

## Characterization of constituents, quality and stability of pomegranate seed oil (*Punica granatum* L.)

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### Abstract

This study aimed to characterize pomegranate seed oil and evaluate its quality and stability parameters against those of linseed oil. The profile of fatty acids and phytosterols and the content of tocopherols were analyzed by gas chromatography and high performance liquid chromatography, respectively. The quality of both oils was assessed as recommended by the American Oil Chemists' Society (AOCS) and stability was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH),  $\beta$ -carotene bleaching (coupled oxidation of  $\beta$ -carotene/linoleic acid) and Rancimat<sup>®</sup> assays. While  $\alpha$ -linolenic acid (52%) was the most abundant fatty acid in linseed oil (LO), punicic acid (55%) was highest in pomegranate seed oil (PSO). Tocopherols and phytosterols (175 and 539 mg/100 g, respectively) were greater in PSO than in LO (51 and 328 mg/100 g, respectively). Both oils met quality standards. The  $\beta$ -carotene bleaching and the DPPH assays showed greater oxidative stability for PSO than for LO. The Rancimat<sup>®</sup> method, on the other hand, indicated low stability for both oils.

**Keywords:** punicic acid; tocopherols; phytosterols; coupled oxidation of  $\beta$ -carotene; DPPH•.

**Practical Application:** Pomegranate seed oil: composition, quality and stability.

### 1 Introduction

Evidence shows that not only the amount of fat consumed, but also the type of fat, particularly certain fatty acids (*Trans*, CLAs, CLnAs), are important factors both in maintaining health and in developing certain diseases (Zock, 2006). Consequently, the role of oils and fats in the human body has been widely researched in recent decades and studies have led to a deeper knowledge of their composition and chemical structure as well as their better characterization (Mancini-Filho et al., 2007). Characterizing each class of food lipids is an essential step in developing tailored applications in the food and health industries. In the light of this, the lipid profile of numerous fruits and their seeds have been characterized and several bioactive compounds have been isolated (Pande & Akoh, 2009).

The pomegranate seed oil (PSO) has attracted increasing interest thanks to the abundance of punicic acid (PA – C18:3 – 9c, 11t, 13c), a positional and geometric isomer of  $\alpha$ -linolenic acid (LNA, C18:3 – 9c, 12c, 15c). Its structure with two *cis* double bonds and one *trans* double bond has been investigated in order to understand its role in physiological processes. According to Elfalleh et al. (2011), the potent antioxidant, anticarcinogenic and antilipidemic properties of PSO makes it a powerful health-promoting agent.

The separation and identification of CFAs (conjugated fatty acids) have gained momentum as numerous studies have proven their *in vitro* and *in vivo* biological activities (De la Fuente et al., 2006). In analyzing CFAs, it is essential that the method chosen

be capable of separating and quantifying these geometric and positional isomers, avoiding additional isomerization during any stage of derivatization (Christie et al., 2007). The identity characteristics of vegetable oils include their fatty acid composition as well as their levels of phytosterols (Mancini-Filho et al., 2007). Phytosterols belong to a class of health-beneficial compounds especially known for their ability to inhibit cholesterol absorption. Tocopherols act as natural preservatives and their presence in seed oils is often correlated with the relative abundance of unsaturated fatty acids (Caligiani et al., 2010).

Some researchers have attributed the health-beneficial effects of CFAs to their antioxidant properties (Ali et al., 2012; Johanningsmeier & Harris, 2011; Tsuzuki et al., 2004; Yu, 2001). However, their *in vitro* antioxidant activity has been investigated in few studies. Elfalleh et al. (2011) advocates that the liposoluble nature of fatty acid conjugates limits the choice of assays for estimating their antioxidant capacity; its assessment has been confined to the DPPH• radical (2,2-diphenyl-1-picrylhydrazyl) test, which can successfully determine the antioxidant capacity of the isomers of the conjugated linoleic acid (CLAs). The same researchers have suggested that the high scavenging activity of CLAs against DPPH• free radicals may contribute to their biological activity. This observation is in agreement with findings by Fagali & Catalá (2008), who also demonstrated the ability of isomers of CLAs to reduce free radicals using a photoemission test (lipid peroxidation of PUFAs was induced by tert-butyl hydroperoxide (TBH) and the reduction in chemiluminescence,

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brought about by adding isomers of CLAs, was monitored) and a spectrophotometric assay (the decrease in absorbance of a solution containing the DPPH• radical through the addition of the isomers of CLAs was monitored).

Studies on isomers of conjugated  $\alpha$ -linolenic acid (CLnAs), such as punicic acid (PA), are, on the other hand, still rare – little is known about the identity, quality and stability of oils containing CLnAs. Pomegranate seed oil (PSO), rich in PA and a promising dietary component with potential health-promoting properties, remains poorly characterized. Therefore, this work aimed to characterize PSO in terms of its profile of fatty acids, tocopherols and phytosterols and evaluate its quality and stability parameters against those of linseed oil (LO), which contains  $\alpha$ -linolenic acid as the major fatty acid.

## 2 Material and methods

### 2.1 Material

The pomegranate seed oil (PSO) was directly imported from Green Source Organics (Boynton Beach, USA). The oil, consisting mainly of punicic acid (PA, C18:3 – 9*c*, 11*t*, 13*c*), an isomer of conjugated  $\alpha$ -linolenic acid, was obtained by the cold pressing of the seeds of pomegranate. Samples of linseed oil (LO), provided by Vital Átman (Uchôa, Brazil) and also obtained by cold pressing, were used for comparison because  $\alpha$ -linolenic acid (LNA, C18:3 – 9*c*, 12*c*, 15*c*) is its major fatty acid as well.

### 2.2 Methods

#### *Fatty acids profile in LO and PSO*

Identification and quantification of fatty acids were performed by gas chromatography using different methodologies.

Two esterification methods were used to prepare methyl esters of fatty acids for chromatographic analysis: an acid/base-catalysed method and a base-catalysed ( $\text{NaOCH}_3$ ) method, as described by Christie et al. (2001). Briefly, the lipid fraction (50 mg of oil), containing fatty acids, was diluted in 1 mL of toluene and 2 mL of sodium methoxide ( $\text{NaOCH}_3$ ) in methanol (0.5 M) was added. The suspension was then heated to 50°C for 10 minutes. In the acid/base-catalysed method, 3 mL of boron trifluoride ( $\text{BF}_3$  – 14%) was added and the suspension was heated once again in water bath (50°C for 10 minutes). Next, the tubes were cooled in water and glacial acetic acid (100  $\mu\text{L}$ ), distilled water (5 mL) and hexane (5 mL) were added; the mixture was stirred by vortex mixing and the upper phase was collected and filtered through anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ). The lower phase was washed again with 5 mL of hexane and the upper phase was collected. It was then evaporated under  $\text{N}_2$ , resuspended in 2 mL of hexane and injected into the chromatograph.

Gas chromatography (GC) analyses were carried out in a Shimadzu GC-2010 chromatograph equipped with a flame ionization detector. Samples were run on a fused silica capillary column (SP-2560, 100 m  $\times$  0.25 mm, 0.2 mm film) under three different chromatographic conditions (CC): (1) Injector and detector temperatures were 250°C and 275°C, respectively. The temperature gradient was as follows: 70°C for 4 min, 70-175°C at 13°C/min,

175°C for 27 min, 175-215°C at 4°C/min and 215°C for 31 min (Kramer et al., 1997); (2) Injector and detector temperatures were 215°C and 250°C, respectively. The temperature gradient was as follows: 100°C for 4 min, 100-175°C at 10°C/min, 175°C for 30 min, 175-220°C at 5°C/min and 220°C for 15.9 min (Cordain et al., 2002); (3) Both injector and detector temperatures were 250°C. The temperature gradient was as follows: 162°C for 32 min, 162-195°C at 1.4°C/min, 195°C for 15 min, 195-235°C at 2°C/min and 235°C for 5 min (Baublits et al., 2007).

Under the three different conditions, helium (1 mL/min) was used as the carrier gas and the split ratio (the amount of sample entering the column) was 1:50. Fatty acids in the samples were identified by comparing their retention times with those for commercial standards (C4-C24 methyl esters - Sigma 18919) and the punicic acid with those reported in the literature.

#### *Content of tocopherols and phytosterols in LO and PSO*

The identification and quantification of tocopherols present in both oils were performed using high performance liquid chromatography (HPLC) and the content of phytosterols was determined by gas chromatography as described below.

The analysis of tocopherols was performed as recommended by Instituto Adolfo Lutz (2005). Samples were prepared as follows: an aliquot of oil was diluted in hexane, filtered through a 0.22  $\mu\text{M}$  membrane and injected into the chromatograph. A Shimadzu HPLC system, consisting of an autosampler and a fluorescence detector (RF-10AXL) (excitation: 295 nm, emission: 330 nm), was used. Samples were run under isocratic conditions on a Shim-pack CLC-Sil column (25 cm) at a flow rate of 1.0 mL/min with a pre-filtered and degassed mobile phase of hexane and isopropyl alcohol (99:1). The identification of  $\alpha$ -tocopherol was based on retention time of synthetic standard ( $\alpha$ -tocopherol - Sigma T3251) and quantification was performed by external standardization. The other tocopherols were identified by comparison with literature data and quantified as equivalents of  $\alpha$ -tocopherol.

Determination of phytosterols was carried out in three steps, as recommended by Almeida (2009): (1) hot saponification using 3% KOH in water bath at 50°C under stirring for three hours; (2) extraction of the unsaponifiable fraction in hexane (10 mL) under vortex mixing for one minute and (3) quantification of phytosterols by gas chromatography. The system consisted of a Shimadzu GC-2010 chromatograph equipped with a DB-5 poly (methylphenyl) siloxane column (5% phenyl, 60 m, 0.25 mm) and a flame ionization detector. The temperature gradient was as follows: 150°C for 0.1 min, 150-300°C at 10°C/min and 300°C for 10 min. The temperatures of the injector and detector were 250°C and 300°C, respectively. Helium (1 mL/min) was used as the carrier gas and the split ratio (the amount of sample entering the column) was 1:50. Identification of the peaks was performed by comparing retention times with those of Sigma standards (campesterol - C5157, stigmasterol - S6126 and  $\beta$ -sitosterol - S9889) and quantification was done by internal standardization using 5 $\alpha$ -cholestane (Sigma C8003) as an internal standard.

### Quality parameters of LO and PSO

The quality parameters of both oils, Acidity Index (AI), Peroxide Index (PI), *p*-anisidine Index (*pI*) and 2-thiobarbituric acid (TBA) value, were evaluated by methods described in Mancini-Filho, et al. (2007), Instituto Adolfo Lutz (2005) and American Oil Chemists' Society (2004).

### Oxidative stability of LO and PSO

The oxidative stability of both oils was assessed using three assays:

- (1)  $\beta$ -carotene bleaching (coupled oxidation of  $\beta$ -carotene/linoleic acid) assay – Basically, the method described in Schubert et al. (1999) monitors the oxidation (discoloration) of a  $\beta$ -carotene emulsion with a spectrophotometer. Briefly, 0.2 mL of  $\beta$ -carotene/chloroform (10 mg/10 mL) solution, 20  $\mu$ L of linoleic acid and 0.5 mL of Tween-40 (emulsifier) was mixed for homogenization and the chloroform was then evaporated under  $N_2$ . Subsequently, 50 mL of distilled water was added and the mixture was vigorously shaken to produce a clear  $\beta$ -carotene/linoleic acid emulsion - its absorbance at 470 nm was between 0.6 and 0.7. In order to determine the antioxidant activity of the oil samples, 5 mL aliquots of the emulsion and 2 mL samples of oil diluted in ethanol (3.5 mg/10 mL) were mixed and kept in water bath at 50°C. Butylated hydroxytoluene (BHT) was used as reference standard at the same concentration of the samples. Absorbance was read at 470 nm on a spectrophotometer (Spectronic® 20 Genesys, Spectronic Instruments Rochester, USA) every 15 minutes over a period of 120 min.
- (2) DPPH free radical scavenging assay – analysis was conducted as described in Brand-Williams et al. (1995) and Blois (1958), with some adjustments. This method is based on the decrease in absorbance of DPPH• (2,2-diphenyl-1-picrylhydrazyl) when it is reduced by an antioxidant, a hydrogen donor. The amount of antioxidant needed to reduce DPPH concentration by 50% (IC50) is then calculated. Briefly, LO and PSO samples were diluted in *n*-butanol to different concentrations. A 0.5 mL aliquot at each concentration was then mixed with 1.5 mL of DPPH• in butanol (3.0 mg/200 mL) to initiate the reaction. After 30 minutes, absorbance was measured at 517 nm on a spectrophotometer (Spectronic® 20 Genesys, Spectronic Instruments Rochester, USA). The DPPH solution (without sample) was used as control and BHT was used as standard. The DPPH scavenging activity of the sample was calculated according to Equation 1 and expressed as percentage.

$$\text{Scavenging activity (\%)} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) \div \text{Abs}_{\text{control}} \quad (1)$$

where  $\text{Abs}_{\text{control}}$  is the absorbance of the control and  $\text{Abs}_{\text{sample}}$  is the absorbance of the sample.

- (3) Rancimat® assay – the test evaluates stability of oils and fats against autoxidation by exposing them to oxidizing conditions and measuring the time until volatile acids are detected (Rancimat induction time). The appearance of these acids is recorded in the measuring instrument as an increase in conductivity (Méndez et al., 1996). The equipment used was a Metrohm 743 Rancimat (Metrohm Instruments, Herisau, Switzerland) with software (1.0) control. Oil samples (5 g) were exposed to oxidation at 110°C (oxygen flow rate of 20 L/h). Induction times (IT) were recorded automatically and expressed in hours (h).

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation ( $n=3$ ). Initially, the levels of fatty acids in both oils (LO and PSO), determined by two different esterification methods, were tested for homogeneity of variances using an F-test. Then, the Student's *t*-test (for independent samples) and the non-parametric Mann-Whitney U-test were used to test data for homogeneous and heterogeneous variances, respectively. The homogeneity of variances of data for quality and oxidative stability of oils was tested using the same tests described above.

The Hartley/Levene's test indicated that the variances of data for oxidative stability of the vegetable oils (LO and PSO) and of the synthetic antioxidant BHT were homogeneous. Statistically significant differences ( $p < 0.05$ ) were evaluated by univariate analysis of variance (ANOVA) and means were compared by Fisher's least significant difference test. All analyzes were performed using Statistica 7 (Statsoft, USA) and Action 2.4 (Estatcamp, Brazil) software.

## 3 Results and discussion

### 3.1 Fatty acids profile in LO and PSO

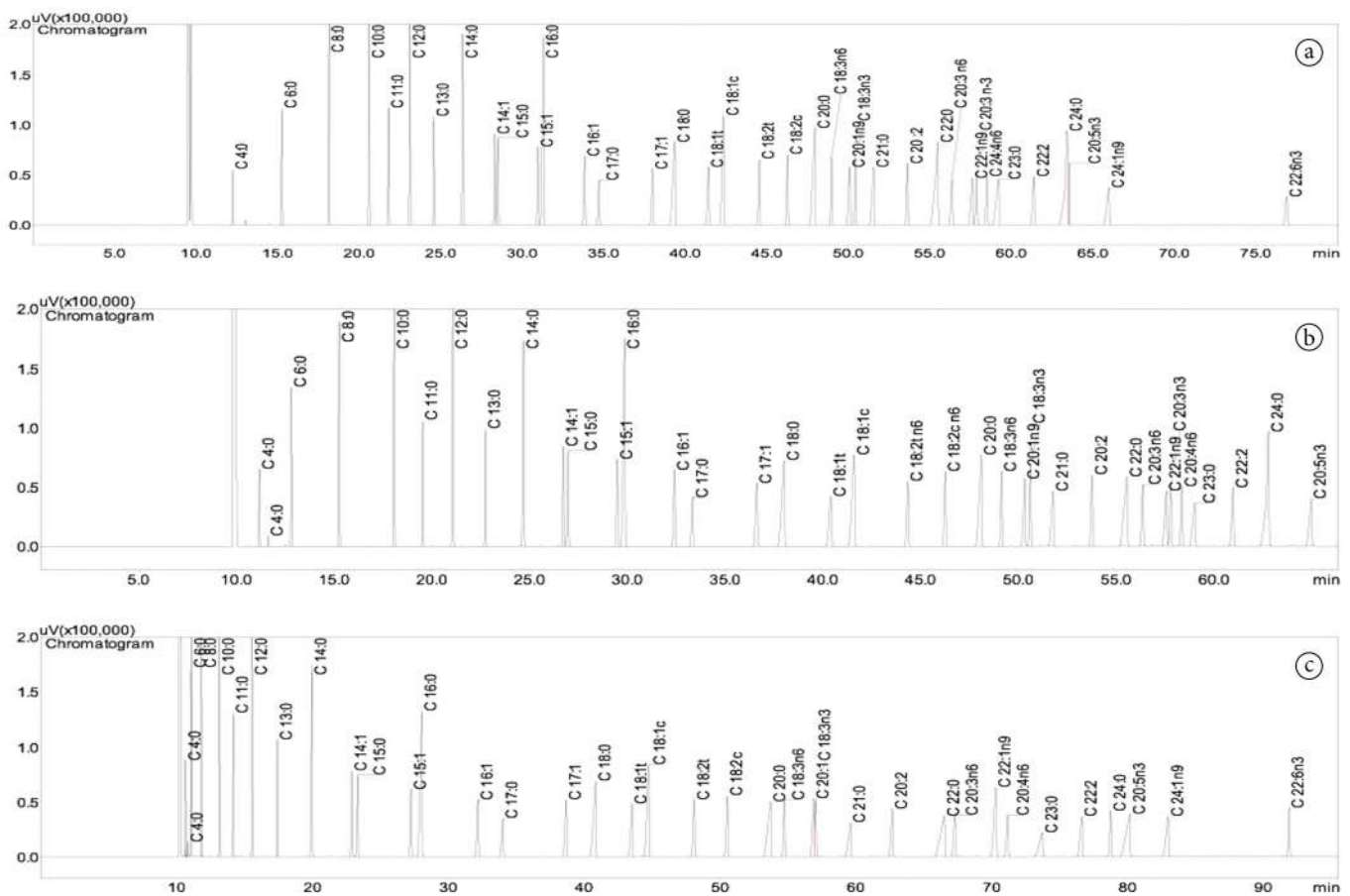
In analyzing conjugated fatty acids (CFAs), it is essential that the method chosen be capable of separating and quantifying these geometric and positional isomers, avoiding additional isomerization during any stage of derivatization (Christie et al., 2007). Converting fatty acids into volatile derivatives, mainly methyl esters, is the first step to analyze their profile by chromatography (De la Fuente et al., 2006). The methods of methylation using KOH or  $\text{NaOCH}_3$  in methanol, at room temperature, have been most reliably used for converting conjugated fatty acids into methyl esters because they do not cause isomerization. Acid-catalyzed methylation, using  $\text{BF}_3$ , HCl or  $\text{H}_2\text{SO}_4$ , on the other hand, favors the isomerization of dienes and trienes (Sassano et al., 2009; Chen et al., 2007; De la Fuente et al., 2006). Kramer et al. (1997) advocates that, although esterification using  $\text{NaOCH}_3$  in methanol does not cause isomerization, it fails to methylate free fatty acids. They also observe that an acid/base-catalysed approach is more successful at esterifying all fatty acids and reducing isomerization. In this study, fatty acids in samples of both oils (LO and PSO) were thus esterified by both an acid/base-catalysed method and a base-catalysed method in order to determine the better methodology for the analysis of fatty acids in PSO. The chromatograms for a standard mix of C4-C24

methyl esters analyzed under three different chromatographic conditions (CC), 1, 2 and 3, are shown in Figure 1.

The chromatographic run under CC 1, described in Kramer et al. (1997), was 80 minutes long and resulted in good separation of, mainly short chain fatty acids (detected in the first 30 minutes). The second set of conditions (CC 2), described in Cordain et al. (2002), also succeeded in separating short-chain fatty acids, but failed to separate all fatty acids in the standard as the run did not last as long (~66 minutes). This shorter chromatographic run time led to poor separation of long chain fatty acids, such as CLnAs (punicic acid), which are found chiefly in PSO. The analysis under the third set of conditions (CC3), described in Baublits et al. (2007), took the longest time (93 minutes) and successfully separated all the fatty acids; peaks of long chain fatty acids were clearly identified. These conditions, therefore, proved to be the most suitable for the analysis of PSO. Baublits et al. (2007) and Pinheiro (2010), who applied these chromatographic conditions to determine the fatty acid profile of meat and milk of ruminants, containing conjugated linoleic acids, reported well-separated peaks of fatty acids. Also, Pinheiro (2010) observed that this set of chromatographic conditions, compared with the other two (described in Cordain et al., 2002 and Kramer et al., 1997), is the most appropriate because it provides satisfactory separation of the isomers of oleic and linoleic acids.

The fatty acid profiles for LO and PSO, determined by two different methods of esterification (acid/base-catalysed and base-catalysed) under chromatographic conditions (CC3), described in Baublits et al. (2007), are shown in Table 1. Good separation of fatty acids in the samples was obtained by using both methods. Esterification with  $\text{NaOCH}_3$  showed a chromatogram with fewer peaks probably because it more efficiently methylated the fatty acids, without the occurrence of isomerization. This is in agreement with observations made by Sassano et al. (2009), Chen et al. (2007) and De la Fuente et al. (2006), who have successfully methylated fatty acids with  $\text{NaOCH}_3$  or KOH in methanol. They also recommend it as the most reliable method as it does not cause isomerization and reduces the loss of short-chain fatty acids.

Table 1 confirms that the major fatty acids in PSO and LO are punicic acid (PA; C18:3 - 9c, 11t, 13c) and  $\alpha$ -linolenic acid (LNA; C18:3  $\omega$ -3), respectively. The results for LO are in agreement with the literature, which reports LNA percentages in LO ranging from about 50 to 58% (Akpolat et al., 2011; Rosa et al., 2010; Tzang et al., 2009; Cintra et al., 2006). Five fatty acids, C16:0 (6.45%), C18:0 (4.35%), C18:1  $\omega$ -9 (18%), C18:2  $\omega$ -6 (12.71%) and C18:3  $\omega$ -3 (58.47%), have been identified in LO (Rosa et al., 2010). Literature on PSO reports variation in fatty acid composition; for instance, PA levels



**Figure 1.** Chromatograms for a standard mix of C4-C24 methyl esters tested under three different chromatographic conditions (CC): (a) 1, (b) 2 and (c) 3.

fall between 37% and 81%. Low levels of other conjugated triene fatty acids in PSO have also been reported, which is consistent with the observations in this study (Elfalleh et al., 2011; Parashar et al., 2010; K yralan et al., 2009; Pande & Akoh, 2009; Jardini & Mancini-Filho, 2007). The acid-base system, however, catalyzed the conversion of punicic acid into other *cis/trans* CLnAs in PSO, causing a reduction in punicic acid levels, from 54.9% to 42.3%, and an increase of all other isomers as well as the formation of two unidentified isomers. Similarly, Sassano et al. (2009) observed a drop in PA levels (from 71.8% to 64.9%) when fatty acids in PSO were methylated using both an acid- and a base-catalysed methods. They also reported a concomitant rise in the percentages of  $\alpha$ - and  $\beta$ -eleostearic acids and catalpic acid.

### 3.2 Content of tocopherols and phytosterols in PSO and LO

The amounts of relevant compounds such as tocopherols in seed oils are often correlated to the relatively abundant levels of unsaturated fatty acids, which represents a further advantage in terms of nutraceutical value (Caligiani et al., 2010). As both linseed

oil (LO) and pomegranate seed oil (PSO) are rich in unsaturated fatty acids, their content of tocopherols was analyzed (Table 2).

Total tocopherol levels were about three times as high in PSO than in LO (Table 2). However, higher values (~300 mg/100 g) of vitamin E in PSO have been reported;  $\alpha$ -tocopherol, found to be in low amounts in the present study, and the  $\gamma$ -tocopherol was the predominant isomer (Elfalleh et al., 2011; Caligiani et al., 2010). Elfalleh et al. (2011) noted that tocopherols in seed oil from the Tunisian pomegranate consisted chiefly of  $\alpha$ -tocopherol (over 53%). They also observed that the high levels of tocopherols in the oil were highly correlated with FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity) values, suggesting the contribution of tocopherols to the antioxidant properties of the oil. Phytosterols are found at different levels in all vegetable oils and account for the largest group of compounds in their unsaponifiable fraction (Mancini-Filho et al., 2007). The levels of phytosterols in LO and PSO are shown in Table 2.

The most abundant phytosterol in both oils was  $\beta$ -sitosterol. Also, the total content of phytosterols was greater in PSO than

**Table 1.** Fatty acids profile (%) in linseed oil (LO) and pomegranate seed oil (PSO) determined using two methods of esterification (acid/base-catalysed and base-catalysed).

Fatty acids	LO			PSO		
	Acid/Base	Base	P-value	Acid/Base	Base	P-value
C16:0	6.03 ± 0.02	6.06 ± 0.01	0.10	2.95 ± 0.01	2.77 ± 0.00	<0.01
C18:0	5.22 ± 0.02	5.27 ± 0.00	0.01	2.57 ± 0.01	2.42 ± 0.00	<0.01
C18:1c $\omega$ -9	21.61 ± 0.03	21.73 ± 0.00	<0.01	6.07 ± 0.02	5.74 ± 0.01	<0.01
C18:1c $\omega$ -7	0.72 ± 0.00	-	NA	0.34 ± 0.00	-	NA
C18:2c $\omega$ -6	14.58 ± 0.00	14.67 ± 0.00	<0.01	7.73 ± 0.03	7.29 ± 0.01	<0.01
C20:0	-	-	-	0.72 ± 0.00	0.39 ± 0.00	<0.01
C20:1c $\omega$ -9	-	-	-	0.42 ± 0.00	0.39 ± 0.00	<0.01
C18:3c $\omega$ -3	51.85 ± 0.08	52.37 ± 0.01	<0.01	0.43 ± 0.00	0.39 ± 0.00	<0.01
C21:0	-	-	-	0.41 ± 0.00	2.91 ± 0.00	<0.01
C18:3 9c11f13c	-	-	-	42.34 ± 0.67	54.90 ± 0.03	<0.01
NI	-	-	-	6.22 ± 0.05	-	NA
NI	-	-	-	1.99 ± 0.05	-	NA
C18:3 9c11f13t	-	-	-	17.29 ± 0.16	16.07 ± 0.01	<0.01
C18:3 9f11f13c	-	-	-	10.19 ± 0.43	6.71 ± 0.00	<0.01
C18:3 9f11f13t	-	-	-	0.33 ± 0.00	-	NA

Chromatographic conditions (CC3) – Baublits et al. (2007); NI = not identified; NA = Not applicable; Results are expressed as mean ± standard deviation (n=3); P-value (paired Student's t test).

**Table 2.** Content of tocopherols and phytosterols in linseed (LO) and pomegranate seed (PSO) oils.

Samples	Tocopherols (mg/100g)				Total
	$\alpha$ -tocopherol	$\beta$ -tocopherol	$\gamma$ -tocopherol	$\delta$ -tocopherol	
LO	0.93 ± 0.01 <sup>b</sup>	18.17 ± 1.90 <sup>a</sup>	32.96 ± 3.37 <sup>b</sup>	1.39 ± 0.01 <sup>b</sup>	53.44 ± 5.26 <sup>b</sup>
PSO	3.81 ± 0.08 <sup>a</sup>	1.03 ± 0.01 <sup>b</sup>	153.21 ± 2.44 <sup>a</sup>	17.04 ± 1.65 <sup>a</sup>	175.09 ± 3.50 <sup>a</sup>
P-value	<0.01	<0.01	<0.01	<0.01	<0.01
Samples	Phytosterols (mg/100g)				Total
	Campesterol	$\beta$ -sitosterol	Stigmasterol	others	
LO	66 ± 2.4 <sup>b</sup>	138 ± 4.0 <sup>a</sup>	11 ± 0.5 <sup>b</sup>	113 ± 3.7 <sup>b</sup>	328 ± 11 <sup>b</sup>
PSO	49 ± 1.6	374 ± 1.4	12 ± 0.7 <sup>a</sup>	104 ± 7.4 <sup>a</sup>	539 ± 11 <sup>a</sup>
P-value	<0.01	<0.01	0.05	0.01	<0.01

Mean ± standard deviation (n = 3); different letters in the same column are statistically different from each other, P-value (paired Student's t test).

in LO, and is consistent with that observed in the literature (Caligiani et al., 2010; Pande & Akoh, 2009).

### 3.3 Quality parameters of LO and PSO

In analyzing oils and fats, indices that characterize their physical and chemical properties are better indicators of quality than percentages of their components. These indices, along with some characteristic reactions, are used to identify and evaluate most oils and fats and the results of the analysis are based on this set of data (Instituto Adolfo Lutz, 2005). Table 3 shows the values of some quality and stability indices for LO and PSO samples.

The Codex Alimentarius (2009) (CODEX-STAN, 210-1999) recommends that the acidity and peroxide indices of cold pressed oils should not exceed 4.0 mg/g and 15 meq/kg, respectively. Overall, the indices for LO and PSO met the standards of quality and showed good stability of both oils.

### 3.4 Oxidative stability of LO and PSO

The rate of reduction in absorbance of  $\beta$ -carotene in the presence of the oils studied (LO and PSO) and the synthetic antioxidant BHT (0.01%) is shown in Figure 2a. The percentage inhibition of the oxidation of  $\beta$ -carotene by both oils and BHT is shown in Figure 2b. The antioxidant BHT was the most stable, followed by PSO (21%) and LO (4%). Using the same methodology, Schubert et al. (1999) reported a higher percentage (approximately 40%) for cold pressed PSO, close to that observed for green tea. They attributed its high antioxidant activity to both fatty acid composition and the presence of flavonoids.

The DPPH scavenging capacity of LO and PSO versus their concentration and the percentage of DPPH reduced by both

oils and BHT at 1 mg are shown in Figures 3a, b, respectively. Pomegranate seed oil (PSO) showed greater concentration-dependent scavenging capacity than LO (Figure 3a), but significantly lower activity compared to the synthetic antioxidant BHT (1 mg) (Figure 3b). While the amount of PSO needed to reduce DPPH concentration by 50% (EC50) was 3.77, the amount of BHT was a mere 0.26 mg; the lower the EC50, the more potent DPPH• scavenger the antioxidant is (Ali et al., 2012). Linseed oil (LO) was incapable of reducing DPPH• radicals at 1 mg (Figure 3b).

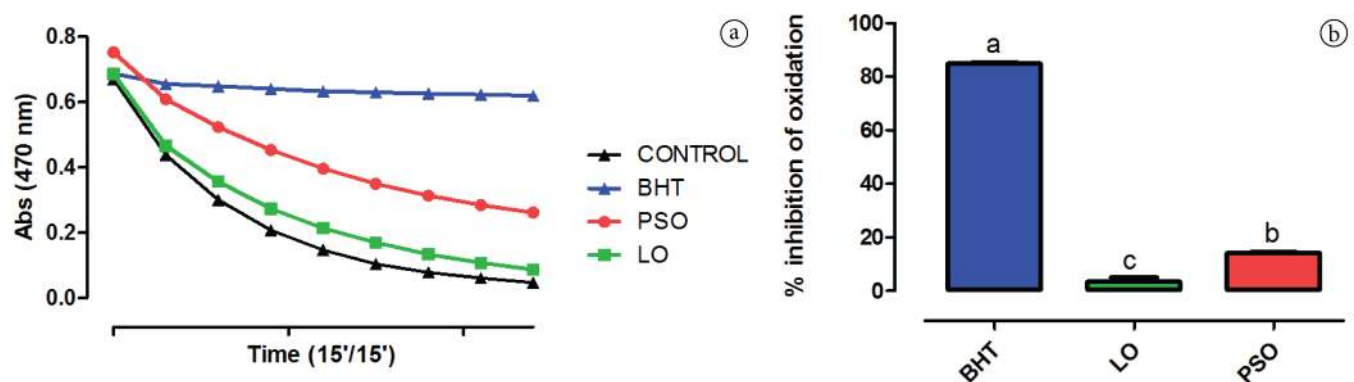
Conjugated fatty acids have been reported as potent antioxidants in animal organisms. However, the *in vitro* antioxidant activities of CLnAs and of the oils rich in them have been scarcely investigated. The DPPH• scavenging capacity results for PSO in this work mirror previous findings from studies using conjugated linoleic acid (CLA) (Ali et al., 2012; Fagali & Catalá, 2008; Yu, 2001). Isomers of CLAs have been shown to have a considerable direct antioxidant capacity and significant DPPH• scavenging properties at different concentrations although they were less effective than vitamin E and BHT (Ali et al., 2012). CLAs provide protection against free radicals, but linoleic acid, one of their non-conjugated isomers, does not (Fagali & Catalá, 2008; Yu, 2001).

Unlike the  $\beta$ -carotene oxidation and DPPH• scavenging results, which were significantly different for LO and PSO, Rancimat® induction times for both oils were similar ( $0.72 \pm 0.03$  and  $0.73 \pm 0.07$  hours, respectively). Free radicals attack unsaturated fatty acid chains at the methylene group between two double bonds, removing a hydrogen atom and producing other free radicals (Mancini & Mancini-Filho, 2005). However, no differences in oxidative stability between the oils studied, rich in conjugated and unconjugated fatty acids, were shown by the Rancimat® assay. The  $\beta$ -carotene/linoleic acid and DPPH•

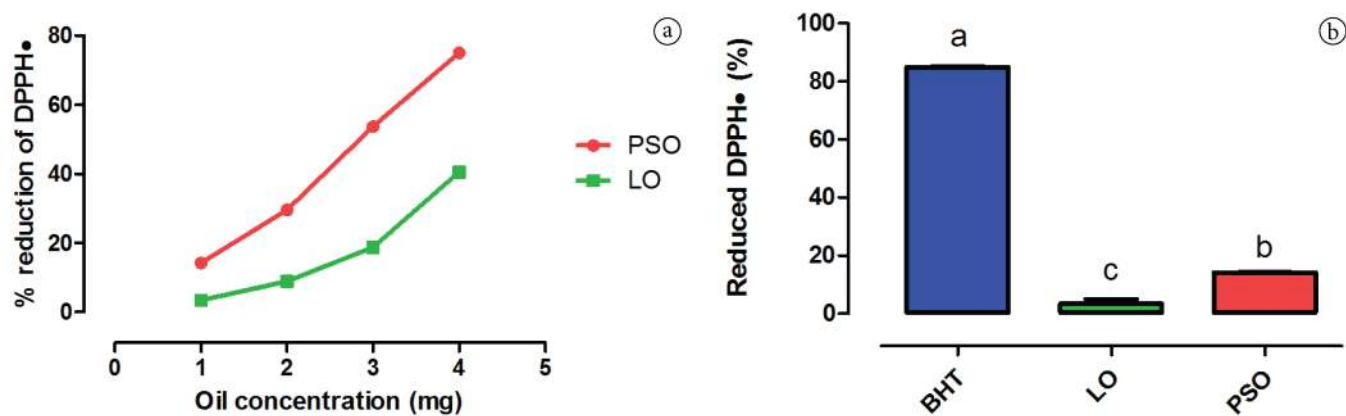
**Table 3.** Quality and stability parameters for linseed (OL) and pomegranate seed (PSO) oils.

Samples	AI (mg/g)	PI (mEq/kg)	IpA	TBA ( $\mu$ g/g)
LO	$1.04 \pm 0.00^a$	$4.40 \pm 0.02^a$	$0.56 \pm 0.33^b$	$0.44 \pm 0.01^a$
PSO	$0.63 \pm 0.00^b$	$3.34 \pm 0.03^b$	$4.84 \pm 1.01^a$	$0.22 \pm 0.01^b$
P-value	<0.01	<0.01	0.01	<0.01

AI (acidity index); PI (peroxide index); IpA (p-anisidine index); TBA (2-thiobarbituric acid value); mean  $\pm$  standard deviation (n = 3); different letters in the same column are statistically different from each other, P-value (paired Student's t test).



**Figure 2.** (a) Kinetics of the inhibition of oxidation of  $\beta$ -carotene by linseed oil (LO), pomegranate seed oil (PSO) and BHT (0.01%) and (b) percentage inhibition by LO, PSO and BHT; One Way ANOVA ( $p < 0.01$ ).



**Figure 3.** (a) DPPH• scavenging capacity of linseed (LO) and pomegranate seed (PSO) oils (b) (%) DPPH• reduced by both oils and BHT at 1 mg. One Way ANOVA (p <0.01).

tests, on the other hand, yielded greater oxidative stability results for PSO than for LO. Yang et al. (2009) compared the oxidative stability of individual CLnA isomers and of LNA, its corresponding non-conjugated acid, by oxidizing both in air at 50°C and monitoring reactions by gas chromatography and the oxygen consumption test, and observed that CLnAs were more susceptible to autoxidation than LNA. Of the three groups of isomers of CLnAs, isomers *t, t, t*-CLnA showed the greatest stability, followed by *c, t, t*-CLnA and *c, t, c*-CLnA. Stability of CLnA geometric isomers in each group was also found to be dependent on the position of the three conjugated double bonds. The presence of CLnAs can contribute to oxidative deterioration of vegetable oils (Kinami et al., 2007). However, PSO met all standards of quality, and most of the indices evaluated were below those of soybean oil, rich in unconjugated fatty acids, reported by Kinami et al. (2007).

#### 4 Conclusions

The base-catalysed esterification method under chromatographic conditions described in Baublits et al. (2007) was found to be the most appropriate methodology to analyse pomegranate seed oil samples by gas chromatography.

The results in this study confirm the presence of high concentrations of conjugated  $\alpha$ -linolenic acid, vitamin E and phytosterols in pomegranate seed oil and show that the oil has good oxidative stability despite the high degree of unsaturation of its fatty acids. Further studies are needed to confirm the effects of pomegranate seed oil in different systems and under physiological conditions.

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