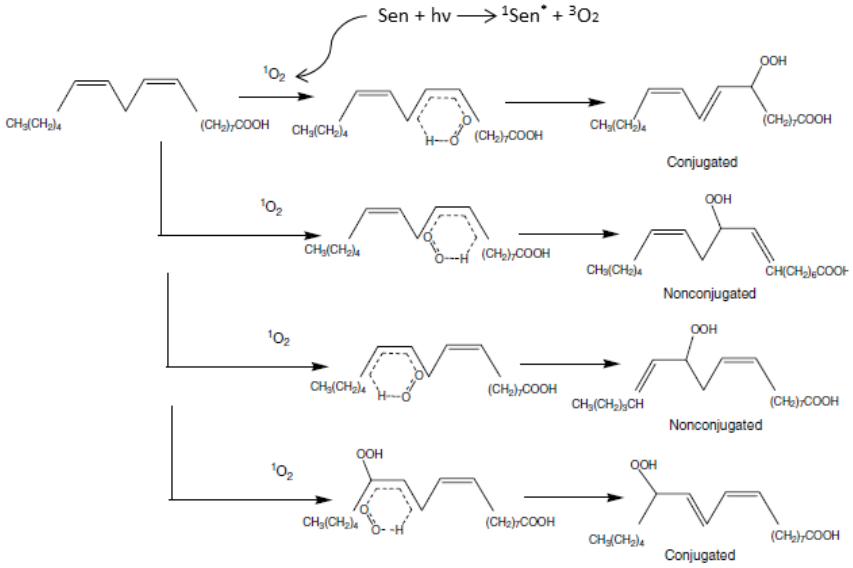

Figure 3: Summary of photooxidation steps mediated by singlet oxygen (Adapted from Choe and Min, 2006)

2.3.3. Oxidation mediated by enzymes

Lipoxygenase (LOX) or Cyclooxigenases (COX) catalyzes the oxidation of PUFA forming LOOH (Frankel, 1993). For example, LOX containing the ferrous iron (LOX-Fe^{2+}), transfers an electron to LOOH, forming a hydroxyl ion (OH^-) and an alkoxyl radical ($LO^{\bullet}$), resulting in a LOX containing ferric iron (LOX-Fe^{3+}) (**Figure 4**). This oxidized form returns to the reduced state (LOX-Fe^{2+}) by abstracting the electron from the hydrogen linked to the double allylic carbon of the fatty acid, forming a carbon radical centered in the fatty acid ($L^{\bullet}$). Then, it occurs the stereospecific insertion of the oxygen molecule (O_2) into the system, forming a peroxyl radical ($LOO^{\bullet}$) centered in the in fatty acid structure. Again, (LOX-Fe^{2+}) reduces $LOO^{\bullet}$ returning to its oxidized form (LOX-Fe^{3+}). In this last reaction, $LOO^{\bullet}$ receives an electron (LOO^-), attracts a proton (H^+), forming another LOOH (Kühn and Borchert, 2002). In the presence of transition metals and oxygen, these LOOH can be decomposed forming secondary products responsible to become vegetable oils unappropriated for consumption (Choe and Min, 2006).

$$LOOH + (LOX\text{-}Fe^{3+}) \longrightarrow LO^{\cdot} + OH^- + (LOX\text{-}Fe^{2+})$$

Figure 4: Summary of oxidation mediated by LOX (Adapted from Choe and Min, 2006).

2.4. Strategies to extend oxidative stability of vegetable oils.

Considering the high potential of n-3 FA rich oils for health proposals, it is necessary to combine strategies to delay the oxidative damage (Spatari et al., 2019). One of the most applied strategies to inhibit the oxidation of PUFA rich oils is the addition of natural antioxidants, since the use of artificial additives are not well accepted by the consumers (Moen, Stoknes and Breivik, 2017) and also does not match with a “health label”. According to the “Polar Paradox”, first reported by Porter (1980) and after discussed by Frankel et al. (1994), polar compounds would exert a better antioxidant activity in bulk oils than in emulsions, in function of their better affinity for the water-lipid interface, where the oxidative reaction is more intense. However, Agnes, Roschel and Castro (2017) evaluating the effect of hydrophilic and lipophilic prooxidants on oxidative stability, concluded that other factors than just polarity must affect the oxidation of bulk oils.

Antioxidants can be radical scavengers, that inhibit the action of the initiators, metal chelators and compounds that regenerates other antioxidants. The efficacy of antioxidants depends on its reduction potential, bond dissociation energy and susceptibly to autoxidation (Martinovic et al., 2019). The combination of molecules that exerts different effects has been applied to extend oxidative stability of fats and oils (Lama-Muñoz et al., 2018; Wang et al., 2020).

In a previous study reported by our group (Espinosa et al., 2015), sinapic acid showed the highest antioxidant activity when present in an emulsion prepared with n-3 FA rich oil. Sinapic acid is a phenolic compound that has been evaluated in vegetable PUFA oils, since it has the ability to delay the oxidation process (Comunian et al., 2016). It acts by donating hydrogen from the aromatic chain to peroxy radical (**Figure 5**), in a similar mechanism of alpha tocopherol, characterized as radical scavenger (Espín, Soler-Rivas and Wichers, 2000). It has been reported that sinapic acid is much more potent radical scavenger than other phenolic compounds (Fan and Eskin, 2015).

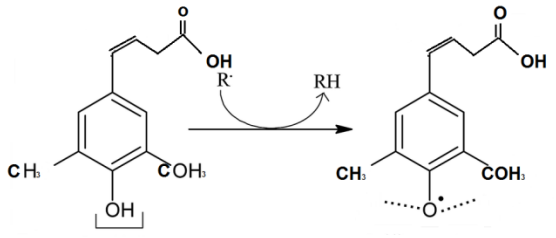

Figure 5: Antioxidant action of sinapic acid (Adapted from Badhani, Sharma and Kakkar, 2015).

Antioxidants that act as radical scavengers can be regenerated by other molecules. Ascorbic acid is a potential regenerator that donates hydrogen to the oxidized molecule due to the presence of enediol group, returning to stable form, and increasing the oxidative stability of the oil (Frankel, 1996; Decker et al., 2005; Carocho and Ferreira, 2013; Moen, Stoknes and Breivik, 2017). Other mechanisms showed by ascorbic acid are metal inactivation, hydroperoxides reduction and oxygen scavenging (Frankel, 1996). Ascorbic acid reduces phenoxyl radical produced in the oxidation of phenolic compounds (Nardini et al., 2002). This reaction depends on the potential of reduction of these two molecules and the chemical characteristics of the solution (Pekkarinen, et al., 1999).

Another strategy often applied to reduce oxidative damage in oils is the addition of metal chelators, such as citric acid. This strategy can be also combined with the radical scavenger compounds. Citric acid is also a natural molecule that has the ability of chelating metal ions, such as iron and copper, forming a heterocyclic ring with metals ions (**Figure 6**), slowing down the oxidation rate (Tian, Decker and Goddard, 2013). Citric acid is stable at temperature below 140ºC, being a good option to keep the oil conserved during storage (Gupta, 2017).

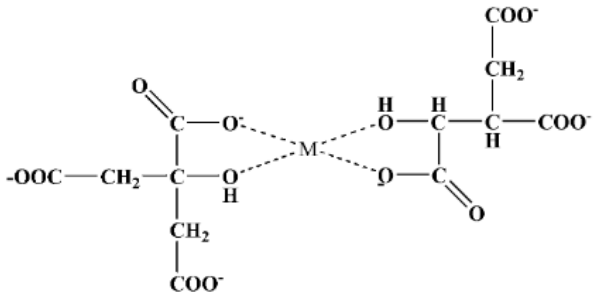

Figure 6: Citric acid chelating metals (adapted from Tian et al., 2013).

Once chelated, these metals, such as iron, cannot react with radicals, forming hydroxyl radicals (·OH), a compound that accelerates the oxidation, as in Fenton and Haber Weiss reaction (**Figure 7**) (Burkitt and Gilbert, 1990). Thus, the combination of a radical scavenger, a potential regenerator and a metal chelator can result in an interesting strategy to reduce the oxidative damage of edible oils.

$$Fe^{3+} + O^{-}_{2} \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + OH^{-}$$

$$O_2^{\cdot-} + H2O2 \xrightarrow{Fe^{2+}/Fe^{3}} O_2 + HO^{\cdot} + OH^{-}$$

Figure 7: Fenton and Haber Weiss reactions (Adapted from Burkitt and Gilbert, 1990).

Independent of the transition metals presence, the decomposition of LOOH is fast at temperatures higher than 70ºC, and the action of antioxidants may not be enough effective (Frankel, 1993). At temperature range from 5ºC to 50ºC, the solubility of oxygen is also elevated accelerating the oxidative process (Cuvelier, et al., 2017). Therefore, besides applying antioxidants, it is also necessary to inhibit the oxygen presence by using vacuum or inert gas during in oils package, for example (Naz, et al., 2004). Due to the photooxidation, there are also some films that protect the oil from light at wavelengths lower than 300 nm (Coltro et al., 2003). The use of dark bottles is recommended to minimize these issues and to increase the shelf life (Zhao, Gong, and Wu, 2018). However, all these strategies are expensive or reduce the visual exposition of the oil toward the consumers.

Lipid oxidation follows the *Arrhenius* model (Tan, et al., 2001). It means that the reaction rate increases according to temperature increase, until the oxygen solubility becomes restrictive (Tan et al., 2001). Although it is also an expensive strategy, it has been recommended to keep PUFA rich oils at refrigeration temperature (4ºC) (Wang, 2014; Wroniak and Rekas, 2016). For this reason, it is usual to find, for example, flaxseed oil being sold in the refrigerated shells in markets. On the other side, controlling only the temperature, is not enough to prevent oxidation of PUFA rich oils during storage and consumption.

Other strategy more recently researched to stabilize PUFA rich oils is to blend with a less unsaturated vegetable oil, usually containing higher proportion of oleic acid (18:1, n-9), known as “High Oleic” oils (Bordón et al., 2019). This strategy is low cost, enhances the oxidative stability and improves the industrial applicability of unconvetional oils, such as *Echium* oil (Bordón et al., 2019). In high oleic oils, more energy is necessary to abstract the hydrogen from the fatty acids mainly due to the absence of 1,4-cis,cis-pentadien structure, increasing the oxidative stability (Smith, King and Min, 2007; Hashempuor-Baltork et al., 2016). In addition, several studies have reported that the olive oil consumption, containing 80% of oleic acid, as one of the factors that contribute to the cardiovascular protection effect associated to the “Mediterranean Diet” (Khan and Shahiddi, 1999; Jurado-Ruiz et al., 2017; Hernaéz et al., 2017).

2.5 Methods to obtain vegetable oils from seeds.

The selection of the extraction process choice to be applied in vegetable oils, especially in case of PUFA rich oils, is extremely important involving cost and stability aspects. Oils can be obtained from mechanical pressing or with solvents, such hexane (Zhao, Wei and Julson, 2016). In the first case, the seeds are just pressured by continuous screw pressing, filtered in case of more stable oils, or submitted to centrifugation, in less stable oils that require less exposure time. Press extraction is usually applied to seeds with high oil content, above

30% (Delfan-Hosseini, et al., 2017). It is simple to be applied, presents low operating cost and results in good quality oil (Delfan-Hosseini et al., 2017). Usually, the hydroperoxide concentration is higher, but the oil is protected by minor components present in the seeds and transferred to the oil, such as phenolic compounds with antioxidant action (Kühn and Borchert, 2002). In the oils obtained by pressure, besides oxidation, hydrolysis of glycerol chain can also take place, resulting in the increase of free fatty acids and, consequently, increase of oil acidity. For this reason, the acidity is one the most important parameters applied to evaluate the quality of this type of oil (Kiritisaks and Shahidi, 2017).

Currently in some countries, such as Spain, the Echium oil production is present and the most applied method is the solvent extraction with hexane, with subsequent vacuum isolation for distillation. Extraction through organic solvent can be considered the most efficient (Castejón, Luna and Señoráns, 2018). Pressing oil associated with solvent cake extraction is already used in the industry for many years, increasding efficacy in oil recovery (Domínguez et al., 1995).

3. HYPOTHESIS

According to the literature review, the following hypotheses were raised in this study:

(1) A mixture of compounds presenting different antioxidant strategies, such as radical scavengers, metal chelators and antioxidants regenerators can exert an antioxidant activity similar or higher than artificial compounds.

(2) Taking the “Polar Paradox” theory into account, it is supposed that a hydrophilic mixture of antioxidants promotes a better protection against oxidation in bulk oils than a lipophilic mixture.

(3) The antioxidant response of the hydrophilic or lipophilic mixture depends on the presence of minor compounds in the bulk oil.

(4) The process applied to extract the oil from the Echium seeds influences the minor compounds composition and the oxidative stability of the oils, being samples extracted by solvents less stable than samples extracted from screw press.

(5) The combination of temperature reduction, hydrophilic antioxidants and addition of less unsaturated oil improves the oxidative stability of Echium oil during the storage time (6 months in sealed flasks) and during the oil consumption (30 days exposed to oxygen at room temperature).

4. OBJECTIVE

In order to evaluate these hypotheses, the objective of this study was firstly to compare the effect of a hydrophilic or lipophilic mixture of natural compounds on oxidative stability of an oil containing a high proportion of proportion of polyunsaturated fatty acids and after to combine the natural strategies to reduce echium oil oxidation during its storage and consumption.

5. DESCRIPTION OF THE CHAPTERS

In order to evaluate the hypotheses raised in this study, the experiments were divided into two parts. In the first part, it was developed a hydrophilic and a lipophilic mixture containing compounds that presented complementary antioxidant mechanisms. In this context, sinapic acid (500 ppm) was mixed with ascorbic acid (250 ppm) and citric acid (150 ppm) to compose the hydrophilic mixture (HM); while α-tocopherol (500 ppm) was mixed with ascorbyl palmitate (250 ppm) and citric acid (150 ppm) to compose the lipophilic mixture (LM). Therefore, three mechanisms were combined to improve the antioxidant activity: radical scavenger, antioxidant regenerator and metal chelator, respectively. The mixtures were added to the flaxseed oil, kept at 40ºC for 15 days, and the oxidative markers were compared with the oil without antioxidants (CONT) and also with the oil added of TBHQ (120 ppm) and EDTA (75 ppm), both artificial compounds. The assays were carried out in the flaxseed crude and stripped oils, aiming to evaluate the influence of the minor compounds naturally present in the bulk oil. The results showed that LM and HM promoted an oxidative protection similar to the artificial antioxidants and that, following the “Polar Paradox” theory, HM seemed to better stabilize crude oil than LM. On the other side, this behavior was not observed using the stripped oil, suggesting the influence of minor compounds on this response, mainly associated to the “association colloids” presence.

Our initial objective was to carry out this first assay already using Echium oil. However, due to the long-demanded time to proceed all steps involved in the Echium seeds importation from England, this first part was performed using another highly polyunsaturated oil (flaxseed) instead of Echium oil. Based on this result, it was selected the HM to be one of the strategies to be applied in the second part.

In the second part of our study, Echium seeds had the oil extracted by two process: using hexane (SOLV) or screw pressing (PRESS). The oils were characterized in terms of fatty acids profile, minor compounds and oxidative stability. It was observed that PRESS samples showed lower values of oxidative markers, possible due to their lower exposition to higher temperatures when compared with the time by which SOLV samples were exposed. For the oils kept under storage condition, oxidative markers were maintained below the legal range and the

reduction of temperature to -25ºC was enough to avoid the oxidation reaction. It was also evident to PRESS samples, that under higher temperature (+25ºC) the addition of full dose HM and 20% of high oleic sunflower oil was necessary to protect the oil from oxidation. Differently, when samples were exposed to oxygen at room temperature, both PRESS and SOLV demanded full dose HM and 20% of high oleic sunflower oil to improve the oxidative stability. Applying the strategies recommended in our study to PRESS and SOLV samples, it was observed 37-41% reduction to hydroperoxide and 40-75% to malondialdehyde concentration in the samples prepared according to the optimized condition, when compared with the standard conditions by which the oil use to be extracted and processed.

The first part of this study was already published in the *European Journal of Lipid Science and Technology,* and the second part will be soon submitted to another scientific journal. For this reason, the present dissertation was formatted in two chapters.

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CHAPTER I

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RESEARCH ARTICLE
Flaxseed Oil
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Combination of Hydrophilic or Lipophilic Natural Compounds to Improve the Oxidative Stability of Flaxseed Oil

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Oxidation of bulk oils involves radical molecules, transitions metals, and association colloids, among other factors. Combining mechanistically different antioxidants is one possible strategy to improve oxidative stability of polyunsaturated fatty acids rich oils. However, antioxidant polarity may play a critical role in the oxidative protection efficacy. Thus, the objective of this study is to formulate a hydrophilic mixture (HM) containing sinapic, ascorbic, and citric acid; and a lipophilic mixture (LM) composed of α-tocopherol, ascorbyl palmitate, and citric acid; and apply these mixtures in crude, and stripped flaxseed oils. The oxidative stability of crude oil samples containing HM and LM is comparable to that of artificial compounds mixture (ethylenediamine tetraacetic acid [EDTA] and terc-butyl hydroquinone [TBHQ]). HM is more effective than LM in crude oil, but not in stripped oil, suggesting a strong influence of minor components on antioxidant activity of natural compounds.

Practical Applications: The increasing demand for foods containing polyunsaturated fatty acids (PUFA) brings the challenge of their chemical stabilization. Here, a strategic mixture of hydrophilic natural antioxidants is effective to stabilize flaxseed oil, with effectiveness comparable to that of artificial antioxidants. Therefore, this HM constitutes a feasible and efficient option to be applied by food producers, especially in formulations that present a health claim.

1. Introduction

Oxidation is the major cause of deterioration of highly unsaturated oils, limiting, for example, the utilization of flaxseed oil.[1,2] Secondary oxidation products are produced from decomposition of hydroperoxides, causing nutrient loss, off-flavors, and color alteration.[3] Furthermore, potentially cytotoxic compounds, such as 4-hydroxy-trans-2-nonenal (HNE), 4-hydroxy-trans-2-hexenal (HHE), acrolein, and malondialdehyde (MDA) can also be formed. These compounds are involved in the progression of many chronic diseases and are applied as biomarkers in clinical studies.[3–5]

Lipid oxidation is affected by several factors, such as oxygen, temperature, light, presence of minor components, and degree of fatty acids unsaturation.[3,6–9] Oils with a high proportion of PUFA, containing 1,4–pentadiene system in their structure, tend to show much lower oxidative stability than those containing a lower proportion of PUFA.[9,10]

One strategy to control oxidation is the use of antioxidants.[11] According to the "Polar Paradox Theory," polar antioxidants tend to be more active in bulk oils while nonpolar antioxidants tend to be more active in oil-in-water emulsions.[12,13] For this reason, in order to provide maximum oxidative stability, antioxidant compounds should preferentially concentrate in the place where oxidation occurs in a higher rate, that is, at the interface of association colloids.[14,15] Therefore, the antioxidant polarity is an important factor to consider when selecting antioxidants to be applied in highly unsaturated lipid systems.[12,13,16]

Synthetic antioxidants are widely used in the food industry, such as EDTA and TBHQ.[11] However, due to their controversial effects on human health, there is an increasing demand for natural antioxidants, for example, vitamins, phenolic compounds, carotenoids, among others.[17] Given the limited number of approved antioxidants for food applications, several strategies have been attempted to improve antioxidant performance. One possible strategy is to combine substances able to exert antioxidant effect by different mechanisms, such as radical scavengers, metal chelators, and molecules regenerator.[18] In this context, radical scavengers, such as tocopherol and phenolic compounds, could be mixed to compounds able to regenerate oxidized molecules, for example, ascorbic acid and metal chelators, for instance citric acid.[19–21] In a previous study carried out by our group, Espinosa et al.[21] showed that among 11 natural phenolic compounds found in red propolis, sinapic acid presented the highest antioxidant activity in emulsions containing oil rich in PUFA. Therefore, considering the

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importance of mixing natural compounds with different antioxidant mechanisms and the influence of antioxidant polarity on its effectiveness, the objective of this study was to evaluate the effect of a hydrophilic or a lipophilic antioxidants mixture on the oxidative stability of crude and stripped flaxseed oil.

1. Experimental Section

1.1. Material and Chemicals

Cold pressed flaxseed oil was purchased from Pazze Indústria de Alimentos Ltda (Panambi/Rio Grande do Sul, Brazil), stored in the dark at room temperature (20 °C). Ascorbyl palmitate, ascorbic acid, α-tocopherol, citric acid, sinapic acid, silicic acid, and activated charcoal were purchased from Sigma-Aldrich (Sigma Chemical, St. Louis, MO, USA). All reagents were HPLC grade.

1.2. Study Design

To compose the LM, α-tocopherol was used as radical scavenger and ascorbyl palmitate as α-tocopherol regenerator. To formulate the HM, sinapic acid was applied as radical scavenger while ascorbic acid was selected as sinapic acid regenerator. In both mixtures, citric acid was used as metal chelator. The proportion of compounds in each mixture was defined according to previous studies.[12,22–29] Bulk oils with high proportion of PUFA naturally contain minor compounds with pro or antioxidant activity. For this reason, the antioxidant effectiveness of the mixtures was evaluated in crude flaxseed oil and in flaxseed oil stripped from its minor compounds. Table 1 shows the proportion of each compound applied to formulate LM and HM. It was included a sample composed only of bulk oil without addition of antioxidants (CONT), as well as a sample containing a mixture of TBHQ and EDTA, which are approved artificial antioxidants (ART). All combinations presented in Table 1 were initially dissolved in ethanol (1 mL) and added to 10 mL of crude or stripped flaxseed oil in 20 mL -amber vials. Afterward, ethanol was evaporated under nitrogen and opened vials were stored at 40 °C for 15 days.[30] After this period, chemical markers of lipid oxidation (peroxide value [PV], malondyaldehyde [MDA], and volatile compounds), fatty acids, sinapic acid, and α-tocopherol contents were evaluated.

Table 1. Concentration of compounds used to prepare hydrophilic and lipophilic antioxidant mixtures.

Treatment	Compound	Concentration [ppm]	Reference
LM	α-tocopherol	500	[23–25]
	Ascorbyl Palmitate	250	[23]
	Citric acid	150	[22,28]
HM	Sinapic acid	500	[50]
	Ascorbic acid	250	[23]
	Citric acid	150	[22,28]
ART	TBHQ	120	[26]
	EDTA	75	[29]
CONT	–	–	–

1.3. Methods

1.3.1. Flaxseed Oil Stripping

Flaxseed oil had its minor compounds stripped according to the methodology proposed by Khan and Shahidi,[6] and modified by Waraho et al.[31] using an open-column chromatographic system (29.5 mm internal diameter, 350 mm in length; Wilmad Labglass, New Jersey, USA) connected to a vacuum system. For the stripping process, 30 g of flaxseed oil was dissolved in 60 mL of hexane, introduced into the column and eluted for 30 min, followed by the addition of 100 mL (three times) and 70 mL of hexane. Eluates were collected in glass flasks immersed in an ice bath. Finally, hexane was vaccum-evaporated (Heildolph Rotavac Valve Tec., Germany) for 30 min at 30 °C. To remove traces of hexane, samples were flushed with nitrogen. Stripped oils were protected from light during all the procedure and stored at −80 °C until use.

1.3.2. Determination of PV

PV was determined according to Shanta and Decker.[32] The absorbance of samples was measured at 510 nm using a UV–vis mini 1240 spectrophotometer. A standard curve was prepared with known concentrations of cumene hydroperoxide (CHP) (0.03–0.93 mg CHP; R^2 = 0.9978) and samples were analyzed in quintuplicate. Results were expressed as meq O_2/L oil.

1.3.3. Determination of MDA Content

MDA concentration was determined by High-Performance Liquid Chromatography (HPLC) (model 1200, Agilent Technologies Inc., Palo Ato, USA) following the protocol of Hong et al.[33] Samples were analyzed utilizing a reverse-phase C18 column (250 mm × 4.6 mm; 5 μm; Phenomenex, Torrance, California, USA) and quantified by fluorescence detection at an excitation wavelength of 515 nm and emission of 553 nm. The HPLC pump delivered an isocratic mobile phase composed of 60% phosphate buffer (50 mmol, pH 7.4) and 40% methanol at a flow rate of 1.0 mL min^{-1}. A standard curve was prepared using 1,1,3,3-tetraetoxipropano (TEP) (24.3–185.9 μg mL^{-1} TEP; R^2 = 0.996), analysis were carried out in quintuplicate and results expressed as mg MDA/L oil..

1.3.4. Fatty Acids Composition

Oil samples (10 mg) were transferred to screw-caped tubes containing 1 mg of tricosanoic acid methyl ester (C23:0) as internal standard, 50 mL of 0.5% BHT ethanol solution, and 1 mL of 0.5 M methanolic NaOH, according to Shirai et al.[34] Samples were placed in a boiling water bath for 5 min, then cooled at room temperature. After that, 2 mL of BF_3 were added to the samples and boiled in water bath for 5 min. Samples were cooled at room temperature and 1 mL of isooctane was added, followed by vigorous homogenization. Then, 5 mL of a saturated NaCl solution was added and samples homogenized gently. The

organic phase was extracted and dried. The samples were re-suspended in 500 μL of isooctane, transferred to vials and analyzed in triplicate. Fatty acids quantification was carried out using a gas chromatography equipped with a G3243A MS detector (Agilent 7890A GC System; Agilent Technologies Inc.). A fused silica capillary column (J&W DB-23 Agilent 122–236; 60 m × 250 mm inner diameter) was used to separate sample compounds. Injection volume was 1 mL. High-purity Helium was used as the carrier gas at a flow rate of 1.3 mL min^{-1} with a split injection of 50:1. The oven temperature was programed from 80 °C to 175 °C at a rate of 5 °C min^{-1}, followed by another gradient of 3 °C min^{-1} to 230 °C, which was maintained for 5 min. The GC inlet and transfer line temperatures were 250 and 280 °C, respectively. GC-MS was performed using 70 eV EI in scan acquisition and recorded as TIC. All mass spectra were acquired over an *m/z* range of 40–500. Fatty acids in samples were tentatively identified by NIST mass library (National Institute of Standards and Technology [NIST]; Gaithersburg, MD, USA) and by comparing the retention times with those of four purified standard mixtures of fatty acid methyl esters (4-7801; 47085-U; 49453-U and 47885-U; Sigma Chemical Co.). Results were expressed as % of fatty acids or mg g^{-1} oil.

1.3.5. Determination of Tocopherol and Sinapic Acid Content

Tocopherol and sinapic acid concentration were performed using HPLC (model 1200, Agilent Technologies Inc.). Compounds separation was carried on a reverse-phase C18 column (Agilent, Santa Clara, California, USA, 150 × 4.6 mm^2; 5 mm). Tocopherol content in oils was determined using a modified method from Gliszczyńska-Świgło & Sikorska.[35] Oils (100 μL) were diluted in 1 mL isopropanol and injected into HPLC, using an isocratic mobile phase consisting of 50% acetonitrile and 50% methanol, at a flow rate of 1 mL min^{-1} and at 30 °C. The eluate was detected using a fluorescent detector set at a wavelength of 295 nm (excitation) and
325 nm (emission). A calibration curve was prepared using standards (α-tocopherol: 0–1046.4 ppm, R^2 = 0.9974 and γ-tocopherol: 0–4317 ppm, R^2 = 0.9996) and analysis were carried out in triplicate. Results were expressed as ppm. To determinate sinapic acid concentration in oils supplemented with the HM, 100 μL oil was diluted in 1 mL of isopropanol, in duplicate and injected in the HPLC using a gradient elution adapted from da Silveira et al.[36] consisting of acetonitrile (A), and an aqueous formic acid solution 0.1% (B). (A) increased from 8% to 92% in 11 min. The flow rate was of 1 mL min^{-1} and the column temperature was maintained at
30 °C. The eluate was detected using a diode array detector set at a wavelength of 325 nm. Identification of sinapic acid was carried out by comparing the retention time and UV–vis spectra of peaks in samples to those of genuine standard, and quantification was performed using a standard curve (97–1147.56 ppm, R^2 = 0.9931). Results were expressed as ppm.

2.3.7. Determination of Volatile Compounds

The volatile content in oil samples was determined according to Gómez-Cortés et al.[37] with some modifications. Oil samples (10 mL) were sealed in 20 mL glass vials (10 mL of headspace). A Combi PAL autosampler was used for automated SPME analysis. The vials were agitated (400 rpm) for 15 min at 50 °C. A 50/30 mm Stableflex 23Ga fiber (DivynilBenzene/Carboxen/Polydimethylsiloxane; DVB/CAR/PMDS – 57298-U; Supelco; Bellefonte, PA, USA) was inserted into the headspace and the vial was agitated for 60 min at 50 °C. The fiber was injected into the GC-MS (Agilent 7890A GC System, Agilent Technologies Inc.), at 250 °C for 60 min, using a splitless mode. During the GC analysis, the fiber thermally cleaned at 250 °C for 60 min. A capillary column (19091S-433UI HP-5 ms Ultra Inert, 30 m, 0.25 mm, 0.25 μm, 5%-Phenyl-methylpolysiloxane, Agilent Technologies) was used. Ultra-pure Helium was the carrier gas at a constant flow of 1 mL min^{-1}. The oven temperature started at 40 °C 5 min^{-1}, increased to 100 °C at 4 °C min^{-1}, to 220 °C at 17 °C min^{-1} and held at 220 °C for 10 min. Volatile compounds identification was carried out using a G3243A MS detector (Agilent 7890 A GC System, Agilent technologies Inc.). The ion source and quadrupole temperatures were 230 and 150 °C, respectively. All mass spectra were acquired in the electron-impact mode with an ionization voltage of 70 eV, adopting a mass range of m/z 35–350. Scan monitoring was used as the data acquisition mode. Compounds were tentatively identified (>70% of match) by using NIST mass library, and analysis were performed in duplicate, except for CONT that was just analyzed one time. Results were expressed as area ratio of the sample to internal standard (mbik).

2.3.8. Statistical Analysis

Results were expressed as mean ± standard error of mean (SEM). Sinapic acid and α-tocopherol contents in the oils (crude and stripped) were compared by Mann–Whitney, while the four treatments were compared by one way ANOVA followed by Tukey test, using software Statistica v. 13.4 (TIBCO Software Inc,

Table 2. Fatty acids composition, PV, and γ-tocopherol content determined in the crude and stripped flaxseed oils.

Fatty acids [%]	Crude	Stripped
16:0	11.58 ± 1.12	11.12 T 0,60
16:1 n7	1.01 T 0.00	0.18 T 0.05
18:0	8.79 T 0.36	9.20 T 0,73
18:1 n9 c	22.48 T 0.89	26.38 T 0.46
18:2 n6 c (LNA)	14.51 T 0.29	16.04 T0,34
18:3 n6 (GLA)	0.42 T 0.00	-
18:3 n3 (ALA)	41.86 T 2.85	36.75 T 1.09
20:0	0.24 T 0.00	0.26 T 0.2
22:0	0.10 T 0.00	0.18 T 0.02
SFA	20.60 T 1.71	20.77 T 1.35
MUFA	22.99 T 1.39	26.56 T 0.50
PUFA	56.58 T 2.93	52.79 T 1.22
PV (meq O_2/L)	1.38 T 0.24	3.85 T 0.27
γ-Tocopherol (ppm)	288.73 T 10.72	–

Round Rode, Texas, USA). An alpha value of 5% was adopted to reject the null hypothesis.

2. Results

Table 2 exhibits the fatty acid composition, PV, and γ-tocopherol concentration of crude and stripped flaxseed oils. No difference was observed in the fatty acids proportion and PV was in accordance with legislation, <15 meq O_2 kg^{-1} oil, [26] confirming that the stripping procedure was adequately performed. In addition, the absence of γ-tocopherol in stripped oil suggested that minor components were removed by the column. Oxidative markers measured in the samples treated with ART, HM, and LM are shown in Figure 1. All treatments showed reduction of PV compared to CONT, indicating that all antioxidants mixtures were effective to protect both crude and stripped oils from oxidation. Figure 1a shows PV for crude flaxseed oil. Among the mixtures, LM samples showed the highest PV ($p < 0.001$), while there was no difference between ART and HM ($p = 0.991$). This result suggests that HM was as effective as ART and promoted a better antioxidant protection than LM. In contrast, for stripped oils (Figure 1b), no difference was found in PV among the mixtures.

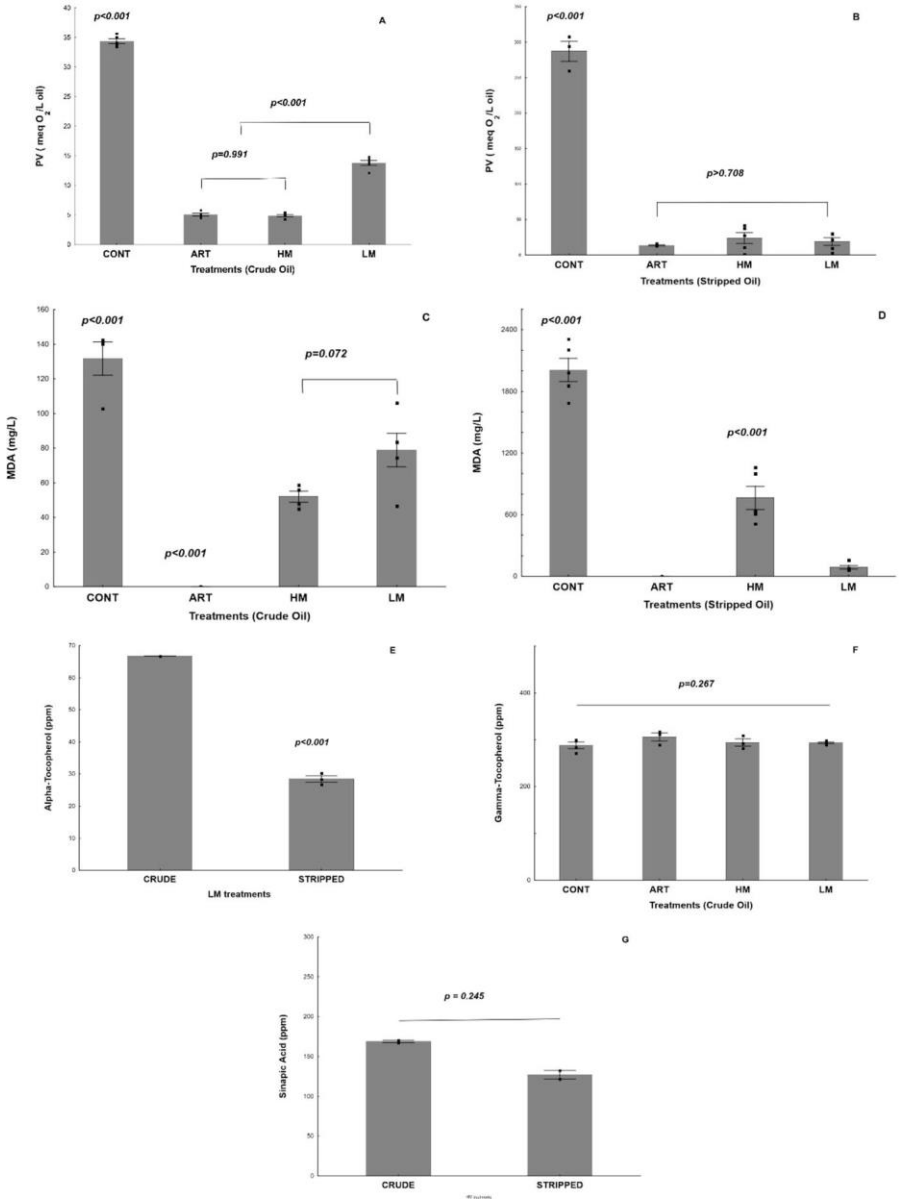

Figure 1. Oxidative markers of crude and stripped flaxseed oils. a) PV of crude oil after oxidation. b) PV of stripped oil after oxidation. c) MDA of crude oil after oxidation. d) MDA stripped oil after oxidation. e) α-Tocopherol content after oxidation of crude and stripped oils. f) γ-Tocopherol content after oxidation of crude oil. g) Sinapic acid content after oxidation of crude and stripped oil. Data presented as mean±SEM. ART: Artificial antioxidant mixture; CONT, Sample without antioxidant; HM, Hydrophilic mixture; LM, Lipophilic mixture; MDA, Malondialdehyde; PV, Hydroperoxides.

MDA concentration in crude and stripped oils is shown in Figure 1c and d, respectively. MDA was not detected in crude flaxseed oil treated with ART (Figure 1c), while HM and LM did not differ from one another. However, there was a trend of lower MDA content in HM samples than in LM samples ($p = 0.072$), showing that the HM could also be more effective at reducing MDA formation in crude oil (Figure 1c). Contrarily, in stripped samples (Figure 1d), the MDA content was higher in HM than in LM ($p < 0.001$), suggesting an influence of minor compounds on the formation of secondary products of lipid oxidation.

Flaxseed oil contains γ-tocopherol in its composition and just traces of α-tocopherol. Thus, the concentration of α-tocopherol was only determined in the oils added of LM (Figure 1e). Compared to the initial added amount of α-tocopherol in LM (500 ppm), it was found a higher reduction in stripped oils (94.31%) than in crude oils (86.64%). As the stripping procedure removes all tocopherol isomers, γ-tocopherol concentration was only determined in crude oil (Figure 1f), with no change observed among the treatments ($p = 0.267$), suggesting that, in our model, γ-tocopherol did not exert any antioxidant action in crude oil stored at 40 °C/15 days. This assumption was confirmed by comparing the γ-tocopherol concentration in crude oils before (292.69 ± 7.04 ppm) and after oxidation (293.79 ± 2.88 ppm) taking into account the mean value of the four treatments ($p = 0.673$). Figure 1g shows the consumption of sinapic acid in crude and stripped oils added of HM. Although no difference in the sinapic acid content ($p = 0.245$) was observed between crude and stripped oils (167.28 ± 3.40 ppm and 139.81 ± 2.27 ppm, respectively), almost half of the initial added concentration (500 ppm) was consumed during oil oxidation. There was not observed sinapic acid peak at 325 nm on crude oil sample, confirming no interference of minor compounds on sinapic acid quantification.

Linoleic (LNA) and α-linolenic (ALA) acids represent the major PUFA in flaxseed oil and their contents are shown in Figure 2. In general, fatty acids concentrations are not used as oxidation markers because a significant increase in primary and secondary oxidation products can be observed without any significant change in fatty acids content. This statement was confirmed in our study with regard to LNA and ALA measured in crude and stripped oils (Figure 2a–d).

Volatile compounds were also measured as secondary oxidation products. All treatments reduced total volatile compounds (Figure 3) when compared with CONT, here adopted as a reference value, for both crude (Figure 3a) and stripped samples (Figure 3b). Although no differences were found in the total volatile compounds content among LM, HM and ART samples, the volatile profile changed. Table 3 presents the major volatiles identified in the samples, being 3.5- octadien-2-one, 2.4-heptadienal, nonanal, and tridecane found both in crude and stripped oils. However, 2.4-heptadienal and 3.5-ocatedien-2-one were only found in CONT groups of crude and stripped samples respectively. Figures 4 and 5 show a typical chromatogram obtained for each sample. Major peaks in crude (Figure 4a–d) and stripped oils (Figure 5a–d) were highlighted.

3. Discussion

Our results indicated that both LM and HM mixtures showed antioxidant activity in crude and stripped oils. However, the mixtures behaved differently according to the type of oil. HM

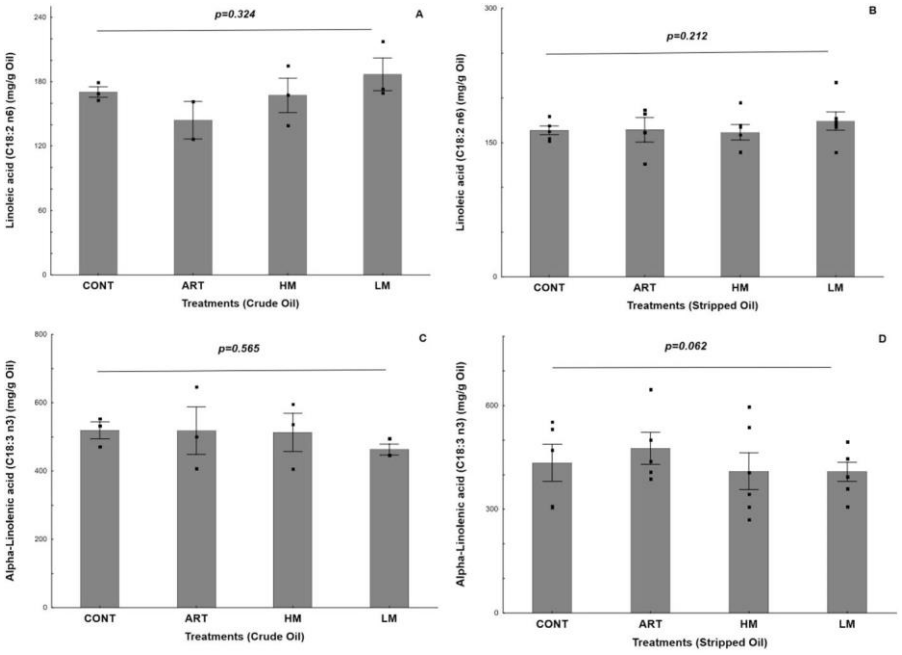

Figure 2. Content of LNA and ALA. a) Linoleic acid content after oxidation of crude oil. b) Linoleic acid content after oxidation of stripped oils. c) α-linolenic acid content after oxidation of crude oil. d) α-linolenic acid content after oxidation of stripped oils. Data presented as mean ± SEM.

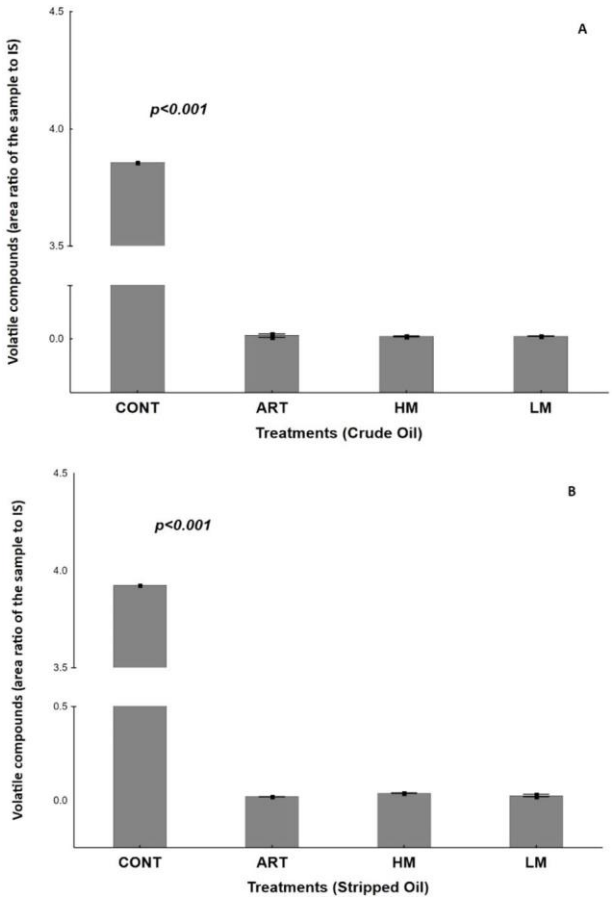

Figure 3. Total volatile compounds. a) Total volatile compounds content in the crude oil. b) Total volatile compounds content in the stripped oil. Data presented as mean±SEM. CONT, Sample without antioxidant; ART, Artificial antioxidant mixture; HM, Hydrophilic mixture; LM, Lipophilic mixture.

showed better antioxidant effect in crude flaxseed oil than LM, as evidenced by the lower PV and trend of lower MDA concentration. In contrast, for stripped oil, there was no difference in PV between LM and HM, but the MDA content was lower in LM than in HM, indicating that LM were, globally, more effective at protecting stripped flaxseed oil.

Results for crude flaxseed oil agree with the "Polar Paradox Theory," which states that polar antioxidants are more effective in bulk oils than nonpolar ones.[13] Although the "Polar Paradox" has been consistently confirmed by several studies,[38–40] others have reported contradicting conclusions. For example, Pereira-Caro et al.[41] observed that the chain length of hydroxytyrosol alkyl esters (C0–C18) did not affect their antioxidant effectiveness in stripped sunflower oils. In another study, Mohanan et al.[2] found that ascorbyl palmitate was better at protecting crude flaxseed oil from oxidation than the more hydrophilic tannic acid.

Oils are complex systems containing minor surface-active compounds, such as hydroperoxides, amphiphilic molecules (phospholipids, mono, and diacylglycerols), natural antioxidants (carotenoids, tocopherols), sterols, transition metals, and traces of water. These minor surface-active compounds have been reported to play a key role in the oxidation rate due to the formation of association colloids.[7,42] Association colloids are structures composed of an aqueous core stabilized by amphiphilic compounds, with the polar head directed to the aqueous phase and the nonpolar tails facing the oil phase.[1] Studies have evidenced that oxidation is most prevalent at the water-oil interface of association colloids, where surface-active compounds accumulate, favoring, or retarding lipid oxidation.[7,39,43] Thus, it is possible that the more polar compounds in HM partitioned into the water phase while those in LM would locate farther from oxidation sites.[1,38] This fact could explain why HM was more effective than LM in crude flaxseed oil.

In contrast, in stripped flaxseed oil, HM, and LM showed equivalent PV. Oils stripped from their minor compounds have their surface-active compounds partially or fully removed, drastically reducing the amount of association colloids.[7,42,43] Thus, it is possible that factors other than polarity may have driven the antioxidant action. For example, it is possible that in the absence of association colloids, antioxidants did not partition according to their hydrophobicity, leading hydrophilic, and lipophilic antioxidants to have comparable effectiveness as radical scavengers, as reported by Laguerre et al.[42] in a study with chlorogenic acid alkyl esters (C2-C16) applied to stripped corn oil.

Table 3. Major volatile compounds found in crude and stripped flaxseed oils treated with ART, HM, or LM (area ratio of the sample to internal standard).

OIL	Treatment	2.4-Heptadienal	3.5 Octadien-2-one	Nonanal	Tridecane	Octadecanoic acid	Nonanoic acid	2-Octenal	Tetradecane	2.4-Hexadienal
Crude										
	CONT	0.611	1.864	0.381	0.596	0.402	–	–	–	–
	ART	ND	0.006 T 0.002	0.003 T 0.001	0.006 T 0.002	–	–	–	–	–
	HM	ND	0.004 T 0.000	0.002 T 0.000	0.002 T 0.002	–	0.003 T 0.000	–	–	–
	LM	ND	0.005 T 0.001	0.002 T 0.000	0.004 T 0.000	–	–	–	–	–
Stripped	CONT	0.209	0.928	0.281	0.443	0.606	0.693	0.613	0.147	–
	ART	0.002	–	0.006 T 0.000	0.013 T 0.001	–	–	–	–	–
	HM	0.015 T 0.000	–	0.002 T 0.000	0.016 T 0.000	–	0.001 T 0.000	–	–	0.001T0.000
	LM	0.013 T 0.004	–	0.004 T 0,001	0.009 T 0.001	–	–	–	–	–

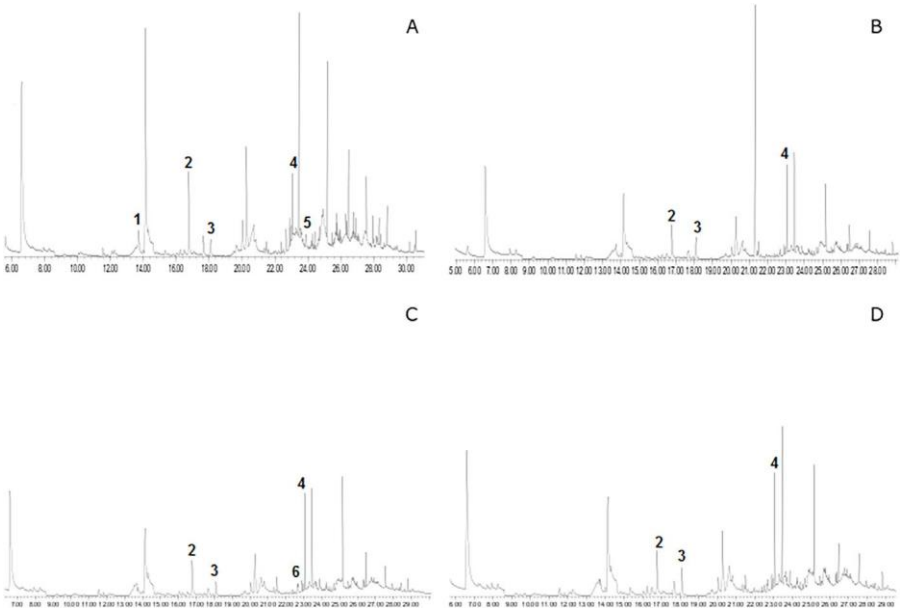

Figure 4. Chromatogram of volatile compounds of crude oil. a) Major peaks found in the CONT samples. b) ART. C: HM. D: LM. 1: 2.4-Heptadienal; 2: 3.5-Octadien-2-one; 3: Nonanal; 4: Tridecane; 5: Octadecanoic acid; 6: Nonanoic acid; 7: Hexanal; 8: 2-Heptenal; 9: 2-Octenal; 10: Tetradecane; 11: 2 (5H) Furanone; 12: 2-Decenal. CONT, Sample without antioxidant; ART, Artificial antioxidant mixture; HM, Hydrophilic mixture; LM, Lipophilic mixture.

Stripped oil samples treated with LM had lower levels of MDA than those treated with HM. It is possible that the absence or lower amount of association colloids impaired the action of hydrophilic antioxidants in inhibting hydroperoxide decomposition, suggesting that other mechanisms are involved in this reaction.[38,39] For instance, Frankel,[44] reported that the hydrogen donating ability of α-tocopherol inhibits the formation of MDA precursors. It was found a higher consumption of α-tocopherol in stripped than in crude oil. Therefore,

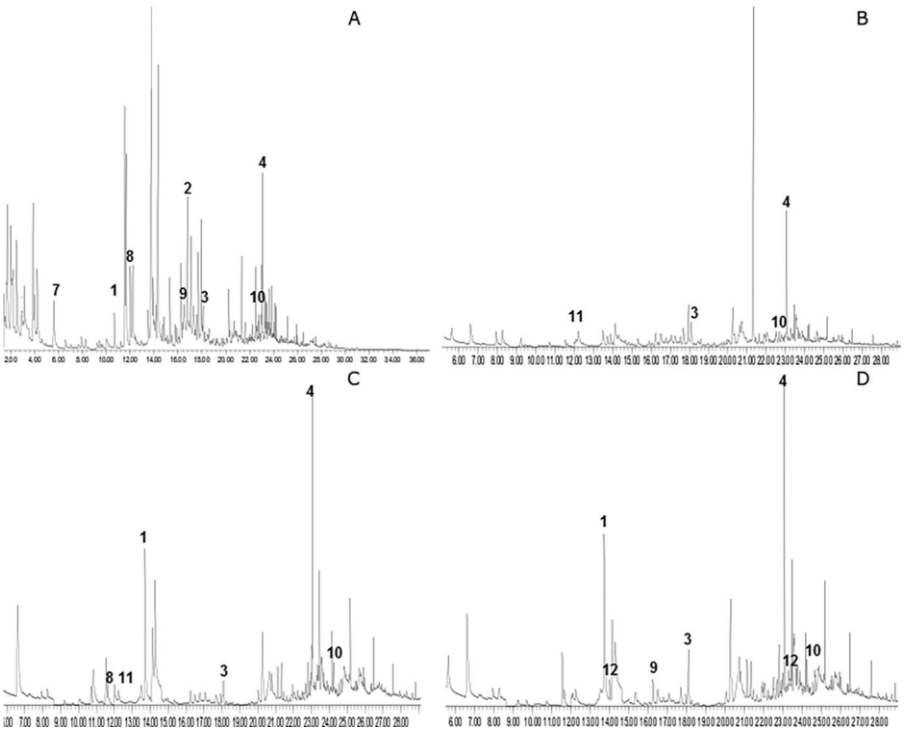

Figure 5. Chromatogram of volatile compounds of stripped oil. a) Major peaks found in the CONT samples. b) ART. c) HM. d) LM. 1: 2.4-Heptadienal; 2: 3.5-Octadien-2-one; 3: Nonanal; 4: Tridecane; 5: Octadecanoic acid; 6: Nonanoic acid; 7: Hexanal; 8: 2-Heptenal; 9: 2-Octenal; 10: Tetradecane; 11: 2 (5H) Furanone; 12: 2-Decenal. ART, Artificial antioxidant mixture; CONT, Sample without antioxidant; HM, Hydrophilic mixtures; LM, Lipophilic mixture.

we suppose that in the absence of association colloids, the higher solubility of α-tocopherol in stripped than in crude oils could have been more significant than polarity for oil oxidative protection.

In our study, γ-tocopherol did not exert any antioxidant protection in crude flaxseed oil, as its concentration did not vary significantly between fresh and oxidized oil samples. In a previous study, Nogueira et al.[45] oxidized flaxseed oil at 60 °C for 15 days and observed total depletion of γ-tocopherol. Thus, the lack of γ-tocopherol depletion can be partially attributed to the lower temperature used in this study. In addition, crude flaxseed oil presents other compounds with antioxidant activity such as β-carotene and plastochromanol-8, a γ-tocotrienol homologue. β-Carotene, that can inhibit autoxidation and shows singlet oxygen chelator properties, is more susceptible to degradation than tocopherols.[46] Plastochromanol-8 has antioxidant capacity similar to that of γ-tocopherol, only differing in size chain length and lipophilicity, and could have been consumed at 40 °C before γ-tocopherol,[46,47] However, specific analysis of these two compounds must be carried out to confirm this hypothesis. Similar depletion of sinapic acid was observed in crude and stripped oils added of HM, suggesting that when considered only antioxidant activity of sinapic acid, it was not influenced by association colloids and minor compounds presence.

Volatile compounds are also secondary products formed from the decomposition of hydroperoxides. Compounds tentatively identified in crude and stripped samples mainly comprised aldehydes, ketones, and alkanes, and have been reported in other studies that analyzed vegetable oils oxidation.[48,49] In general, nonanal and tridecane showed up in all oxidized samples, independent on the applied treatment. However, it was observed that the volatile profile varied among samples. These differences suggest that the type of antioxidant (HM or LM), presence of association colloids, and oil minor compounds affect the pathways of volatile compounds formation, influencing the type and amount of volatile compounds formed from flaxseed oil oxidation. Despite of the profile differences, total volatile compounds content indicated that HM and LM had comparable effectiveness at protecting both crude and stripped oils. This study contributed to a practical application aiming to replace artificial by natural antioxidants in foods, but more analysis must be done to better clarify the chemical mechanisms involved in these results.

4. Conclusions

HM containing three natural organic acids showed better antioxidant protection of crude flaxseed oil than LM. Moreover, HM presented comparable efficacy to ART in preventing the formation of primary and secondary oxidation products. Our results also suggested that combining different mechanisms of the already approved natural compounds could be a good strategy to provide oxidative stability of oils rich in PUFA, with a high potential to replace artificial antioxidants.

Abbreviations

4-HHE, 4-hydroxy-hexanal; 4-HNE, 4-hydroxy-nonenal; ALA, α-linolenic acid; ART, artificial antioxidant mixture; CONT, sample without antioxidant; HM, hydrophilic mixture; LM, lipophilic mixture; LNA, linoleic acid; MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; PV, peroxide value.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

association colloids, antioxidants, flaxseed oil, polar paradox, polyunsaturated fatty acids

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CHAPTER II

Combination of natural strategies to improve oxidative stability of echium seed oil

Running title: Improvement of the oxidative stability of echium oil

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Abstract

BACKGROUND

Omega-3 fatty acids (n-3 FA) promote several health benefits. Echium seed oil (ESO) is considered an alternative source to supply n-3 FA, since marine sources are limited. ESO have around 60% of polyunsaturated fatty acids, however its composition makes it high susceptible to oxidation. Thus, it is essential to combine strategies aiming to protect ESO from oxidative damage. This study was aimed to optimize antioxidant strategies (storage temperature, concentration of a mixture of antioxidants, and concentration of high oleic oil) to increase the oxidative stability of ESO.

RESULTS

ESO was extracted by screw press (PRESS) alone or screw press followed by hexane (SOLV). The oxidative stability of these samples was analyzed in sealed flasks for 180 days, or in open flasks for 30 days at 25 ºC. As the latter showed more limited oxidative stability than the former, the focus of the strategy optimization was directed towards open samples. Better results were obtained by blending 20% of high oleic sunflower oil and the full dose of the antioxidant mixture. In this optimized condition, it was observed 37-41% reduction in the PV (peroxide value) and 40-75% in the malondialdehyde (MDA) concentration compared with control oils where no strategy was applied.

CONCLUSION

The chemical composition of the oils seemed to play an important role to determine successful antioxidant strategies. It can be concluded that ESO extracted by PRESS alone showed better oxidative stability than SOLV, keeping good stability during all the storage period (180 days), but when in contact with oxygen, the full antioxidant mixture and 20% high oleic oil addition, was necessary to improve the oxidative stability.

Keywords: stearidonic acid, omega-3 fatty acids, natural antioxidants, factorial design.

1. INTRODUCTION

Omega-3 fatty acids (n-3 FA), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), promote several health benefits, including reduction of risk for cardiovascular diseases[1]. Since humans have limited ability for EPA and DHA synthesis, these must be obtained from the diet, with fish, algae and krill oils being the main commercial sources of n-3 FA. However, dietary EPA and DHA intakes are constrained by food choice and limited viable systems to supply the growing demand for n-3 FA[2,3]. For these reasons, new alternative sources of n-3 FA have been investigated[4].

Among these, *Echium plantagineum* is an herbaceous plant from *Boraginaceae* family which produces seeds with around 30% oil[5]. Echium seed oil (ESO) contains about 15% of oleic acid, 10% of γ-linolenic acid (GLA), 13% of linoleic acid (LNA), 30-35% of α-linolenic acid (ALA), and 14-16% of stearidonic acid (SDA)[6]. It distinguishes from other vegetable sources of n-3 FA due to its high content of SDA, which is the product of ALA desaturation by Δ6-desaturase in the pathway of EPA and DHA biosynthesis[7]. Considering that Δ6-desaturase is the rate limiting step to convert ALA to EPA, several studies have demonstrated that the consumption of SDA-rich oils, such as echium seed oil promotes higher plasma and cell concentrations of EPA (approximately 16% conversion to EPA) than the consumption of ALA-rich oils (approximately 6% conversion to EPA) [7,41-43].

ESO has been recently authorized as a novel food ingredient for human consumption in USA and European Union[11,12]. Although it is considered an excellent alternative to supply n-3 FA, the great proportion of polyunsaturated fatty acids (PUFAs) (>60%) in ESO composition makes it highly susceptible to lipid oxidation, leading to the formation of fishy odors and potentially toxic compounds, inhibiting the oil consumption[13,14]. Thus, it is essential to combine strategies aiming to protect ESO from oxidative damage, increasing its commercialization and use by the individuals.

Usually, oil extraction techniques include solvent extraction, such as hexane, followed by vacuum-evaporation for complete elimination of the solvent[5. Alternatively, oils can be physically extracted using continuous screw press[15]. Different extraction methods affect yields and oil composition, mainly in terms of minor polar compounds content[5,15]. Moreover, the oil will undergo different processing conditions, such as varying temperatures and oxygen exposure that can strongly affect its oxidative stability during storage.

The utilization of antioxidants is one strategy used to inhibit lipid oxidation[16]. Although artificial compounds have been more applied as antioxidants in foods, these compounds can be potentially mutagenic and carcinogenic[17]. Thus, natural compounds have been preferred, especially to be added in oils showing health-claims. In this context, it was

suggested that sinapic acid, a plant-derived phenolic compound, protected ESO in both microcapsule[18] and oil-in-water emulsion systems, showing antioxidant activity similar to that of TBHQ[19]. In another study, it was observed that a hydrophilic mixture containing sinapic, ascorbic and citric acids promoted better oxidative stability of crude flaxseed oil than a lipophilic mixture composed of α-tocopherol, ascorbyl palmitate and citric acid[20].

According to Decker et al.[21], the degree of unsaturation represents 30% of the contribution to increase the oxidation rate. Thus, the combination of ESO with high oleic acid oils, such as sunflower oil, can also be an interesting alternative to increase its oxidative stability. Finally, considering that the oxidative reaction follows the Arrhenius model[22], one other possible strategy to retard lipid oxidation in ESO could be storage at low temperatures.

However, studying the effect of all the above-mentioned strategies on oxidation rates of the oil would normally require an extensive number of experiments, resulting in time-consuming and high cost experimental designs. Multivariate factorial designs are excellent tools for studying a great number of factors simultaneously with a reduced number of experiments, allowing for evaluation of main and interaction effects between the variables[23]. Therefore, this study aimed to investigate the interaction effect between three natural strategies to improve the oxidative stability of echium seed oil obtained by two methods usually employed by the industry: screw press alone, and screw press followed by solvent extraction.

2. MATERIAL AND METHODS

2.1. Material

Were purchased 100Kg of *Echium Plantagineum* seeds from De Wit Speciality oils (ED De Wall, Netherlands), and kept under refrigeration (8 ºC) in the dark until extraction. 2L of Refined monounsaturated high oleic sunflower oil was purchased from Bunge Alimentos S/A (São Paulo, Brazil), with no antioxidant addition, and kept under refrigeration (8 ºC) until use. The major fatty acids in the high oleic sunflower oil were: hexadecenoic acid: 4.12%, stearic acid: 2.91%, oleic acid: 81.34%, and linoleic acid 9.46%. Sinapic acid, ascorbic acid and citric acid were purchased from Sigma-Aldrich (Sigma Chemical, St. Louis, MO, USA). Hexane P.A used for oil extraction was obtained from Synth (São Paulo, SP, Brazil). Isooctane, isopropanol, methanol, hexane and butanol HPLC grade were obtained from Merck & Co. (Whitehouse Station, NJ, USA).

2.2. Methods

2.2.1. Oils extraction and blend formulation

Echium seed oil was extracted using a continuous screw press (Scott Tech, ERT 50, Vinhedo, SP, Brazil). In the extraction, 70 kg of seeds resulted in 14.2 kg (22% oil). During the extraction process (approximately 3 h), the oil temperature did not exceed 50 °C. After extraction, the crude oil was centrifuged (IEC, Centra GP8R, London, UK) at 2,200 rpm for 15 min at room temperature (25 ºC), transferred to 5 L-amber flasks and stored at -25 ºC until experiments. This oil was identified as pressed oil (PRESS). The echium seed cake resulting from the continuous screw press (55 kg) was subjected to solvent extraction in a pilot-scale extractor (Ecirtec Ltd., Bauru, SP, Brazil), using hexane with three re-extractions (ratio 1:2 m/v each), for 5 hours. During the extraction, the temperature was kept around 45-55 ºC, and yielded 7% oil (3.85 kg). After the extraction, the crude oil was centrifuged at 2,200 rpm for 15 min at room temperature (25 ºC). Then, 2.4 kg of the seed cake oil was blended with 4.6 kg of pressed oil (30:70 m/m), transferred to 5 L-amber flasks and stored at -25 ºC until experiments. This oil blend was designated as press-solvent oil (SOLV).

2.2.2. Experimental design

We investigated the isolated and interaction effects of three strategies on the oxidative stability of PRES and SOLV. The first strategy consisted of applying a mixture of natural antioxidants containing 500 ppm of sinapic acid, 250 ppm of ascorbic acid and 150 ppm of citric acid, totalizing 900 ppm or the half concentration, as previously described by[20]. The second strategy consisted of blending PRESS or SOLV with 10% or 20% of high oleic oil, while the third one consisted of storing the oils under different temperatures: -25 ºC or 0ºC or 25 ºC (Supplementary table 1 and 3).

Before samples preparation, the oils were stored for no more than 4 days under -25ºC. For sample preparation, 50 ml of PRESS or SOLV were transferred to amber flasks, and received no treatment (no antioxidant and no high oleic oil), the mixture of antioxidants (900 ppm) or high oleic oil (20%), or both (mixture of antioxidants and high oleic oil). These samples were stored in sealed flasks for 180 days at – 25 ºC or 25 ºC, simulating the storage condition (Supplementary table 1 and 3). One flask of oil was collected every 30 days for analysis.

Alternatively, 50 ml of PRESS or SOLV were transferred to amber flasks, and received no treatment, the mixture of antioxidants (900 ppm), or high oleic oil (20%), or both (the mixture of antioxidants and high oleic oil). These samples were stored in open flasks for 30 days at 25 ºC, simulating the consumption condition. One flask of oil was collected every 10 days for analysis (Supplementary table 2 and 4).

2.2.3. Determination of fatty acids composition

Determination of fatty acids composition was carried according to Shirai et al. [24]. Oils (10 mg) were transferred to test tubes containing 1 mg of tricosanoic acid methyl ester as internal standard (C23:0), 50 µL of a 0.5 % butylated hydroxytoluene (BHT) solution and 1 mL of a methanolic NaOH solution (0.5 M). Then, samples were placed in a boiling water bath for 5 min, followed by addition of 2 mL of boron trifluoride diethyl etherate ($BF_{3)}$ and new boiling for 5 min. After cooling, 1 mL of isooctane was added and the mixture was vigorously homogenized. Then, 5 mL of a saturated NaCl solution was added and the samples were gently homogenized. The organic phase was extracted, dried, re-suspended in 500 µL of isooctane, and injected into a gas chromatography coupled with a triple quadrupole mass spectrometer (GC-MS Agilent 7890A GC System, Agilent Technologies Inc., Santa Clara, USA).

Fatty acids were separated on a fused silica capillary column (J&W DB-23 Agilent Inc. Santa Clara, USA) with 60 m × 0.250 mm dimensions. Injection volume was 1 µL in the split mode (1:50) and the GC inlet temperature was 250ºC. High-purity Helium was used as the carrier gas at a flow rate of 1.3 ml/min. The oven temperature was programmed to rise from 80 ºC to 175 °C at a rate of 5 °C/min, followed by another gradient of 3 °C/min until 230 °C, which was maintained for 5 min. The transfer line temperature was 280 °C. All mass spectra were obtained by electron impact (70 eV), in the scan mode (40 – 500 m/z). Compounds were identified by comparison of the retention time of fatty acids in the samples with those of standards, and also based on a comparison of their mass spectra with those given in the spectral database of the National Institute of Standards and Technology (NIST, Goitnarsbury, MD, USA). Experiments were carried out in triplicate and the results were expressed in g kg^{-1} oil.

2.2.4. Determination of primary oxidation products

The peroxide value (PV) was determined according to Shanta and Decker[25]. Briefly, the samples were prepared by vortexing 300 µL of oil in 1.5 ml of an isooctane:isopropanol (3:1 v/v) solution. Then, 200 µL of this mixture was transferred to 2.8 mL of methanol:butanol (2:1 v/v) and added of 30 µL of aqueous ammonium thiocyanate:ferrous solution (3.94 mol/L). The absorbance of the samples was measured after 20 min reaction at 510 nm using a UV–VIS mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was prepared with known concentrations of cumene hydroperoxide (CHP) (0.02 – 1.28 mg CHP; $R^2 = 0.9933$) and results were expressed as meq O_2 kg oil^{-1}. Experiments were carried out in triplicate.

2.2.5. Determination of secondary oxidation products

2.2.5.1. Malondialdehyde

Malondialdehyde (MDA) concentration was determined by High Performance Liquid Chromatography (HPLC Agilent 1260, Agilent Technologies Inc., Santa Clara, USA) following the protocol described by Hong et al.[26]. Briefly, oil samples (50 µL) were dissolved in 450 µL of an isooctane:isopropanol solution (3:1 v/v). This extract was mixed with 1 mL of a thiobarbituric acid solution (15% w/v thichloroacetic acid + 0.375% w/v thiobarbituric acid and 0.25 mol/L HCl) and heated at 95 ºC for 15 min. Then, the samples were cooled, centrifuged (10,000 rpm for 10 minutes), filtered using a 0.22 µm PVD filter and injected into the HPLC. MDA was analyzed on a reverse phase C18 analytical column (250 mm x 4.6 mm; 5 µm) (Phenomenex, Torrence, CA, USA). An isocratic mobile phase composed of 60% phosphate buffer (50 mmol, pH 7.4) and 40 % methanol was used at a flow rate of 1.0 mL/ min. The injection volume was 30 µL and the column temperature was maintained at 30 ºC. Detection was performed by fluorescence with emission and excitation wavelengths at 515 nm and 553 nm, respectively. A standard curve was prepared using 1,1,3,3-tetraetoxipropano (TEP) (30 – 1449.45 mmol/L TEP; R^2 = 0.998) and results were expressed as mg MDA kg^{-1} oil. Experiments were carried out in triplicate.

2.2.5.2. Volatile compounds

Volatile compounds were determined according to Gómez-Cortéz et al.[27], with some modifications. Oil samples (10 mL) added of 4-methyl-2-pentanone as internal standard (IS) were sealed in a glass vial with 10 mL headspace and extracted using Headspace Solid Phase Microextraction (SPME) in a Combi PAL autosampler (Zwingen, Switzerland). The vials were shaken (400 rpm) for 15 min at 50 ºC, then, a 50/30 µm Stableflex DVB/CAR/PMDS fiber (Divynil Benzene/Carboxen/Polydimethylsiloxane, Supelco, Bellefonte, PA, USA) was exposed in the headspace for 60 min at 50 ºC. The fiber was thermally desorbed into a GC-MS (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara, CA, USA), at 250 ºC for 30 min, using splitless mode. Volatiles separation was performed on a fused silica capillary column (5%-Phenyl-methylpolysiloxane, HP-5, 30 m x 0.25 mm dimensions) (Agilent Technologies Inc., Santa Clara, CA, USA). High-purity Helium was the carrier gas at a constant flow of 1.0 mL/min. The oven temperature was programmed to increase from 40 ºC until 100 ºC at 4ºC/min, and then to 220 ºC at 17 ºC/min, which was maintained for 10 min. All mass spectra were acquired using electron impact ionization (70 eV) in the scan mode (35–350 *m/z*). Compounds were identified by comparison of the retention time of volatiles in the samples with those of standards, and also based on a comparison of their mass spectra with those given in the spectral database of the National Institute of Standards and Technology (NIST, Goitnarsbury, MD, USA). The

results were expressed as an area ratio of volatiles peak area/IS peak area. Assays were carried out in duplicate.

2.2.6. Determination of tocopherols

Tocopherols content (α-, β- + γ- and δ-tocopherols) was determined by HPLC (Agilent 1260, Agilent Technologies Inc., Santa Clara, USA) using a modified method from Gliszczynska-Swiglo and Sikorska[28]. Samples were diluted in isopropanol, filtered through 0.22 µm PVDF filters and injected into a ZORBAX Agilent C18 column (150 mm × 4.6 mm; 5 µm, Agilent Technologies Inc., Santa Clara, USA). An isocratic mobile phase consisting of 50% acetonitrile and 50% methanol was used at a flow rate of 1 mL/min and at 30 ºC. Detection was carried out by fluorescence at a wavelength of 295 nm (excitation) and 325 nm (emission). Identification was performed by comparison of retention time of the compounds in the samples with those of tocopherol standards. Quantification was carried out using standard curves, and the results were expressed in g kg^{-1} oil. Experiments were performed in duplicate.

2.2.7. Statistical analysis

Results were expressed as mean ± SEM. Differences in fatty acids and initial oxidative quality between the two types of oil (PRESS and SOLV) were compared by T-test for independent groups. Initially, the parameter applied to quantify the oxidation was selected based on behavior of the samples during the experimental time as plotted in Figures 1 to 4, supplementary material. Based on these selected chemical markers, regression models were adjusted to the experimental data for samples analyzed for 180 days that followed the 2^3 design. Coefficients of the models were evaluated by T-test and the quality of the adjustment measured by ANOVA, coefficient of determination and residual plot. Data from 2^2 design were analyzed by factorial ANOVA, followed by Tukey post hoc test. The same procedure was used to 2^3 design for PRESS samples. Control and optimized values were compared by T-test or Mann-Whitney U-test. The statistical software Statistica v. 13.4 (TIBCO Software Inc, Round Rode, Texas, USA) were used to perform all analyses. GraphPad Prism (San Diego, CA, USA) was applied to plot the graphs. An alpha value of 0.05 was adopted to reject the null hypothesis in this study.

3. RESULTS

Table 2 shows the results for the chemical and oxidative characterization of PRESS and SOLV oils. The results suggested that the oil extraction method influenced its composition.

SOLV showed higher ALA, GLA and SDA content than PRESS oil, while LNA was higher in PRESS oil. Among the investigated tocopherols, only γ-tocopherol was found in ESO. With respect to the oxidative quality, the oils showed comparable levels of triacylglycerol hydrolysis, as evidenced by their similar acidity values. However, SOLV had higher PV and MDA concentration than PRESS samples. SOLV also showed higher total phenolic compounds and γ-tocopherol contents. Although the profile of major volatiles has been different between the samples, the total volatiles (TV) concentration did not differ between them.

The oxidative stability of both SOLV and PRESS under *storage condition* (2^3 factorial design), and *consumption condition* (2^2 factorial design), was evaluated by following three chemical markers of oxidation: PV, MDA and γ-tocopherol contents. TV compounds were also determined in the samples under *storage condition*. Based on the behavior of these markers over the oxidation progress (Figs. 1 – 4, supplementary material), the highest PV and concentration of MDA and TV, and the lowest concentration of γ-tocopherol, were selected as end-point data to which regression models were adjusted (Tables 1- 4, supplementary material).

Table 3 shows the models adjusted to data from PRESS oil under *storage condition*. Among the chemical markers of oxidation, no models could be adjusted to MDA and TV, and the model adjusted to γ-tocopherol presented *p* value of lack fit <0.001. Only PV showed a significant model with no evidence of lack of fit, in which temperature was the only factor influencing the oxidative stability. In order to evaluate the effect of all the studied factors, a factorial ANOVA (2^3) was also applied to the data (Fig. 1). Fig. 1A confirmed that temperature was the most important factor modulating PV, with the higher the temperature, the higher PV. For MDA (Fig. 1B), the temperature had no effect. It was assumed that greater concentrations of γ-tocopherol indicated higher preservation of this compound, thus, better oxidative stability of the oil. Hence, the worst strategy for γ-tocopherol was observed upon addition of the full dose of antioxidant and 20% high oleic oil at 25 ºC (Fig. 1C). Finally, only temperature increase promoted a raise in TV compounds concentration (Fig. 1D), although this increase has not been significant. Based on these results, it can be suggested storage under -25 ºC as the best strategy to improve the oxidative stability of PRESS oil during 180 days, as summarized in Fig. 1E.

Factorial ANOVA applied to PRESS oil under *consumption condition* (30 days storage at 25 ºC) is shown in Figure 2. Probability values obtained for the two main factors and their interactions is shown in Table 5, supplementary material. Both MDA concentration and PV were reduced with increasing high oleic oil or antioxidant concentration (Figs. 2A and 2B), while none of the strategies influenced γ-tocopherol preservation in the samples (Table 5, supplementary material). Thus, differently from the *storage condition*, the utilization of the full dose of antioxidants and/or 20% of high oleic oil showed to be the best strategy to improve oxidative stability of PRESS oil under *consumption condition* (Fig. 2C).

Since the levels of PV in PRESS samples in the *storage condition* were below the legal limits of 15 meq O_2 kg^{-1} oil fixed for unrefined oils[32] (Table 1, supplementary material), regardless of the strategy applied, as final recommendation, it is suggested the utilization of the strategy rendering the higher oxidative stability of PRESS oils in the *consumption condition.*

For the SOLV oil under *storage condition* (Table 4), except for MDA, all regression models were significant and had good quality of adjustment. Pareto Charts of the standardized effects and contour plots are shown in Figure 3. The addition of 20% high oleic oil was the most effective strategy to decrease PV (Fig. 3A). Moreover, the interaction between temperature and the antioxidant concentration indicated that the lowest PV occurred at -25 °C, regardless of the antioxidant concentration (Fig. 3B). However, reduced PV was also achieved at 25 ºC using the full dose of antioxidants (Fig. 3B), indicating that this could be an alternative stabilization condition instead of the use of freezing temperatures.

For γ-tocopherol, it was observed that the interaction between temperature and the antioxidant concentration was the most relevant to increase γ-tocopherol preservation (Fig. 3C), whereas the increase of high oleic oil concentration contributed negatively to its preservation (Fig. 3C). Hence, higher γ-tocopherol preservation was obtained at -25 ºC in absence of high oleic oil and antioxidants (Fig. 3D), or at 25 ºC with addition of the full dose of antioxidants (Fig. 3D) and absence of high oleic oil. Finally, TV compounds content increased with increasing the antioxidant concentration at -25 ºC, while high oleic oil had no effect (Fig. 3E). In agreement with the previous chemical markers, the interaction between temperature and the antioxidant concentration strongly influenced the reduction of TV levels (Fig. 3F). Thus, the lowest TV compounds concentration was achieved at -25 °C in absence of antioxidants, but low levels were also observed at 25 ºC upon addition of the full dose of antioxidants.

In order to find an optimal condition of stabilization benefiting all the chemical markers simultaneously, i.e., minimum PV and TV levels, and higher γ-tocopherol preservation, the desirability function was applied (Table 5). It suggested that the optimal strategy combination was no addition of antioxidants, storage temperature of -25 °C and 5% addition of high oleic oil (shown in Fig. 3G).

SOLV samples under *consumption condition* were analyzed using factorial ANOVA (Table 6, supplementary material and Figure 4). It was observed that increasing simultaneously antioxidant and high oleic oil concentrations promoted the highest reduction in PV and MDA (Figs. 4A and 4B). Conversely, for γ-tocopherol, increasing antioxidant concentration adversely affected its preservation (Fig. 4C), while high oleic oil showed no effect. As no addition of the antioxidant mixture drastically increased PV content (Table 4, supplementary material), the best strategy combination to stabilize SOLV oil under *consumption condition* was addition of 20% of high oleic oil and full dose of antioxidant (Fig. 4D).

Likewise PRESS oil, the levels of PV in SOLV oil from *storage condition* were below to the legal limits of 15 meq O_2 kg^{-1} oil fixed for unrefined oils[32] (Table 3, supplementary material), regardless of the strategy applied. Thus, as final recommendation, it was suggested the utilization of the combination rendering the higher oxidative stability of SOLV oil in the *consumption condition.*

4. DISCUSSION

The great oxidative instability of highly polyunsaturated oils, such as ESO, still hampers their commercialization and consumption. In the present study, it was hypothesized that the optimization of antioxidant strategies will increase the oxidative stability of ESO obtained by screw press alone (PRESS), and of an ESO blend composed of 70% of screw press-extracted oil and 30% of cake seed solvent-extracted oil (SOLV).

With respect to the initial chemical characteristics of the oils, SOLV and PRESS samples had distinct chemical composition and oxidative quality, with SOLV samples showing higher concentration of polyunsaturated fatty acids, total phenolic compounds and tocopherols. SOLV oil also showed lower oxidative quality, as evidenced by the higher PV and MDA concentration. These differences could be attributed to the presence of seed cake solvent-extracted oil in SOLV samples, and suggest that the technique and the matrix fraction (seed or seed cake) utilized for the oil extraction influence its composition. Solvent-based techniques can penetrate in the matrix cell, promoting a greater abstraction of lipids and other minor compounds with either antioxidant or pro-oxidant properties. In addition, the utilization of the echium seed cake (which was crushed) for oil extraction may have favored the solvent penetration and enhanced the extraction of those compounds. Thus, as the oil has not been refined, it was assumed that the chemical composition of seed cake oil was more complex compared to the oil obtained by screw press alone. Similar results have been reported by Chouabi et al.[29], who found higher concentration of total phenolic compounds in solvent-extracted red pepper seed oil than in the cold pressed oils. Moreover, Petropoulos et al.[30] observed that cardoon seed cake solvent-extracted oil showed greater total phenolic compounds than oil seeds, which was attributed to the greater portion of total phenols remaining in seed cakes after mechanical pressing of seeds. However, the high temperature combined with extended extraction time in solvent extraction led to higher oxidation of the seed cake oil, explaining the lower oxidative quality of SOLV oil. This result is in agreement with Chouabi et al.[29], who also observed lower oxidative quality of solvent-extracted red pepper seed oil compared to cold pressed samples.

It was observed that the effect of the strategies applied varied greatly according to the oil characteristics (PRESS or SOLV), storage condition and oxidation marker. In general, SOLV

oils under both *consumption and storage* conditions showed higher PV, MDA and TV concentrations than PRESS oils (Tables 1-4, supplementary materials), which is probably related to the lower initial oxidative quality of SOLV samples[31].

Regardless of the applied oxidation condition in the factorial design, both PRESS and SOLV oils in the *storage condition* showed PV within the average value defined by the Codex Alimentarius Commission[32] (15 meq O_2 kg oil^{-1} for unrefined oils) after 180 days, even in the control oil, where no antioxidant strategy was applied. This was expected due to the limited oxygen headspace in these samples[33], as has been already reported for olive oil by Iqdiam et al.[34], and suggests that for long-term storage in sealed flasks, endogenous γ-tocopherol could efficiently protect ESO from oxidative damage.

With respect to the effect of the studied factors on the oxidative stability of PRESS and SOLV in the *storage condition,* temperature was the most important factor to control oxidation in PRESS oils. As the rate of lipid oxidation is exponentially related to temperature, the shelf life of a food lipid decreases logarithmically with increasing temperature[35], which explains the increase in PV, TV, and the drop in γ-tocopherol concentrations with increasing temperature from -25 ºC to 25 ºC. The effect of temperature on the oxidative stability of unsaturated oils has been well described in literature, with the higher the storage temperature, the higher the oxidation rate, in agreement with our results[35–37].

Although temperature have also influenced SOLV in the *storage condition*, its effect was overcame by high oleic oil addition (20%). Blending polyunsaturated oils with different proportions of monounsaturated oils aims to reduce their levels of polyunsaturated fatty acids. Oleic acid, the main monounsaturated fatty acid in high oleic oils, is 40 and 80 times less reactive than LNA and ALA, respectively, because these fatty acids have one and two active bis-allylic methylene groups between two double bonds that can lose a hydrogen atom very readily. As a result, lipid free radicals able to initiate and/or propagate oxidation are more rapidly formed in LNA and ALA than in oleic acid[35]. Thus, the more unsaturated the fatty acid, the more bis-allylic methylene groups appears in its structure, and the less stable is the oil[35]. For this reason, the use of high oleic oil has been reported as a simple and low cost method to prepare more stable oil products[36,38,39].

Interactions between full dose of the antioxidant mixture and high oleic oil, and full dose of the antioxidant mixture and temperature were also important to modulate the oxidative stability of SOLV oil in the *storage condition,* showing the importance of combining strategies to optimize the oxidative stability. Previous studies have demonstrated the efficacy of combining antioxidant strategies as the ones applied in this study to stabilize highly unsaturated oils such as fish oil and perilla oil[34,40]. A strength of this study was that the use of a factorial design allowed determining different possible combinations for optimizing SOLV oil stability. For example, it was observed that the simultaneous addition of high oleic oil and full dose of the

antioxidant mixture, allowed the oils to be stored at 25 ºC instead of -25 ºC. Probably, at such low temperature, oxidation rates are already sufficiently slow, so addition of the antioxidant mixture did not contribute to increase oxidation inhibition. This is an important finding from the food industry and commercial points of view because cold chain is usually a bottleneck for food preservation due to its high cost.

When it comes to SOLV and PRESS oils in the *consumption condition*, PV and MDA concentration were much higher than those found in oils in the *storage condition.* Moreover, under certain experimental circumstances, the oils under *consumption condition* showed PV above the average set by the Codex Alimentarius Commission[32] (15 meq O_2 kg oil^{-1} for unrefined oils) after 30 days. These results were expected due to the large oxygen supply in *consumption* samples[31,33], and emphasize the necessity of antioxidant strategies to be applied in ESO afterward opening the packaging. It was observed a consistent positive effect of high oleic oil addition for reducing PV and MDA concentration, in agreement with our previous findings, which might be attributed to the above-discussed reasons. The interaction between high oleic oil (20%) and full dose of the antioxidant mixture was positive for increasing the oxidative stability of SOLV oil, except for γ-tocopherol, while in PRESS oil this interaction reduced the oxidative stability. This find suggests that the different chemical composition of these oils, caused by the presence of seed cake solvent-extracted oil in SOLV samples, may have influenced oxidation pathways. However, further studies are necessary to confirm this hypothesis.

5. CONCLUSION

Our study showed that optimal conditions of oxidative stabilization differed under storage (sealed flasks) or consumption condition (open flasks). The chemical composition of the oils seemed to play an important role in determining successful antioxidant strategies. Overall, ESO extracted by screw press alone showed better oxidative stability than the ESO blend composed of screw pressed oil and seed cake solvent-extracted oil. Both PRESS and SOLV exhibited good stability during 180 days of storage, however, after oxygen exposition, the addition of full dose of the antioxidant mixture, containing sinapic, ascorbic and citric acids, and of 20% of high oleic oil, improved the oxidative stability of the oils compared with samples without these treatments. In this optimized condition, it was observed 37-41% reduction in PV and 40-75% in the MDA concentration compared with the control where no antioxidant strategy was applied.

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Table 1. Levels of the factors applied in the 2^3 and 2^2 factorial designs.

		Range of variation		
Factors	**Description**	**-1**	**0**	**+1**
F1†	Antioxidants‡ (%)	0	half dose	full dose
F2	Temperature (°C)	-25	0	+25
F3†	High Oleic Oil (%)	0	10	20

† Factors and levels applied in the 2^2 factorial design.

‡ Full dose of the antioxidant mixture: sinapic acid (500 ppm), ascorbic acid (250 ppm) and citric acid (150 ppm); half dose of the antioxidants: sinapic acid (250 ppm), ascorbic acid (125 ppm) and citric acid (75 ppm).

Table 2. Fatty acids composition and quality of SOLV and PRESS oils.

	Oil sample[†]		
	SOLV	**PRESS**	***p* value**[‡]
Fatty acids (g 100 g^{-1} oil)			
Palmitic acid (C16:0)	10.67 ± 0.57	9.99 ± 1.72	0.727
Stearic acid (C18:0)	6.23 ± 0.26	5.04 ± 0.14	0.014
Oleic acid (C18:1 n9)	16.60 ± 0.48	14.74 ± 1.93	0.402
Linoleic acid (C18:2 n6) - LNA	12.60 ± 0.43	25.48 ± 1.29	0.001
α-Linolenic acid (C18:3 n3) - ALA	29. 86 ± 0.67	28.26 ± 0.42	0.040
ϒ-Linolenic (C18:3 n6) - GLA	9.09 ± 0.23	7.45 ± 0.37	0.021
Stearidonic acid (C18:4 n3) -SDA	14.22 ± 0.35	11.36 ± 0.61	0.016
Gondoic acid (C20:1 n9)	0.68 ± 0.04	0.46 ± 0.02	0.009
Oil quality			
Acidity (mg KOH g^{-1} oil)	1.42 ± 0.03	1.44 ± 0.01	0.552
Antioxidant activity (μM TE)[§]	40.89 ± 3.36	88.83 ± 2.42	<0.001
Total phenolic compounds (mg GAE kg^{-1} oil) [§]	37.07 ± 2.21	13.42 ± 0.16	<0.001
Malondialdehyde (mg kg^{-1} oil)	15.70 ± 1.88	2.70 ± 0.13	0.002
Peroxide value (meq O_2 kg^{-1} oil)	4.54 ± 0.17	0.84 ± 0.03	<0.001
γ-Tocopherol (g kg^{-1} oil)	3.25 ± 0.12	1.86 ± 0.03	<0.001
Volatile compounds (area ratio *10^3)			
2,4-Heptadienal	1.78 ± 0.07	2.98 ± 0.23	0.038
3,5-Octadien-2-one	27.76 ± 2.74	24.80 ± 0.84	0.411
1-Hexanol	49.18 ± 11.18	45.30 ± 0.26	0.761
3-Hepten-2-one	4.72 ± 0.33	-	0.005
2,4-Decadienal	-	1.95 ± 0.20	0.010
2-Octenal	5.00 ± 0.40	6.62 ± 0.08	0.058
Total	88.44 ± 14.71	81.65 ± 1.61	0.861

[†] Oil samples: SOLV oil - obtained by mixing oil from hexane extraction with oil from continuous screw press); PRESS oil - obtained by continuous screw press.

[‡] Probability value obtained by t test for independent groups (n=2-4 replicates).

[§] Antioxidant activity measured by DPPH methodology. TE: trolox equivalent. GAE: gallic acid equivalent.

Table 3. Regression models adjusted to the chemical markers of oxidation determined in PRESS oil under *storage condition*.

Chemical Marker	Regression Model†	Quality parameters
Peroxide value	$\hat{y} = 0.64 + 0.75X_2$ SD mean: ± 0.05 SD coefficients: ± 0.05	*p* value of the lack of fit: 0.059 R^2 adjusted : 0.75
MDA	$\hat{y} = 4.37$ SD mean: ± 0.14	Non-significant model
γ-Tocopherol	$\hat{y} = 0.69 – 0.04X_1\text{-}0.03X_2\text{-}0.09X_3\text{-}0.05X_1X_2\text{-}0.03X_1X_3\text{-}0.03X_2X_3\text{-}0.02X_1X_2X_3$ SD mean: ± 0.0009 SD coefficients: ± 0.0011	*p* value of the lack of fit: <0.001 R^2 adjusted : 0.68
Total volatile compounds	$\hat{y} = 52.732$ SD mean: ± 3.93	Non-significant model

† X_1: antioxidant concentration; X_2: temperature; X_3: high oleic oil concentration.

Table 4. Regression models adjusted to the chemical markers of oxidation determined in SOLV oil under *storage condition* and optimized conditions for oxidative stabilization.

Chemical Marker	Regression Model†	Quality parameters	Optimized Value
Peroxide value	$\hat{y}$= 6.53 -0.47X_1 + 0.53 X_2 -1.16X_3 -0.75X_1X_2 SD mean: ± 0.08 SD coefficients: ± 0.10	*p* value of the lack of fit: 0.074 R^2 adjusted : 0.72	6.03 meqO_2/Kg oil
MDA	$\hat{y}$ = 6.53 SD mean: ± 0.08	Non-significant model	6.53 mg/Kg oil
γ-Tocopherol	$\hat{y}$ = 0.68 -0.04X_3 +0.06X_1X_2+0.04X_2X_3 SD mean: ± 0.007 SD coefficients: ± 0.008	*p* value of the lack of fit: 0.174 R^2 adjusted : 0.69	0.77 g/Kg oil
Total volatile compounds	$\hat{y}$ = 68.77+16.05 X_1 -15.18 6X_1X_2 SD mean: ± 1.75 SD coefficients: ± 2.05	*p* value of the lack of fit: 0.079 R^2 adjusted : 0.51	41.18 ratio x10^{-3}
Optimized condition for oxidative stability	Antioxidant concentration: 0 ppm Temperature: -25 ºC High oleic oil concentration: 5%		

† X_1: antioxidant concentration; X_2: temperature; X_3: high oleic oil concentration.

Figure captions

Figure 1. Effect of antioxidant concentration, temperature, and high oleic oil on the oxidative stability of PRESS oil under *storage condition*. Factorial ANOVA for: A) PV; B) MDA; C) γ-tocopherol; D) total volatile compounds; E) concentration of chemical markers of oxidation in the optimal stabilization condition *vs.* in the control oil. PV: peroxide value (meq O_2 kg^{-1} oil); MDA: malondialdehyde (mg kg^{-1} oil); TOC: γ-tocopherol (g kg^{-1} oil); TV: total volatile compounds (area ratio*10^3).

Figure 2. Effect of antioxidant and high oleic oil concentrations on the oxidative stability of PRESS oil under *consumption condition* (at 25 ºC). Factorial ANOVA for: A) PV; B) MDA; C) concentration of chemical markers of oxidation in the optimal stabilization condition *vs.* in the control oil. PV: peroxide value (meq O_2 kg^{-1} oil); MDA: malondialdehyde (mg kg^{-1} oil).

Figure 3. Effect of antioxidant concentration, temperature, and high oleic oil on the oxidative stability of SOLV oil under *storage condition*. Pareto Charts and contour plots for: A and B) PV; C and D) γ-tocopherol; E and F) total volatile compounds; G) concentration of chemical markers of oxidation in the optimal stabilization condition *vs.* in the control oil. PV: peroxide value (meq O_2 kg^{-1} oil); TOC: γ-tocopherol (g kg^{-1} oil); TV: total volatile compounds (area ratio*10^3).

Figure 4. Effect of antioxidant and high oleic oil concentration on the oxidative stability of SOLV oil under *consumption condition*. Factorial ANOVA for: A) PV; B) MDA; C) γ-tocopherol; D) concentration of chemical markers of oxidation in the optimal stabilization condition *vs.* in the control oil. PV: peroxide value (meq O_2 kg^{-1} oil); MDA: malondialdehyde (mg kg^{-1} oil); TOC: γ-tocopherol (g kg^{-1} oil).

Figure 1.

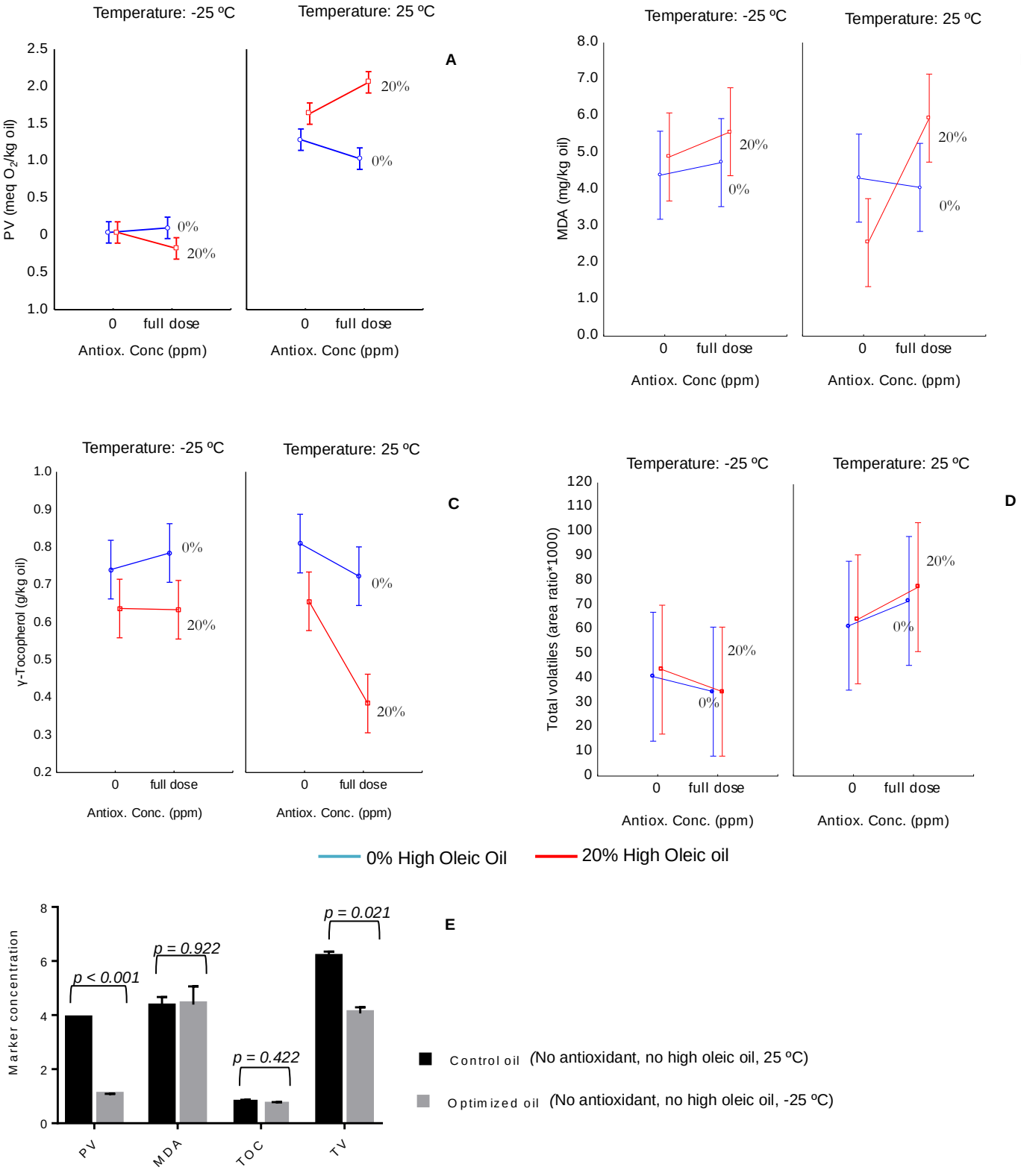

Temperature: -25 ºC
Temperature: 25 ºC
A
PV (meq O2/kg oil)
2.5
2.0
1.5
1.0
0.5
0
0.5
1.0
0%
20%
0
full dose
Antiox. Conc (ppm)
B
MDA (mg/kg oil)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Antiox. Conc. (ppm)
C
γ-Tocopherol (g/kg oil)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
D
Total volatiles (area ratio*1000)
120
110
100
90
80
70
60
50
40
30
20
10
0
0% High Oleic Oil
20% High Oleic oil
E
Marker concentration
8
6
4
2
0
p < 0.001
p = 0.922
p = 0.422
p = 0.021
PV
MDA
TOC
TV
Control oil (No antioxidant, no high oleic oil, 25 ºC)
Optimized oil (No antioxidant, no high oleic oil, -25 ºC)

Figure 2.

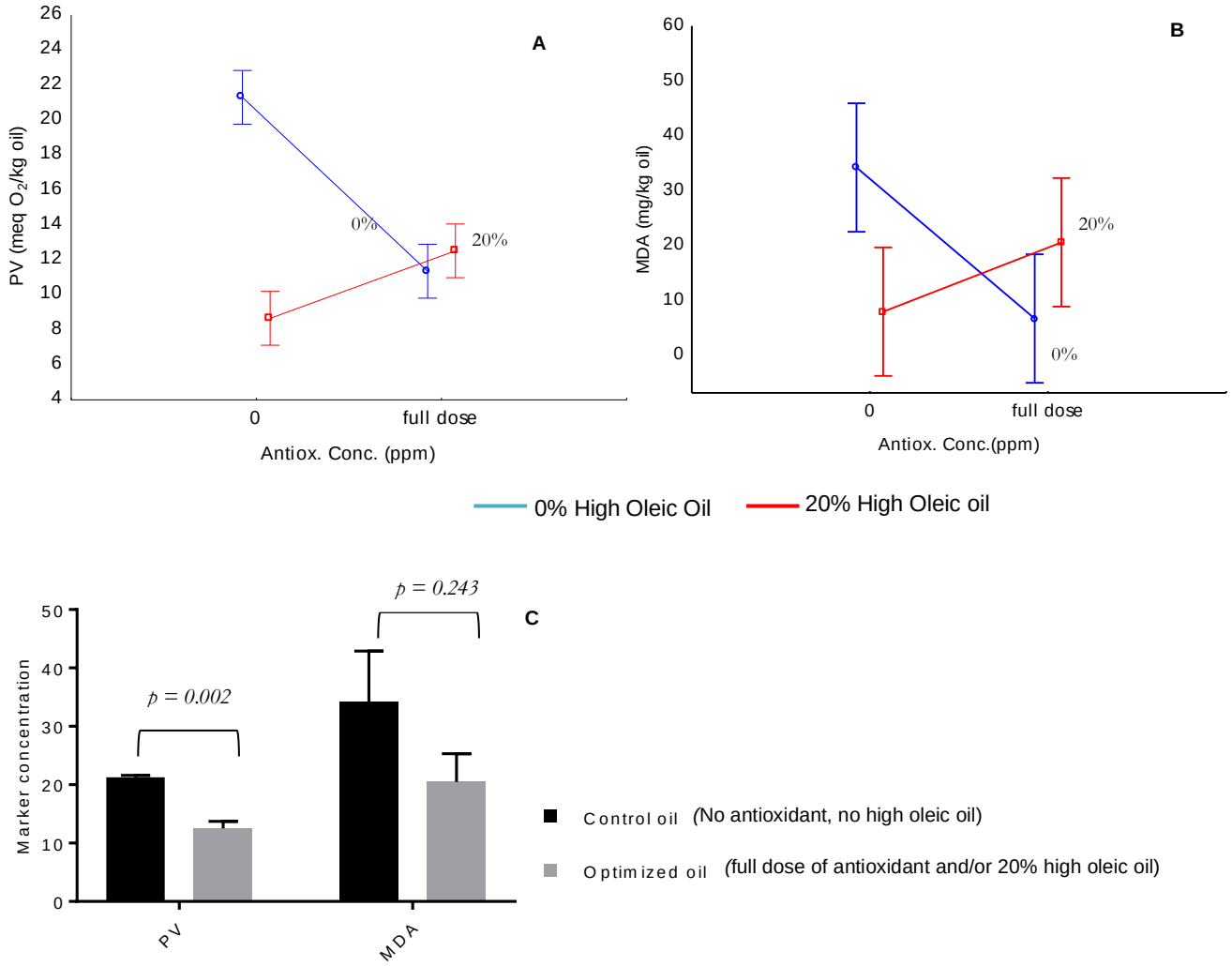

A
PV (meq O_2/kg oil)
26
24
22
20
18
16
14
12
10
8
6
4
0%
20%
0
full dose
Antiox. Conc. (ppm)
B
MDA (mg/kg oil)
60
50
40
30
20
10
0
20%
0%
0
full dose
Antiox. Conc.(ppm)
0% High Oleic Oil
20% High Oleic oil
C
p = 0.243
p = 0.002
Marker concentration
50
40
30
20
10
0
PV
MDA
Control oil (No antioxidant, no high oleic oil)
Optimized oil (full dose of antioxidant and/or 20% high oleic oil)

Figure 3.

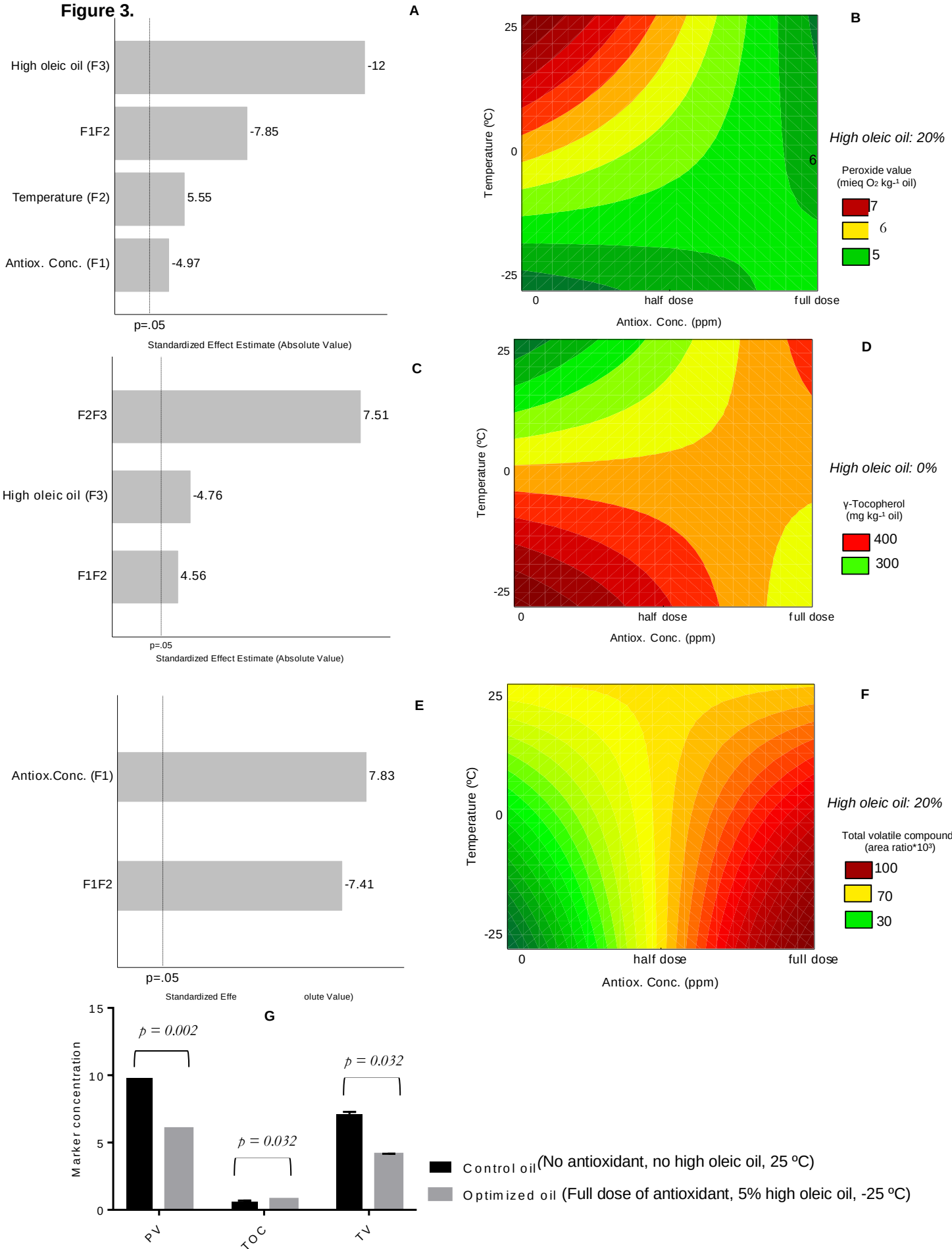
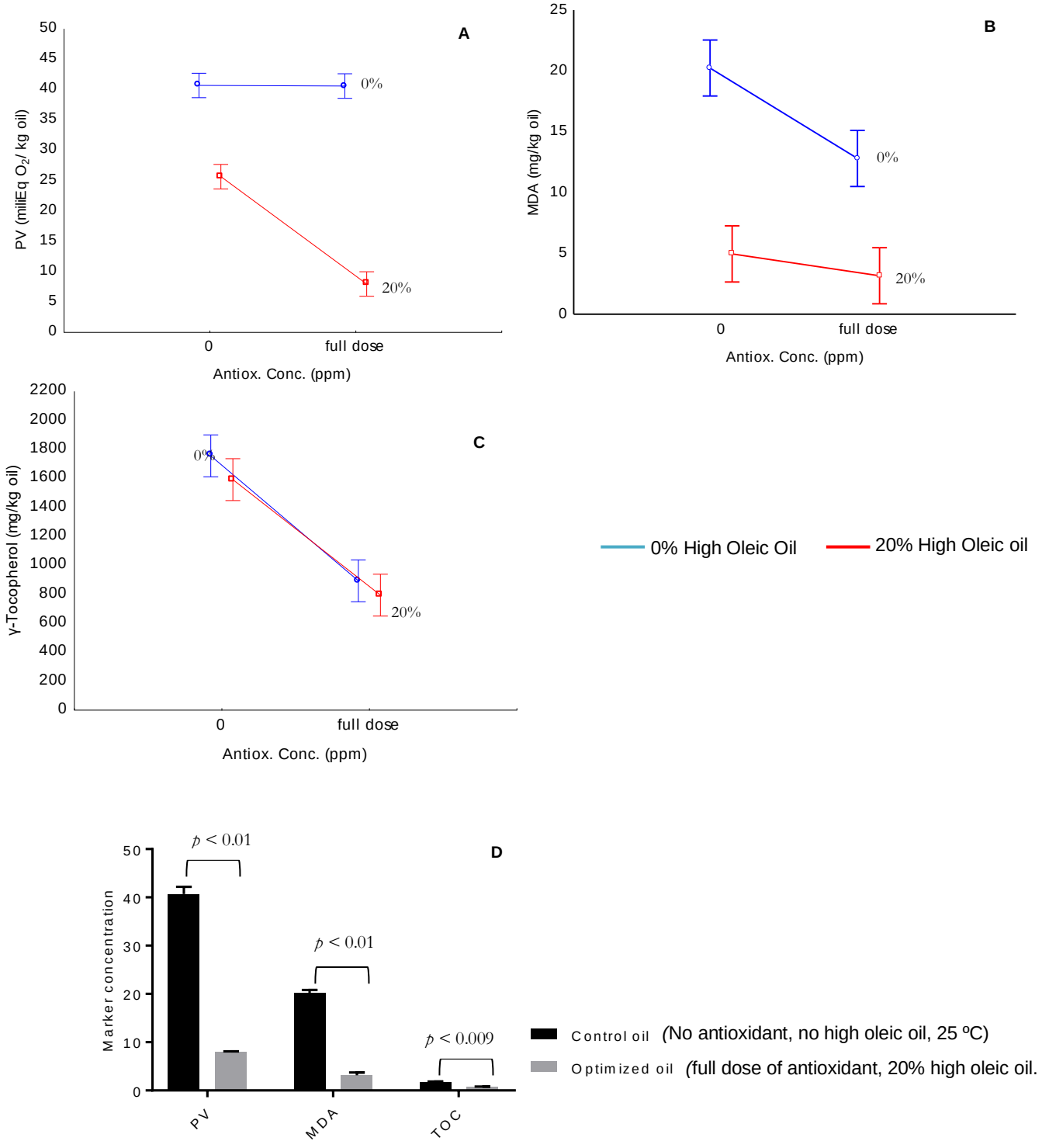

A
High oleic oil (F3)
-12
F1F2
-7.85
Temperature (F2)
5.55
Antiox. Conc. (F1)
-4.97
p=.05
Standardized Effect Estimate (Absolute Value)
B
25
0
-25
Temperature (ºC)
6
0
half dose
full dose
Antiox. Conc. (ppm)
High oleic oil: 20%
Peroxide value
(mieq O2 kg-1 oil)
7
6
5
C
F2F3
7.51
High oleic oil (F3)
-4.76
F1F2
4.56
p=.05
Standardized Effect Estimate (Absolute Value)
D
25
0
-25
Temperature (ºC)
0
half dose
full dose
Antiox. Conc. (ppm)
High oleic oil: 0%
γ-Tocopherol
(mg kg-1 oil)
400
300
E
Antiox.Conc. (F1)
7.83
F1F2
-7.41
p=.05
Standardized Effe
olute Value)
F
25
0
-25
Temperature (ºC)
0
half dose
full dose
Antiox. Conc. (ppm)
High oleic oil: 20%
Total volatile compounds
(area ratio*10³)
100
70
30
G
15
10
5
0
Marker concentration
p = 0.002
p = 0.032
p = 0.032
PV
TOC
TV
Control oil (No antioxidant, no high oleic oil, 25 ºC)
Optimized oil (Full dose of antioxidant, 5% high oleic oil, -25 ºC)

Figure 4.


A
PV (miliEq O2/ kg oil)
0%
20%
0
full dose
Antiox. Conc. (ppm)
B
MDA (mg/kg oil)
0%
20%
0
full dose
Antiox. Conc. (ppm)
C
γ-Tocopherol (mg/kg oil)
0%
20%
0
full dose
Antiox. Conc. (ppm)
0% High Oleic Oil
20% High Oleic oil
D
p < 0.01
p < 0.01
p < 0.009
Marker concentration
PV
MDA
TOC
Control oil (No antioxidant, no high oleic oil, 25 ºC)
Optimized oil (full dose of antioxidant, 20% high oleic oil.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Factorial design 2^3 applied to PRESS oils evaluated under *storage condition* (sealed flasks for 180 days).

Assay	Independent factors (coded and uncoded values)			Chemical markers of oxidation‡			
	Antioxidant concentration (ppm) †	Temperature (ºC)	High oleic oil concentration (%)	PV§	MDA§	γ-Tocopherol¶	Total volatiles§
1	-1 (0)	-1 (-25)	-1 (0)	1.04 ± 0.05	4.39 ± 0.68	0.74 ± 0.04	40.65 ± 2.33
2	+1 (full dose)	-1 (-25)	-1 (0)	1.12 ± 0.14	4.73 ± 0.44	0.78 ± 0.00	34.53 ± 3.21
3	-1 (0)	+1 (25)	-1 (0)	3.87 ± 0.08	4.31 ± 0.36	0.81 ± 0.06	61.50 ± 2.05
4	+1 (full dose)	+1 (25)	-1 (0)	2.93 ± 0.02	4.05 ± 0.39	0.72 ± 0.01	71.62 ± 29.07
5	-1 (0)	-1(-25)	+1 (20)	1.04 ± 0.06	4.89 ± 0.27	0.64 ± 0.01	43.55 ± 5.58
6	+1(full dose)	-1(-25)	+1 (20)	0.84 ± 0.03	5.57 ± 1.19	0.63 ± 0.01	34.53 ± 3.21
7	-1 (0)	+1 (25)	+1 (20)	5.85 ± 0.74	2.55 ± 0.37	0.66 ± 0.01	64.11 ± 9.28
8	+1(full dose)	+1 (25)	+1 (20)	9.39 ± 0.39	5.94 ± 0.07	0.38 ± 0.06	77. 27 ± 7.31
9	0 (half dose)	0 (0)	0 (10)	1.20 ± 0.10	3.39 ± 0.58	0.75 ± 0.04	35.97 ± 4.65
10	0 (half dose)	0 (0)	0 (10)	1.46 ± 0.03	3.94 ±1.25	0.75 ± 0.01	55.66 ± 2.01
11	0 (half dose)	0 (0)	0 (10)	1.63 ± 0.07	4.30 ±1.04	0.74 ± 0.00	60.58 ± 4.02
n	-	-	-	3	3	2	2
p¥	-	-	-	*<0.001*	*0.024*	*0.048*	*0.006*

† Full dose of antioxidant mixture added to the oil: sinapic acid (500 ppm), ascorbic acid (250 ppm) and citric acid (150 ppm); half dose of antioxidant mixture added to the oil: sinapic acid (250 ppm), ascorbic acid (125 ppm) and citric acid (75 ppm).

‡ Chemical markers of oxidation are expressed as mean ± SEM. PV (peroxide value): meq O_2 kg^{-1} oil; MDA (malondialdehyde): mg kg^{-1} oil; γ-tocopherol (g kg^{-1} oil) and total volatile compounds (compound area/IS area ratio x 10^3).

§ Higher values observed within 180 days (See Fig. 1, supplementary material).

¶ Lower value observed within 180 days (See Fig. 1, supplementary material).

¥ The highest probability value below 0.05 obtained by Factorial ANOVA

Supplementary Table 2. Factorial design 2^2 applied to PRESS oil evaluated under *consumption condition* (open flasks for 30 days at 25 ºC).

Assay	Independent factors (coded and uncoded values)		Chemical markers of oxidation‡		
	Antioxidant concentration (ppm)†	High oleic oil concentration (%)	PV§	MDA§	γ-Tocopherol¶
1	-1 (0)	-1 (0)	21.29 ± 0.31	34.16 ± 8.74	0.76 ± 0.04
2	+1 (full dose)	-1 (0)	11.35 ± 0.48	6.59 ± 0.88	0.81 ± 0.00
3	-1 (0)	+1 (20)	8.66 ± 0.13	7.84 ± 1.74	0.41 ± 0.26
4	+1 (full dose)	+1 (20)	12.52 ± 1.20	20.50 ± 4.83	1.03 ± 0.04
n	-	-	3	3	2
p¥	-	-	0.002	<0.001	>0.05

† Full dose of antioxidant mixture added to the oil: sinapic acid (500 ppm), ascorbic acid (250 ppm) and citric acid (150 ppm); half dose of antioxidant mixture added to the oil: sinapic acid (250 ppm), ascorbic acid (125 ppm) and citric acid (75 ppm).

‡ Chemical markers of oxidation are expressed as mean ± SEM. PV (peroxide value): meq O_2 kg^{-1} oil; MDA (malondialdehyde): mg kg^{-1} oil ; γ-tocopherol (g kg^{-1} oil) and total volatile compounds (compound area/IS area ratio x 10^3).

§ Higher values observed within 180 days (**See Fig. 2 of supplementary material**).

¶ Lower value observed within 180 days (**See Fig. 2 of supplementary material**).

¥ The highest probability value below 0.05 obtained by Factorial ANOVA

Supplementary Table 3. Factorial design 2^3 applied to SOLV oils evaluated under *storage condition* (sealed flasks for 180 days).

Assay	Independent factors (coded and uncoded values)			Chemical markers of oxidation‡			
	Antioxidant concentration (ppm) †	Temperature (ºC)	High oleic oil concentration (%)	PV§	MDA§	γ-Tocopherol¶	Total volatiles§
1	-1 (0)	-1 (-25)	-1 (0)	6.50 ± 0.22	13.08 ± 1.57	0.80 ± 0.10	52.30 ± 1.93
2	+1 (full dose)	-1 (-25)	-1 (0)	7.87 ±1.45	15.64 ± 2.27	0.77 ± 0.01	117.40 ± 19.85
3	-1 (0)	+1 (25)	-1 (0)	9.82 ± 0.00	15.57 ± 2.27	0.61 ± 0.06	71.22 ± 1.13
4	+1 (full dose)	+1 (25)	-1 (0)	7.70 ± 0.36	10.30 ± 0.65	0.78 ± 0.12	86.73 ± 3.39
5	-1 (0)	-1(-25)	+1 (20)	5.52 ± 0.34	12.29 ± 1.12	0.73 ± 0.04	45.75 ± 1.28
6	+1(full dose)	-1(-25)	+1 (20)	5.25 ± 0.21	10.67 ± 0.80	0.52 ± 0.01	105.57 ± 48.14
7	-1 (0)	+1 (25)	+1 (20)	7.31 ± 0.37	9.51 ± 1.03	0.64 ± 0.01	73.97 ± 8.22
8	+1(full dose)	+1 (25)	+1 (20)	4.54 ± 0.17	9.78 ± 2.28	0.74 ± 0.00	61.94 ± 4.95
9	0 (half dose)	0 (0)	0 (10)	5.51 ± 0.35	9.84 ± 0.33	0.64 ± 0.02	41.14 ± 6.14
10	0 (half dose)	0 (0)	0 (10)	6.04 ± 0.47	11.55 ± 1.26	0.64 ± 0.02	47.80 ± 3.48
11	0 (half dose)	0 (0)	0 (10)	5.74 ± 0.15	13.69 ± 1.42	0.68 ± 0.01	52.67 ± 13.59
n	-	-	-	2-3	3	2	2
p¥	-	-	-	*0.044*	*0.032*	*0.017*	*0.042*

† Full dose of antioxidant mixture added to the oil: sinapic acid (500 ppm), ascorbic acid (250 ppm) and citric acid (150 ppm); half dose of antioxidant mixture added to the oil: sinapic acid (250 ppm), ascorbic acid (125 ppm) and citric acid (75 ppm).

‡ Chemical markers of oxidation are expressed as mean ± SEM. PV (peroxide value): meq O_2 kg^{-1} oil; MDA (malondialdehyde): mg kg^{-1} oil; γ-tocopherol (g kg^{-1} oil) and total volatile compounds (compound area/IS area ratio x 10^3).

§ Higher values observed within 180 days (See Fig. 3 of supplementary material).

¶ Lower value observed within 180 days (See Fig. 3 of supplementary material).

¥ The highest probability value below 0.05 obtained by Factorial ANOVA.

Supplementary Table 4. Factorial design 2^2 applied to SOLV oil under *consumption condition* (open flasks for 30 days at 25 ºC).

Assay	Independent factors (coded and uncoded values)		Chemical markers of oxidation‡		
	Antioxidant concentration (ppm)†	High oleic oil concentration (%)	PV§	MDA§	γ-Tocopherol¶
1	-1 (0)	-1 (0)	40. 68 ± 1.52	20 24 ± 0.59	1.76 ± 0.09
2	+1 (full dose)	-1 (0)	40.58 ± 0.68	12.81 ± 1.30	0.89 ± 0.01
3	-1 (0)	+1 (20)	25.66 ± 0.51	4.96 ± 1.27	1.59 ± 0.04
4	+1 (full dose)	+1 (20)	7.98 ± 0.14	3.16 ± 0.57	0.79 ± 0.04
n	-	-	3	3	2
p¥	-	-	*<0.001*	*0.022*	*<0.001*

† Full dose of antioxidant mixture added to the oils: sinapic acid (500 ppm), ascorbic acid (250 ppm) and citric acid (150 ppm); half dose of antioxidant mixture added to the oils: sinapic acid (250 ppm), ascorbic acid (125 ppm) and citric acid (75 ppm).

‡ Chemical markers of oxidation are expressed as mean ± SEM. PV (peroxide value): meq O_2 kg^{-1} oil; MDA (malondialdehyde): mg kg^{-1} oil; γ-tocopherol (g kg^{-1} oil) and total volatile compounds (compound area/IS area ratio x 10^3).

§ Higher values observed within 180 days (See Fig. 4 of supplementary material).

¶ Lower value observed within 180 days (See Fig. 4 of supplementary material).

¥ The highest probability value below 0.05 obtained by Factorial ANOVA.

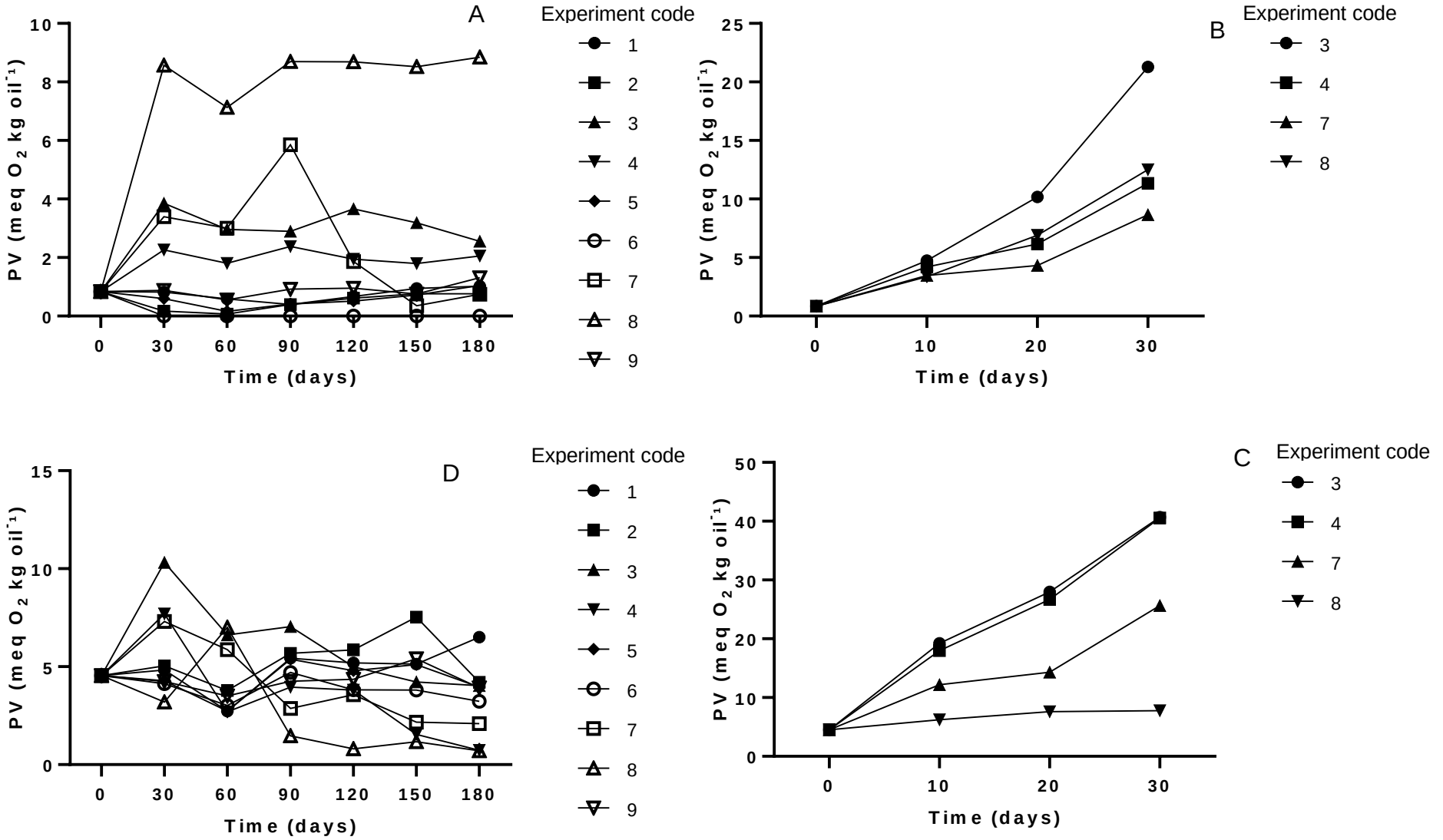
Supplementary Table 5. Probability values of chemical markers of oxidation determined in PRESS samples under *consumption condition* (open flasks for 30 days at 25 ºC).

Chemical marker of oxidation	*p* value
Peroxide value	Antioxidant concentration (F1): 0.002 High oleic oil concentration (F3): <0.001 F1 x F3 (interaction): <0.001
MDA	Antioxidant concentration (F1): 0.181 High oleic oil concentration (F3): 0.258 F1 x F3 (interaction): 0.004
γ-Tocopherol	Antioxidant concentration (F1): 0.071 High oleic oil concentration (F3): 0.642 F1 x F3 (interaction): 0.097

Supplementary Table 6. Probability values of chemical markers of oxidation determined in SOLV samples under *consumption condition*, i.e., uncapped flasks for 30 days at 25 ºC.

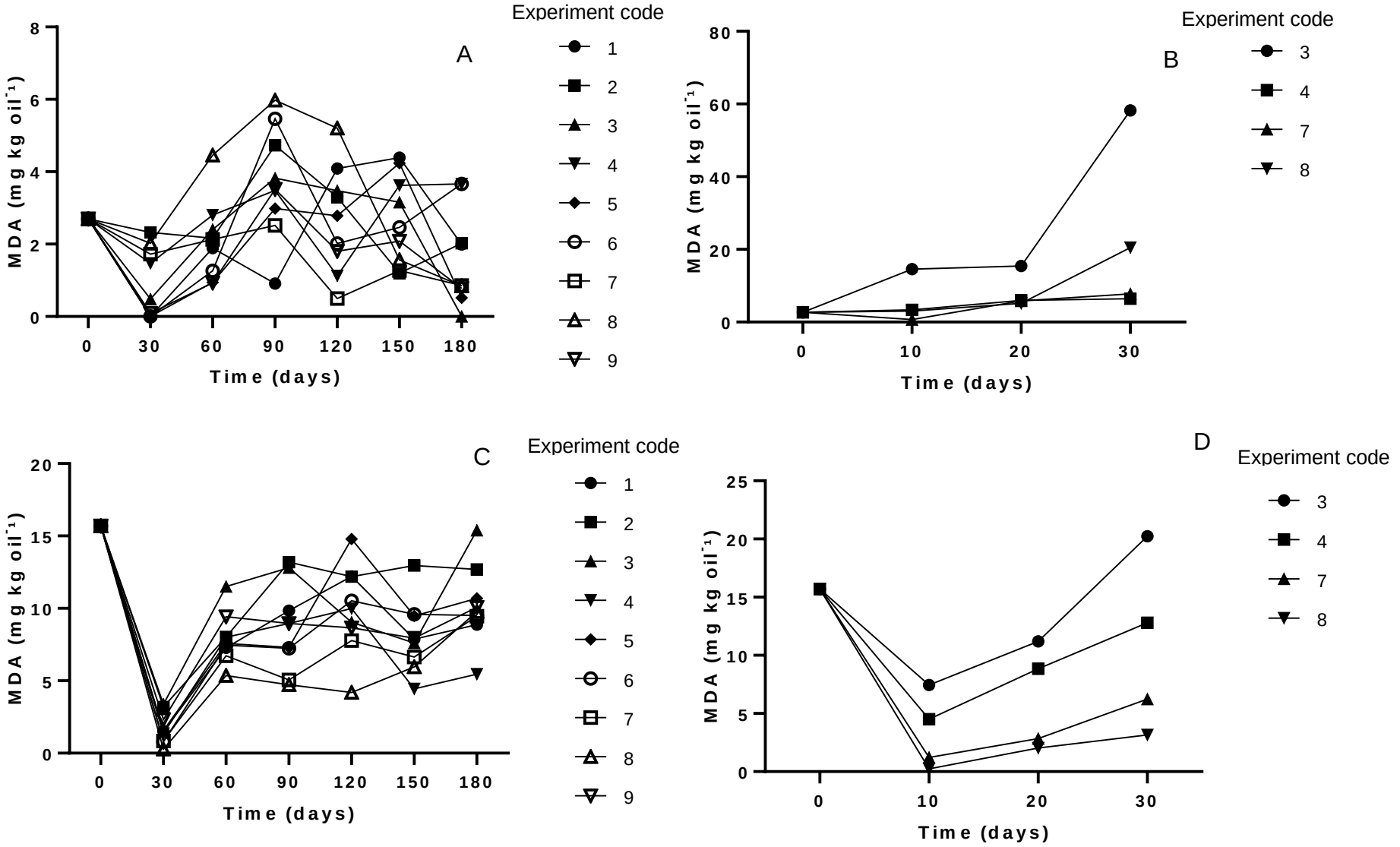
Chemical marker of oxidation	*p* value
Peroxide value	Antioxidant concentration (F1): <0.001 High oleic oil concentration (F3): <0.001 F1 x F3 (interaction): <0.001
MDA	Antioxidant concentration (F1): 0.002 High oleic oil concentration (F3): <0.001 F1 x F3 (interaction): 0.022
γ-Tocopherol	Antioxidant concentration (F1): <0.001 High oleic oil concentration (F3): 0.065 F1 x F3 (interaction): 0.568

Supplementary Figure 1.



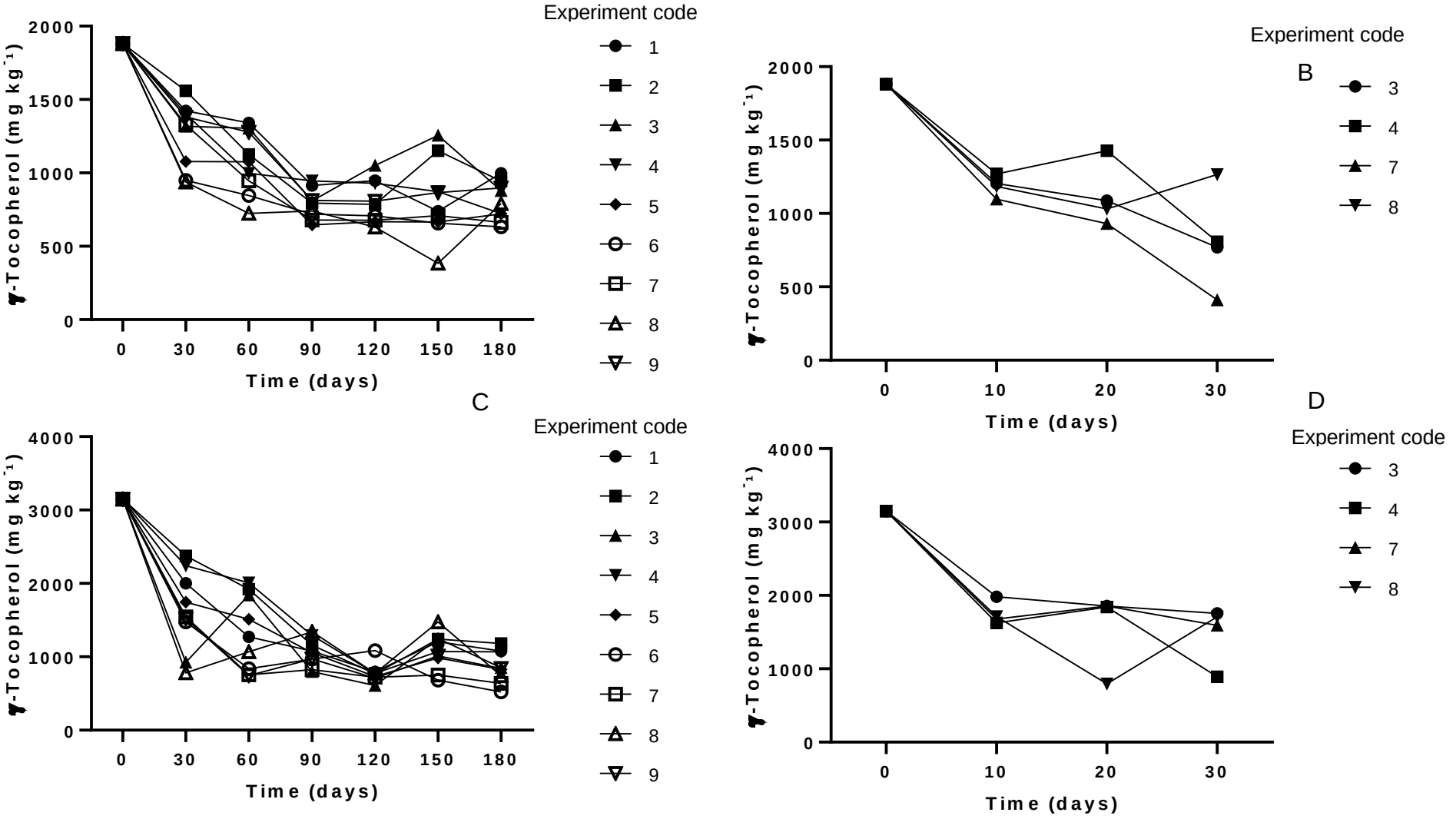
Supplementary Figure 1. PV determined in the experiments of the factorial designs: (A) PRESS oil under *storage condition*; (B) PRESS oil under *consumption condition*; (D) SOLV oil under *storage condition;* (C) SOLV oil under consumption condition.

Supplementary Figure 2.



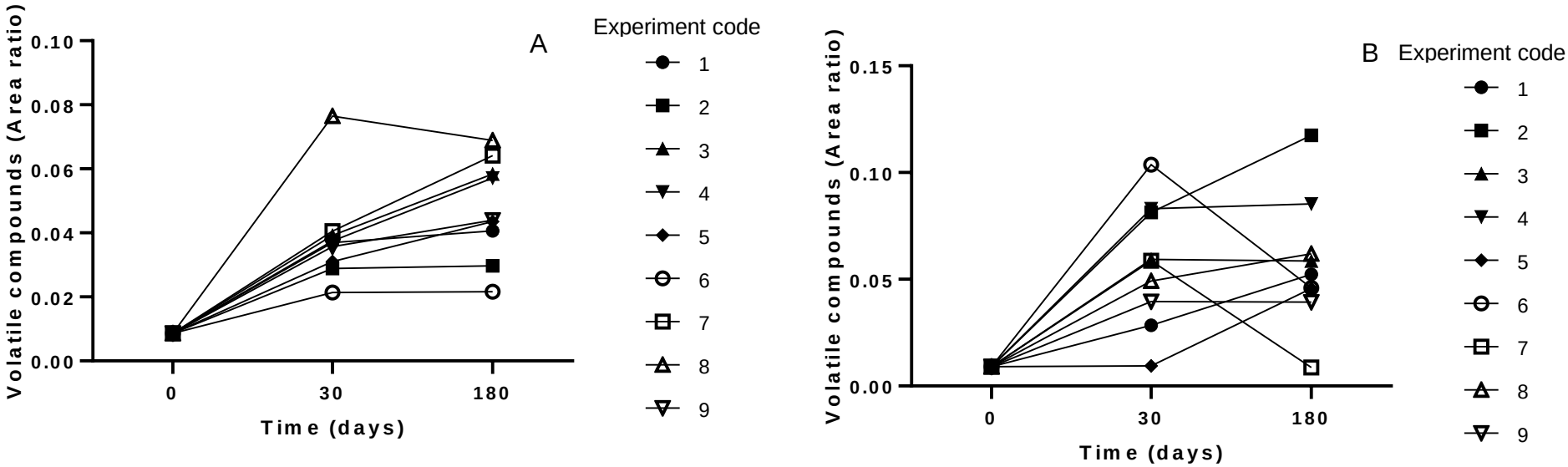
Supplementary Figure 2. Malondialdehyde determined in the experiments of the factorial designs: (A) PRESS oil under *storage condition*; (B) PRESS oil under *consumption condition*; (C) SOLV oil under *storage condition;* (D) SOLV oil under consumption condition.

Supplementary Figure 3.



Supplementary Figure 3. γ-tocopherol determined in the experiments of the factorial designs (A) PRESS oil under *storage condition*; (B) PRESS oil under *consumption condition*; (C) SOLV oil under *storage condition;* (D) SOLV oil under consumption condition.

Supplementary Figure 4.



Supplementary Figure 4. Total volatile compounds determined in the experiments of the factorial designs: (A) PRESS oil under *storage condition*; (B) PRESS oil under *consumption condition*.

7. CONCLUSION

The results obtained from the two parts of our study showed that the combination of compounds showing different antioxidant strategies, such as radical scavengers, metal chelators and antioxidant regenerators promoted an antioxidant activity similar to those showed by artificial compounds (TBHQ and EDTA). It was also observed that for crude flaxseed oil, the mixture of hydrophilic antioxidants was more effective than the lipophilic mixture, confirming the "Polar Paradox" theory. But this behavior was changed in stripped oil, according to the "association colloids" absence. In addition, the oil extracted from Echium seeds by screw press showed better oxidative stability than the oil extracted by solvent. Both Echium oils showed a good stability during the storage for 6 months. After oxygen exposition, the addition of full dose of mixture of hydrophilic antioxidants, containing sinapic, ascorbic and citric acids, and the mixture with 20% of high oleic sunflower oil, improved the oxidative stability when compared with samples without these treatments.

8. NEXT STUDIES

Some limitations still need to be investigated in order to become the Echium oil enough stable for food application, such as for example:

- It is necessary to better understand the biochemical reactions involved in the initial oxidative reactions that take part when seeds are being pressed.
- The oil obtained from screw press extraction must be sensory evaluated.
- Other solvents, such as ethanol, can be applied instead of hexane to obtain a more environmentally friendly process and the final oil must be submitted to the next steps of refining.
- It is necessary to make Echium seeds viable in Brazil
- More studies involving the cardiovascular protection of Echium oil must be carried out, mainly using human and animal models.