CHIA (*Salvia hispanica* L.) OIL STABILITY: STUDY OF THE EFFECT OF NATURAL ANTIOXIDANTS

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ABSTRACT

The chia seed (*Salvia hispanica* L.) is globally popular and valued for its nutritional and health attributes. Chia oil is mainly composed of triglycerides, in which polyunsaturated fatty acids (PUFAs, linoleic and α-linolenic acids) are found in high amounts. Although it seems evident that such fatty acid composition is favorable from a nutritional point of view, a higher content of linoleic and linolenic acids results in poorer oxidative stability and shorter shelf life of the oil. The aim of this study was to evaluate the combined effects of the storage condition (300 days under fluorescent light - 800 Lux - or in the dark, both at room temperature) with the addition of natural antioxidants (rosemary extract, RE; tocopherol, TOC; ascorbyl palmitate, AP). In the dark, the combined addition of AP and TOC significantly reduced lipid oxidation and improved oil shelf life. Moreover, this combination maintained an acceptable quality of at least up to 300 storage days. Results from this work highlight the influence of illumination condition on chia oil oxidative stability, suggesting that this oil should be stored in containers with light-barrier properties, and probably added to the antioxidants examined in the current study.

KEY–WORDS: chia oil, oxidative stability, natural antioxidant, storage.

1.INTRODUCTION

Chia (*Salvia hispanica* L.) is an annual herbaceous plant that belongs to the Lamiaceae family, which is native from southern Mexico and northern Guatemala. Chia seed contains about 0.32 g oil /g seed; 0.28 g fiber /g seed, 0.21 g protein /g seed and 0.05 g ash /g seed, and chia oil contains the highest proportion of α-linolenic acid (0.6 g /g oil) from any known vegetable source (Ayerza & Coates 2004). The antioxidant capacity of this oil is relatively low because the phenolic compounds present in the seed are mostly hydrophilic in nature (Da Silva Marineli *et al.* 2014). Polyunsaturated fatty acids (PUFA) play a major role in the prevention and treatment of non-communicable diseases (NCDs), also known as chronic diseases, such as: hypertension, coronary artery disease, diabetes and cancer (Fereidoon 2009; Poudyal *et al.* 2012). Although it seems clear that such fatty acid composition is favorable from a nutritional point of view, a higher content of PUFAs results in poorer oxidative stability and shorter shelf life of the oil. When PUFAs are exposed to environmental factors such as air, light and temperature, oxidation reactions produce undesirable flavors, rancid odors, discoloration and other forms of spoilage. Natural or synthetic antioxidants can increase the shelf life of food products by retarding lipid oxidation through different action mechanisms. The synthetic antioxidants of the food industry, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertbutyl hydroquinone (TBHQ), have widespread use as food additives; yet, their effects on human health and metabolic routes are being questioned (Shahidi & Zhong 2005; Iqbal & Bhanger 2007). As a result, there is a great interest in obtaining and using antioxidants from natural sources, such as simple phenols, phenolic acids, carotenoids, anthocyanins, flavonoids, vitamins and spice extracts (Maestri *et al* 2006). Among these, rosemary extract (*Rosmarinus officinalis* L) has been evaluated in many studies on different lipid matrices (Hras et al 2000; Erkan *et al* 2008; Ixtaina *et al* 2012; Martinez *et al* 2013[a]; Martinez *et al*

2013[b]; Chen *et al* 2014). This is a *GRAS* additive and its major active antioxidant component is carnosic acid (Terpinc *et al* 2009). Ascorbic acid has strong reducing properties due to the presence of the enediol group. The tocopherols abound naturally in unrefined vegetable oil and have been extensively tested. Numerous studies have evaluated antioxidant effectiveness in different lipid matrices by accelerated oxidation tests; however, work carried out under real storage conditions is scarce (Gómez Alonso *et al* 2007; Let *et al* 2007; Olmedo *et al* 2008; 2009 Arcoleo *et al* 2009; Ixtaina *et al* 2012; Martinez *et al* 2013[a]).

This work aimed at evaluating the effectiveness of natural antioxidants alone and/or in combination during ten-month storage in the dark and at room temperature in the oxidative stability of chia oil obtained by cold pressing.

2. MATERIALS AND METHODS

2.1 Materials

Chia seeds were obtained from commercial plantations in the province of Salta. Chia oil extraction was carried out in a single step with a Komet screw press, pilot plant scale type (Model CA 59 G, IBG Monforts, Germany). The moisture content of the seeds was adjusted to 0.11 g/g dry basis, the pressing temperature was 30 °C, the screw speed was 20 rpm and the restriction die was 6 mm (Martínez *et al* 2012). The oil obtained was filtered through a filter press and stored until use in amber glass bottles at -20 °C under nitrogen atmosphere.

Natural mixed tocopherols (GUARDIAN™ TOCO 70 IP A), rosemary extract (GUARDIAN™ 12, fat soluble), ascorbyl palmitate (GRINDOX™ 562), citric acid (GRINDOX™ 373) and a commercial mixture of ascorbyl palmitate (10%) and tocopherols (10%) (GRINDOX™ 497)

were obtained from Danisco (Copenhagen, Germany). The content of the main rosemary antioxidative components (carnosic acid) was 1% and the relative percentage of tocopherol isomers was analysed by HPLC (purity 70%: α= 8,8%, β= 1,55%, γ= 61,82% and δ= 27,82%). Tertbutyl hydroquinone (TBHQ), a synthetic antioxidant, was used as a positive control due to its known ability to stabilize edible oils and consequent wide use in the food industry.

2.2 Methods

2.2.1 Total seed oil content

Three samples (10 g each) of dry chia seeds were used to determine total oil content in accordance with AOCS official method Ba 3-38 (AOCS, 2009).

2.2.2 Oil analysis

Acidity index (AI), peroxide (PV), K_{232} (conjugated dienes, CD) and K_{270} (conjugated trienes, CT) values were evaluated using standard AOCS (2009) methods. Chlorophyll and carotenoid content was determined at 670 and 470 nm, respectively, in cyclohexane via specific extinction values using the method of Mínguez- Mosquera *et al.* (1991).

The oxidative stability (OSI) was measured following the Rancimat (Metrohm, Switzerland) method (Cd 12b-92 AOCS, 2009) by using 3g oil aliquots. Airflow rate was set at 20L/h and temperature of the heating block was maintained at 100 °C. Results corresponded to the break points in the plotted curves and were expressed as induction time (IT) in hours.

To evaluate radical scavenging capacity (RSC), 100 mg of chia oil in 1 mL toluene was vortexed (20 s, ambient temperature) with 3.9 mL toluene solution of the free stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radical ($DPPH^{\cdot}$) at a concentration of 10^{-4} mol/L according to Martinez *et al* (2008). Against a blank of pure toluene, the absorption at 515 nm was

measured in 1 cm quartz cells using an UV-visible spectrophotometer (Perkin-Elmer Lambda 25, Shelton, CT, USA). RSC toward $DPPH^{\cdot}$ was estimated by mean of the following equation:

$DPPH^{\cdot}$ = {1 – [(Absorbance of control – Absorbance of test sample) / Absorbance of control]} x 100

Where $DPPH^{\cdot}$ expresses the amount of the radical that remains in the medium after the antioxidants in the oil sample are depleted (Espín *et al.* 2000). RSC was expressed as IC50, reflecting the depletion of the free radical to 50%. A lower IC50 value indicates higher antiradical activity.

For fatty acid (FA) composition determinations, 0.5 g of oil was subjected to alkaline saponification by reflux (45 min) using 30 mL 1 N KOH in methanol. Unsaponificable matter was extracted with n-hexane (3 x 30 mL). FA were converted to methylesters (FAME) by reflux (45 min) using 50 mL 1 N H_2SO_4 in methanol and analysed by gas chromatography (GC) (Perkin-Elmer, Shelton, CT, USA) using a fused silica capillary column (30 m x 0.25 mm i.d.x 0.25 lm film thickness) CP Wax 52 CB (Varian, Walnut Creek, CA, USA); carrier gas N_2 at 1 mL/min; split ratio 100:1; column temperature programmed from 180 °C to 240 °C (10 min) at 4 °C/min; injector and detector temperatures at 250 °C, FID. The identification of FAME was carried out by comparison of their retention times with those of reference compounds (Sigma–Aldrich, St. Louis, MO, USA) (Martinez *et al. 2013 a*).

Iodine value (IV) was calculated from fatty acid percentages (Torres & Maestri, 2006) by using the formula:

IV = (% Palmitoleic acid x 1.001) + (%Oleic acid x 0.899) + (% Linoleic acid x 1.814)

The relationship between monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) was also calculated:

MUFA/PUFA= (% Palmitoleic acid + % Oleic acid) / (% Linoleic acid + % Linolenic acid)

Tocopherols were analyzed by HPLC (Perkin-Elmer, Shelton, CT, USA) according to the procedure of Lazzez *et al*. (2008) with some modifications. Samples of 1 g oil were placed into 10 mL volumetric flasks. A quantity of n-hexane was added, swirling to dissolve the sample and making up to volume with the same solvent. An aliquot of 20 µL of this solution was injected onto a Supelcosil LC-NH2-NP column (25 cm x 4.6 mm, Supelco, Bellefonte, PA, USA). The mobile phase was n-hexane/ethyl acetate (70/30 v/v) with a flow rate of 1 mL/min. UV detection at 295 nm was performed. Individual tocopherols were identified by comparison of their retention times with those of authentic standards (CN Biomedicals, Costa Mesa, CA). Individual tocopherols were quantified by the external standard method. The linearity of the response was verified by fitting to line results of each tocopherol individual of twenty standard solutions with known concentrations. The concentration was expressed as mg tocopherol / kg of oil.

The total phenol content (TPC) in oil was determined by the Folin-Ciocalteau method according to Torres *et al* (2009). Briefly, phenolic compounds were analysed in 20 g aliquots of oil. They were dissolved in 10 mL of n-hexane and extracted three times with 12.5 mL of methanol/water (60 : 40 v/v) by stirring over a magnetic plate for 15 min. The pooled extracts were washed twice with 10 mL of n-hexane, and solvents were removed in a rotating evaporator (Buchi, Flawil, Switzerland) at 30 °C under vacuum. To

a suitable dilution of the extracts, Folin–Ciocalteu reagent (Fluka, Buchs, Switzerland) was added and the absorbance values of the solutions at 725 nm (total phenols) were measured. Total phenol content is given as mg gallic acid /kg oil. In the case of *σ*-diphenols, concentration was calculated from the reaction with $Na_2MoO_4.2H_2O$ and reading at 350 nm (Gutfinger 1981) expressed as mg caffeic acid/kg oil

Squalene determinations were done from 200 mg oil aliquots according the procedure of Maestri *et al.* (2015). The unsaponifiable fractions in oil were determinate according Martinez et al. 2006. An aliquot of oil (1g) was saponificated by reflux (45 min, 20 mL KOH in methanol 1M) and then extracted with n-hexane (3 × 30 mL). Preparative TLC plates (0.5 mm silica gel; Merck, Darmstadt, Germany) were used to separate fractions (toluene/acetone (95:5, vol/vol). Sterols and methylsterols were run without further treatment using a VF-5ms (Varian, Walnut Creek, CA) capillary column (30 m × 0.25 mm i.d.) coated with a 0.25 µm layer of 5% phenyl, 95% polydimethylsiloxane; carrier gas N2 at 1 mL/min1; column temperature programmed from 240°C (1 min) to 290°C at 2°C min–1; injector and detector temperatures 300°C; FID. GC–MS used an HP 5 (Hewlett-Packard, Palo Alto, CA) fusedsilica capillary column (30 m × 0.25 mm i.d.) coated with a 0.25 µm layer of 5% phenyl methyl siloxane, and helium (flow rate 1 mL/min) as carrier gas. The column, injector, and detector temperatures were as for GC analysis. Sterols and methylsterols were identified by comparison of the mass spectral data with those of authentic reference compounds. Hydrocarbons were analyzed by GC and GC–MS. Briefly, hydrocarbons purified by TLC as described above were analyzed by GC using a VF-5ms capillary column. The column temperature was programmed from 70 to 300°C at 4°C min–1, injector and detector temperatures 320°C, carrier gas N2 at 1 mL/min, FID. GC–MS used an HP 5 capillary column and helium (flow rate 1 mL/min) as carrier gas. The column, injector, and detector temperatures were as for GC analysis. Hydrocarbons were identified

by their retention times and comparison of the mass spectral data with those of authentic reference compounds.

2.2.3 Rancimat analysis

Rosemary extracts (RE), tocopherols (TOC), ascorbyl palmitate (AP), citric acid (CA) and blends were added separately to chia oil aliquots at different concentrations. The additives were dissolved in oil by using a shaker 5 min (Martinez *et al* 2013[a]). Tocopherols were in range of 50 to 800 mg/kg; rosemary extract ranged from 1000 to 16000 mg/kg; ascorbyl palmitate from 50 to 700 mg/kg; citric acid 100 mg/kg and TBHQ 100 and 200 mg/kg. Several combinations were evaluated: ascorbyl palmitate and tocopherols (50-700 mg/kg), rosemary extract (8000 mg/kg) and citric acid (100 mg/kg), rosemary extract (8000 mg/kg) and ascorbyl palmitate (200 mg/kg), rosemary extract (8000 mg/kg) and tocopherols (200 mg/kg). Oil oxidative stability was evaluated by the Rancimat method, using 3 g of oil sample warmed at 100 °C with an air flow of 20 L/h. Oil stability was expressed in terms of induction time (IT) (h) and the effectiveness of all tested antioxidants and their blends was expressed as the protection factor (PF):

$$PF: \frac{IT_{ant}}{IT_0}$$

where IT_{ant} is the induction time of the samples treated with antioxidant, and IT_0 is the induction time of the control system (without antioxidant).

Upon combining two antioxidants, the resulting IT were used for each concentration to calculate synergism between antioxidants using the equation proposed by Bishov *et al.* (1977).

$$\%\text{Syn: } \frac{(IT_{blend}-IT_0) - [(IT_1 - IT_0) + (IT_2 - IT_0)]}{[(IT_1 - IT_0) + (IT_2 - IT_0)]} \times 100$$

where IT_1 and IT_2 are the induction time of the individual antioxidants, IT_{blend} is the induction time of the mixture of antioxidant 1 and 2 in the same concentration as that alone and IT_0 is the induction time of the control system (without antioxidant).

2.2.4 Experimental design for storage stability test

Antioxidants (RE, AP, TBHQ, TOC, CA) or their mixtures were added to oil samples selected on the basis of Rancimat analyses, and taking into account Argentine Food Code and Codex Alimentarius standards. Briefly, the additives (RE, AP, TBHQ, TOC, CA) were dissolved in 50 mL-oil aliquots by using a shaker (approximately 5 min) until a homogeneous oil was achieved. The mixtures (oil plus additive) were transferred separately to transparent glass bottles (250 mL) each containing 200 mL chia oil. The bottled oils (final volume 250 mL) were mixed thoroughly and then placed in a thermostated chamber at 25 ± 1 °C kept in the dark by wrapping each bottle with an aluminum foil. For each treatment three sets of bottled oils were prepared. For control treatments, oil samples without added antioxidants were used, three of them in the dark (OC) and three without aluminum foil to evaluate effect of photooxidation on chia oil (LC). Bottled oils were stored for ten months under illumination (800 Lux). Every fifteen days, each individual oil sample was withdrawn from the chamber for scheduled analysis. This is a typical dynamic storage assay,

compared with static storage assay (Ixtaina e*t al.* 2012). In dynamic assays the head space volume increases over time. Acidity (A), peroxide value (PV), K_{232} and K_{270} values were evaluated (AOCS 2009) every fifteen days in each treatment and control. Additionally, with the aim of analyzing possible changes in the characteristic profile of oil, the following determinations were performed on controls (LC and DC) at 0, 165 and 300 days of storage: fatty acid (FA) composition and Iodine value, pigments, quantification of tocopherols and radical scavenging capacity (DPPH).

2.2.5 Statistical analysis

Statistical differences among treatments were estimated from ANOVA test at the 5% level ($P < 0.05$) of significance, for all parameters evaluated. Whenever ANOVA indicated a significant difference, a pair wise comparison of mean by least significant difference (Fisher LSD) was carried out.

3. RESULTS AND DISCUSSION

3.1 Chia oil analysis

Table 1 shows results from chia oil analysis. Acidity (A), peroxide value, conjugated dienes and trienes values (K232 and K270) are similar to those observed in cold-pressed chia oil (Martínez *et al*. 2012), much lower than the maximum values established by the Codex Alimentarius (2001) for non-refined oils, indicating that the oil extraction method employed did not affect adversely those indicators of hydrolytic and oxidative rancidity. Linolenic acid is the major fatty acid (61.8 g/100 g oil) followed by linoleic (20.1 g/100 g oil) and oleic (7.18 g/100 g oil) acids. Such composition leads to high unsaturation degree (iodine value, 212). This fact, together with a relatively low tocopherol and phenolic compound concentration , accounts for the low oxidative stability of chia oil (< 3.04 h). According to Wong (1995),

285 vegetable oils are susceptible to photooxidation during storage under light, especially when
286 photosensitizers, such as chlorophylls, are present. Although chia oil has very low
287 chlorophyll content (4.66 mg/kg oil), such concentration may be sufficient to induce
288 photochemical oxidation (Suzuki *et al.* 1984; Martinez *et al.* 2013a).
289 Regarding carotenoid pigments, Warner and Frankel (1987) have shown that, in soybean
290 oil, the presence of β-carotene at concentrations between 5 and 20 mg/kg oil has a
291 protective effect against oxidative damage induced by light. Considering the fatty acid
292 composition of chia oil, it can be assumed that carotenoid content (5.41 mg/kg oil) is not
293 enough to provide protection against photooxidative degradation.
294 The unsaponifiable fraction analysis shown that sterols were represented by β-sitosterol (60
295 mg/100 mg), 4-6-cholestadien-3β-ol (30 mg/100 mg) and campesterol (9,7 mg/100 mg), the
296 triterpene alcohols and methylsterols were not detected The hydrocarbon fraction was
297 represented mostly by alkanes, the main were octadecane (36,42 mg/100 mg), hexadecane
298 (29,25 mg/100 mg), docosane (16,33 mg/100 mg) tetradecane (7,96 mg/100 mg) and
299 nonadecane (2,68 mg/100 mg). Regarding squalene, since there are not previous studies in
300 chia oil, it was analyzed but no significant levels of this hydrocarbon were detected.
301
302 *3.2 Natural and synthetic antioxidant performance on chia oil*
303 In order to evaluate the antioxidant efficiency of natural and synthetic substances (individual
304 or in combination) in chia oil, oxidative stability (Rancimat test) was studied under different
305 conditions. Table 2 shows a summary of antioxidant conditions for chia oil and their
306 protection factors (PF). Considering RE, significant differences can be found ($p \leq 0.05$)
307 between PF of all concentrations tested, but from 10000 (mg/kg oil) a marked decrease is
308 evidenced in the solubility of the extract in oil (turbidity). In addition to this technological
309 drawback, the increase in oxidative stability was not proportional to the increase in the

concentration of ER, hence we used 8000 (mg/kg oil) of RE. A similar behavior was reported when RE was incorporated to walnut and almond oils (Martinez *et al.* 2013[a], Martinez *et al.* 2013[b]). Tocopherols showed the lowest antioxidant performance (PF 1.39, 800 mg/kg oil). For PA, the highest FP was achieved at 800 mg/kg oil; however, the maximum level has been set to be 200 mg/kg oil (Codex Alimentarius). At this concentration, PF was 3.55 ± 0.08. When RE was combined with CA 100 (mg/kg oil), the blend showed a significant additional protective effect ($p \leq 0.05$) compared to that of RE alone. When achieved, synergism was relatively low despite being positive (17.25%). Some authors reported that the combination of PA and TOC could be positive for some oils (Marinova & Yanishlieva 1990). In this work an equal part blend of each antioxidant was evaluated. For 250 mg/kg oil TOC and 250 mg/kg oil PA, the maximum PF (9.49 ± 0.05) was achieved. The synergism effect varied from 141 per cent (300 mg/kg oil TOC + 300 mg/kg oil PA) to 42 per cent (400 mg/kg oil TOC + 400 mg/kg oil PA). In order to compare natural and synthetic antioxidants, TBHQ was employed at 100 and 200 mg/kg oil in chia oil. PF were 3.79 ± 0.07 and 6.64 ± 0.01 respectively, showing that the antioxidant capacity of synthetic additives could be higher than that of natural antioxidants for chia oil.

3.3 Storage stability test of chia oil

Considering the results obtained from the study of antioxidant performance in chia oil and regulations in force on the level of additives, Table 3 shows concentration of each antioxidant and storage condition. The oxidative stability of chia oil stored in the dark without adding any antioxidant shows a similar oxidation rate than that stored under light. Carotenoids do not show a statistically significant difference between both lighting conditions; yet, chlorophylls show a decrease of 80 % and 63 % under light and dark conditions, respectively. The antioxidant activity of this edible oil decreased at 300 days of

storage in both lighting conditions. This could be attributed to the fact that tocopherol content in chia oil decreased by approximately 30 %. The relative proportion of the different fatty acids was not significantly affected during storage. Particularly, acidity did not differ significantly ($p \leq 0.05$) between treatments, showing no significant increase in time. Considering that the maximum allowed for this parameter in chia oil is around 1 g oleic acid/100g oil, in this test, at 165 days of storage, CL condition presented a 0.22 value. This evidences that chia oil is stable against the hydrolytic degradation of glycerides, even under exposure to light. Figure 2 shows that the combination of ascorbyl palmitate (AP) and tocopherols (TOC) at 200 mg/kg oil each is more effective in the stabilization of chia oil than the addition of TBHQ at 200 mg/kg oil stored in the dark (0.66 and 2.35 meq O_2/kg oil at 300 days of storage, respectively). However, the addition of ascorbyl palmitate (AP) at 200 mg/kg oil, tocopherols (TOC) at 200 mg/kg oil and rosemary extract (RE) at 8000 mg/kg oil did not show good antioxidant capacity, exceeding 15 meq O_2/kg oil (Codex Alimentarius, 2001) from approximately 135-150 days of storage (Figure 1). Although found in static tests, similar trends were reported by Ixtaina *et al.* 2012 for rosemary extract and for the addition of tocopherol in the preservation of chia oil. It should be noted that light exerts a significant effect on the generation of primary oxidation products, accelerating photooxidation probably due to the presence of photosensitizers such as chlorophylls (Wong, 1995; Frankel, 2005) in sufficient quantity to promote photochemical production of singlet oxygen (Suzuki *et al.* 1984). Regarding K232 and K270, parameters show the same trend as that found in PV. In addition, pigment content in chia oil decreased significantly at 165 days of storage.

4. CONCLUSIONS

The nutritional benefits gained by consuming chia oil are mainly ascribed to the high content of ω3 and ω6 fatty acids; yet, they also show a technological disadvantage in terms of product stability. Although chia oil contents naturally antioxidant substances to prevent

oxidation, when exposed to environmental factors such as light and the oxygen, its chemical quality could be altered. In response to this, it was found that the protective effect of the combination of natural antioxidants PA and TOC (50:50) was more marked than that achieved with TBHQ, one of the most widely used synthetic antioxidants. This result represents an interesting alternative for these unconventional oils from crops being reexamined in the country and in the continent as a novel alternative to the oil industry.

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