

Antioxidant Activity of Asparagus Ethanol Extract against TBHQ and Tocopherols

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ABSTRACT

Asparagus (Asparagus officinalis L.) is considered an important crop in the region of Caborca, Sonora, due to its high production and its natural antioxidant (phenolic compounds) content. The use and effect of natural antioxidants on Refined soybean oil (RBD) were evaluated by the Oxidative Stability Index (OSI) using the Rancimat Method. Asparagus phenolic compounds were extracted with 95: 5 (v: v) ethanol: water. The antioxidant activity of the hydroalcoholic extract obtained was compared to Tertiary Butyl Hydroquinone (TBHQ) and to a mix of tocopherols in soybean oil free of synthetic antioxidants, which complied with the chemical properties and composition of pure edible soybean oil. The total phenol content in asparagus extract was similar to what was reported in other fruit and asparagus in various countries. The soybean's oil with higher concentrations of asparagus ethanol extract than 0.3% (w/w) showed statistically higher induction times ($P < 0.05$) than natural (tocopherols) and synthetic antioxidants (TBHQ) at the tested concentrations, which strongly supports the concept of using asparagus as a source of natural antioxidants.

Keywords: *Asparagus officinalis* L., Natural antioxidants, Oxidative stability index, Soybean oil.

INTRODUCTION

The addition of antioxidants to food is a regular practice used to delay fat oxidation. Although synthetic antioxidants are widely used in the food industry, their use has been widely questioned due to toxicology issues related to diabetes, cancer, hypertension and coronary diseases (Ixtaina *et al.*, 2012). As a result, the necessity to find new and safe antioxidants for use as additives has increased and has resulted in an important topic within the food industry.

Lipid peroxidation can not only deteriorate food flavor and odor, but it can also cause emphysema, mutagenesis, carcinogenesis and hearth diseases (Addis, and Warner 1991; Takahashi, 1992). There is a growing concern about finding a more comfortable diet that

not only contains nutrients but also includes bioactive components that have been proven to produce beneficial effects on human health. The nutritional value of vegetable-based food used as a source of minerals, essential vitamins carbohydrates, proteins and fiber is highly recognized (Fukushi, *et al.*, 2010; Hafizur *et al.*, 2012). Epidemiologic studies have reported an inverse association between vegetable consumption and the reduction of chronic diseases, such as various types of cancer and heart disease.

Phytochemical studies on plants have shown that the active components responsible for this protecting effect are the antioxidants, among others. Therefore, food components' bioactivity is evaluated by its antioxidant capacity (Bazzano *et al.*, 2002; Shreiner, 2004).

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The main bioactive components of asparagus are phenols (flavonoids and hydroxycinnamic acids) and saponins, though other compounds such as sterols, oligosaccharides, amino acids and carotenoids can also contribute to the functional and antioxidant properties of this vegetable (Chin *et al.*, 2002; Jang *et al.* 2004). Asparagus is also rich in fiber, which also has potential benefits (Heredia *et al.* 2003).

Numerous essays are available regarding food that is rich in oils and in biological systems to evaluate antioxidant activity and the capacity to trap free radicals. Nevertheless, there are no universal methods with the ability to measure the antioxidant capacity of all oxidation compounds (primary and secondary products) in a precise and quantitative manner (He, *et al.* 2012).

One of the methods used successfully to measure the Oxidative Stability Index (OSI) of natural and synthetic antioxidants is the Rancimat method (Hasenhuettl, and Wan, 1992; Gámez-Meza *et al.* 1999). This method measures the increment of electric conductivity that occurs when fat and oil are oxidized into shorter free fatty acids (formic acid), especially under accelerated conditions of heat and aeration.

The purpose of this research was to evaluate and compare the antioxidant activity of an asparagus ethanol extract (*Asparagus officinalis* L.) against the synthetic antioxidant TBHQ (tertiary butyl hydroquinone) and natural antioxidants tocopherols by determining their OSI values using Rancimat equipment.

MATERIALS AND METHODS

Vegetative Material

Asparagus (*Asparagus officinalis*) from the Caborca, Sonora agriculture region was collected, stored at 20 °C, and processed in the research laboratory at the University of Sonora Caborca-North Branch.

Soybean Oil Used in Essays

Refined soy oil (RBD) was obtained from the Mexican Company “Derivados de Oleaginosas del Valle” from Obregón city, Mexico and used as a control. The soybean oil used in the antioxidant essays was characterized determining its tocopherols content, peroxide value, and *p*-anisidine value and fatty acid profile.

Peroxide Value and *p*-Anisidine Value

Quantification of the oxidation's primary products was determined by the peroxide value using AOCS official method Cd 8-53 (Firestone, 2012) and, for the secondary products, through determining the *p*-anisidine value (AOCS Cd 18-90).

Fatty Acid (FA) Composition

Fatty acid profile was determined by a quantitative separation of the mix of saturated and unsaturated fatty acids with 8 to 24 carbons after their conversion to methyl esters using the Ce 2-66, A.O.C.S method. We used a Varian 3800 CX gas chromatograph (with a flame ionization detector) coupled with Varian Star 2000. A capillary column SP-2560 was used with a stationary phase 100% of biscyanopropyl polysiloxane (100 m×0.25 mm di×0.2 μm particle size, Supelco, Inc. Bellefonte, PA 16823-0048 USA). The column's temperature program was from 140-210°C (4°C min⁻¹) and 210-220°C (1°C min⁻¹). Helium was used as carrier gas with a flux of 20 cm/sec. The injector and detectors temperature was maintained at 250°C. The content of fatty acids was calculated using heptadecaenoic acid (C-17:0) as internal standard (Sigma Chemical Co., ST Luis MO). The peak areas' identification was determined through comparison against the standard's time retention.

Tocopherols Quantification in Soybean Oil

Tocopherol quantification was done by HPLC using a Varian 9050 chromatograph equipped with an ultraviolet detector (Varian 3400) and a Lichrosorb Si60 column (25 cm×73 4 mm×5μ particle size, Supelco, Inc., Bellefonte, PA 16823- 0048 USA). The mobile phase was a mix of hexane: isopropanol (99:1), with a flux of 3.5 mL min⁻¹. Quantification was determined at 292 nm. Two grams of the sample was diluted in 25 mL de hexane (Warner, and Mounts, 1990; Chase, *et al.* 1994). A sample of 20 μL was injected directly into the chromatograph twice. The peak areas were identified and quantified by comparing against α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol (Sigma Chemical Co., St. Luis MO). This method determined the amount of tocopherols in natural soybean oil since some of these antioxidants in combination with the ones added to the oil could have a synergetic or pro oxidant effect.

Asparagus Extract

The asparagus sample (1,500 g) was first rinsed with tap water followed by a distilled water rinse. The sample was divided into two parts, the green Edible Part (EPC: 1,068 g) and the white Non-Edible Part (EPNC: 432 g). Both parts were cut into 1 cm pieces and placed inside separate Ziploc bags and then frozen at -23°C. The samples were lyophilized at -46°C and a 0.370 mBar for 6 days in a LABCONCO lyophilizer. Next, the sample was pulverized using a porcelain mortar and pestle. Asparagus phenolic compounds were extracted three times following the Onyenko and Hettiarachchy protocol (Onyenko, and Hettiarachchy 1991), which uses 95% ethanol as an extraction solvent (1:5, Solid: Liquid) for 2 h periods, with agitation and covered from light on a Labnet agitator. After this, the sample was centrifuged for 15 minutes (2,500 g) in an

IEC CL 31R Multispeed Thermo Electronic centrifuge.

The obtained supernatants were collected and filtered, using filter paper Whatman No.4. Finally, the asparagus extracts were concentrated on a rotavapor (Buchi Switzerland, model R-215) at reduced pressure, 130 rpm and 60°C.

Lyophilized Asparagus, Asparagus Ethanol Extract and Soybean Oil's Pro-oxidant Mineral's Quantification

The content of metallic compounds was analyzed on lyophilized asparagus, asparagus ethanol extract and on the soybean oil. For the ethanol extract, 5 mL were incinerated and then digested with chloridic acid. After this, the presence of Mg, Ca, Cu and Fe was determined by atomic absorption spectrophotometry. For the lyophilized asparagus, 100 g of the edible part was used. This sample was placed on a stove for 2 to 4 hours at 450-500°C. The dried sample (1.0 g) was digested with chloridic acid on a hot plate. Phosphorous was analyzed by the official protocol AOAC 958.01 (2016), using a spectrophotometer adjusted to 650 nm (UV VIS Lambda PE 2S, Perkin-Elmer, Norwalk, CT). Magnesium, calcium, copper and iron were detected by atomic absorption spectrophotometry (PE-3100; Perkin-Elmer). Wavelengths used for copper and iron were 324.7 and 248.3 nm, respectively. For the soybean oil, 5 g were used for the same determination (Mg, Ca, Cu, and Fe). The sample was digested using chloridic acid followed by atomic absorption spectrophotometry. All the experiments were performed in glassware to minimize metal contamination.

Asparagus Ethanol Extract's Total Phenol Quantification

Colorimetric analysis using Folin-Ciocalteus reagent (Singleton and Rossi, 1965) was performed to determine the total phenol content of the asparagus ethanol extract. Sixty milliliters of water were added to an



aliquot of 1 mL of the extract. After this 5 mL of Folin-Ciocalteu's reagent and 15 mL of 20% sodium carbonate were added. After 5 minutes at 25°C, the absorbance was registered at 750 nm (spectrophotometer UV-VIS HACH DR 5000 model). A catechin standard curve was used for the total phenol quantification.

Ethanol Extract's Antioxidant Test

Asparagus ethanol extract dilutions (0.1, 0.3 and 0.5%) in 95% ethanol were prepared and mixed with 25 mL of emulsifier (canola oil monoglycerides) and soybean oil to have a final oil extract and emulsifier mix 10:5:5. The official protocol of AOCS Cd 12b-92 on Rancimat equipment 892 professional model was followed to test the mix antioxidative efficiency.

An amount of 3.5 g of oil with the different extract dilutions were placed in reaction cuvettes with an air flux of at 20 L h⁻¹ and a temperature of 110°C. The induction time (h) for each sample was automatically measured by the equipment and it represented the time in which the mix of oil, antioxidants and emulsifier initiated its deterioration producing polar compounds, which were responsible for its conductivity changes.

To compare antioxidative activity of the ethanol extracts obtained in this study against synthetic and natural commercial antioxidants (tocopherol and TBHQ), OSI test was ran using the Rancimat equipment and the same oil quantity. For these determinations, tocopherol and TBHQ were used at 0.02%, which is used at the industrial level. The control for this test was soybean oil without any additives.

Statistical Analysis

Results were analyzed through variance analysis $P > 0.05$ (ANOVA) and difference among means was determined by Tukey test.

All the analyses were carried out using JMP IN ver. 3.2.1 (Statistical Graphic Corp.).

RESULTS

Asparagus ethanol extract characteristics were associated mainly to its chemical composition. The extract showed a green-reddish color due to its high chlorophyll and anthocyanin content; it exhibited a viscous texture and an asparagus characteristic odor. Violet coloration of the tip is due to anthocyanin synthesis in the epidermal tissues (Siomos *et al.* 2010).

The peroxide value of the oil used in the study was 1.0 ± 0.09 mEq kg⁻¹ of oil and the *p*-anisidine value was 0.75. These values indicate that the soybean oil was among the range of recently refined oil and there were no antioxidants present in the oil. The recommended values for this type of oil is a value less than 1.0 mEq kg⁻¹ of oil for peroxide and less than 2 for *p*-anisidine (Egan *et al.* 1991, Bachari-Saleh, *et al.*, 2013). Peroxides are the primary oxidized products produced, which on further oxidation would degrade to aldehydes, ketones, esters, etc., which are the secondary oxidized products. Peroxide Value (PV) is a measure of oxidation during storage and the freshness of lipid matrix (Malheiro, *et al.*, 2013). In addition, it gives important information about lipid autoxidation. PV measures only the earlier stage of oxidation and primary oxidation products (Srivastava, and Singh *et al.* 2015). Unsaturated fatty acids are the primary targets of thermal oxidation as well as autoxidations, leading to formation of secondary oxidation products (Latha, and Nasirullah, 2014). *p*-Anisidine Value (*p*-AV) is a reliable indicator of oxidative rancidity in fats and oils. *p*-AV measures the secondary oxidation product (aldehyde and ketone, etc.) produced during oxidative degradation of oils, which is usually estimated by using isooctane as the solvent under 350 nm of wavelength (Firestone, D. 2012).

Table 1. Soybean oil's fatty acid profile (mg 100 mg⁻¹ oil).

Fatty acids	Soybean oil	Reported ^a
Palmitic acid (C16:0)	10.63 ± 0.23	7.0 - 12.0
Stearic acid (C18:0)	3.78 ± 0.11	2.0 - 5.5
Oleic acid (C18:1)	23.64 ± 0.23	20.0 - 40.0
Linoleic acid (C18:2)	53.53 ± 0.85	40.0 - 57.0
Arachidic acid (C20:0)	0.25 ± 0.12	0.0 - 1.0
Linolenic acid (C18:3)	7.83 ± 0.65	5.0 - 11.0

^a NOM, 2005 (36).

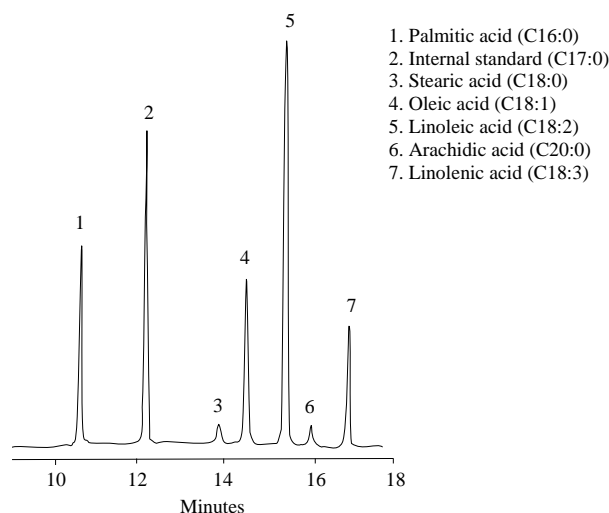


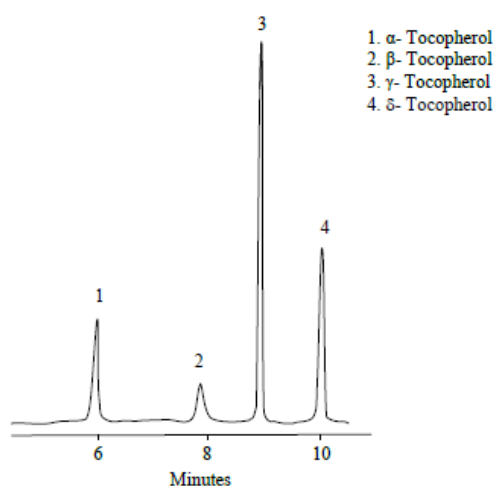
Figure 1. Partial soybean's oil fatty acids profile chromatography. Capillary column SP-2560 (100 m×0.25 mm di×0.2 mm (micrometers) particle size Supelco). Columns' temperature program: 140 to 210°C (4°C min⁻¹) and from 210 to 220°C (1°C min⁻¹). Carrier gas: Nitrogen at 20 cm seg⁻¹, Injector and detector's temperature: 250°C.

The fatty acid profile and chromatograms are shown in Table 1 and Figure 1, respectively. The linoleic acid value presented 53.53 mg 100 mg⁻¹ of oil and the linolenic acid value 7.83 mg 100 mg⁻¹ of oil, which represents a total of polyunsaturated fatty acids (AGPI) more than 60 (Bachari-Saleh, *et al.*, 2013). These AGPI levels confirmed that the soybean oil is composed of fatty acids highly susceptible to oxidation, which directly affects oxidation stability and color. These two factors are critical for its acceptance as edible oil (Bachari-Saleh, *et al.*, 2013; Parry and Yu, 2004). Natural antioxidants, including vitamin E and phenolic compounds can effectively prevent lipid oxidation on edible oils as much as the commonly used synthetic antioxidants (TBHQ, BHT and BHA).

Table 2 shows the content of tocopherols and Figure 2, the chromatogram obtained. The soybean's tocopherol analysis showed a total concentration of 883.89±4.62 ppm. These results correspond with the Medina *et al.* (2000) report (Medina-Juárez, *et al.* 2000). The γ -tocopherol (considered an antioxidant) was found in higher concentration (630.42±8.59 ppm) than α -tocopherol (vitamin), which was found in a lower concentration (71.23±2.19 ppm). Vitamin E (tocopherols) has different antioxidant properties, which are helpful for health conditions, and representing is competitively given to a group of fat-soluble compounds (Prateek, *et al.* 2019). Soybean oil is considered the main source of tocopherol among vegetable edible oils. To avoid the reduction of this characteristic, it is important to take into account the addition

Table 2. Soybean oil tocopherol content (ppm).

Tocopherol	Concentration	Reported ^a
α	71.23 \pm 2.19	86
β	44.62 \pm 0.32	NR
γ	630.42 \pm 8.59	624
δ	137.62 \pm 7.39	188
Σ	883.89 \pm 4.62	

^aMedina-Juárez (2002) (30), NR: Not Reported.

Figure 2. Partial soybean's oil tocopherols profile chromatography. Lichrosorb Si60 column (25 cm \times 4 mm \times 5 mm particle size, Supelco), Ultraviolet detector (292 nm), Mobile phase: Hexane: Isopropanol (99:1), Flow rate: 3.5 mL min⁻¹.

of natural antioxidants as flavonoids during processing (Zaunschirm, *et al.*, 2018).

The phenol compound's antioxidant activity depends on the presence of catalytic metals. In this study, the mineral content (iron, copper, calcium, magnesium, and phosphorus) in soybean oil used in each oxidation essay was similar to the ones reported by Medina *et al.* (2002) eliminate (Medina-Juárez, 2002) in its content value and composition (Table 3).

The content of iron and copper in lyophilized asparagus was between the normal values reported in food (tomatoes, cauliflower, onion and cabbage) by (Moreiras 2013). Different factors including asparagus variety, spear parts, and diameter were found to affect the mineral content in

asparagus. Usually, mineral content is iron, copper, magnesium, and sodium, zinc, copper, and manganese (Chitrakara, *et al.* 2019).

It is important to underline the decrement of copper and iron observed in lyophilized asparagus and ethanol extract (21.31 and 50.7% respectively), probably due to the fiber removal, which is rich in minerals.

The pro oxidant activity can be associated with the flavonoids capacity to subject themselves to a catalyzed autoxidation by transition metals to produce peroxide hydrogen and to form hydroxyl radicals (Rui, *et al.* 2015). In this study, the soybean oil control's OSI value (7.10 h) was significantly less ($P < 0.05$) than the OSI of

Table 3. Mineral content (ppm) of soybean oil and asparagus (*Asparagus officinalis* L.) ethanol extracts.^a

Sample	Minerals				
	Iron	Copper	Calcium	Magnesium	Phosphorus
Lyophilized asparagus	59.14 ± 2.26 ^a	23.16 ± 2.01 ^a	31.12 ± 0.21 ^a	11.00 ± 0.33 ^a	74.23 ± 3.14 ^a
Asparagus ethanol extract	46.53 ± 3.25 ^b	11.42 ± 1.12 ^b	18.12 ± 0.19 ^b	5.36 ± 0.20 ^b	60.00 ± 2.45 ^b
Soybean oil	1.12 ± 0.28 ^c	0.69 ± 0.12 ^c	18.39 ± 2.31 ^b	8.66 ± 1.16 ^c	0.02 ± 0.01 ^c

^a Values shown in columns are the media of three repetitions. Different super script letters in columns show statistically significant ($P < 0.05$) values.

all soybean oil plus ethanol extract (at all tested concentrations).

Phenolic compounds are considered secondary metabolites produced by plants. They impart important physiological benefits to humans such as antioxidant, cancer chemo-preventive, etc. (Hughes *et al.*, 2017).

The asparagus total phenol content determined was 5.07 ± 0.49 mg/asparagus dry weight expressed as catechin equivalents. The obtained result is concurrent with (Rui, *et al.* 2015), who used different solvents in his study (methanol, ethanol and acetone). In our study, ethanol was used since it was considered an extract to be used in the food antioxidant (oils) area. The obtained results are important, given the phenol content being similar to what (Molina 2012) reported for different types of fruit (mango, grapes and blackberry).

Asparagus total phenol ethanol extract's induction times are shown in Table 4. The control (soybean oil) was the one that had the lowest induction time due to its fatty acids composition and to its tocopherol. The oil with asparagus ethanol extract at 0.1% (p/p) was similar ($P > 0.05$) to the oil with tocopherols, so, it can be used as a substitute for this compound group. The oil with extract concentrations higher than 0.3 and 0.5% (p/p) showed induction times significantly higher ($P > 0.05$) than natural (tocopherols) and synthetic (TBHQ) antioxidants at the tested concentrations. This fact is very important, given its role as a natural antioxidant from asparagus.

CONCLUSIONS

Asparagus (*Asparagus officinalis* L.) has natural antioxidants content (phenolic compounds), which makes this crop an interesting research subject due to its potential as a natural antioxidant source in the food and oil industry. In this study, the phenol compounds' extraction using ethanol and the addition of such phenol compounds

**Table 4.** Synthetic, natural, and edible asparagus extract antioxidant activity (test Rancimat).

Treatment ^a	Induction time ^b (hours at 110°C)
Soybean oil (Control)	7.10 ± 0.18 ^a
Soybean oil+0.1% AEETPC	12.40 ± 0.32 ^b
Soybean oil+0.3% AEETPC	25.60 ± 0.98 ^c
Soybean oil+0.5% AEETPC	> 48.00 ^d
Soybean oil+0.02% w/w tocopherol	13.50 ± 1.10 ^b
Soybean oil+0.02% w/w TBHQ	22.23 ± 0.58 ^e

^a AEETPC: Asparagus Ethanol Extract Total Phenols Content; TBHQ: Tertiary Butyl Hydroquinone.

^b Average values ± three repetitions standard. The media with different letters as a super script are statically different (P < 0.05).

to the soybean oil for the oxidation tests (OSI) through Rancimat equipment was easy and fast. Soybean oil with asparagus ethanol extract at 0.3 and 0.5% concentrations showed higher induction times than natural (tocopherols) and synthetic (TBHQ) antioxidants at the tested concentrations. Based on these research results, we confirmed that fresh asparagus consumption is important for human health, given that they are a natural source of antioxidants. Asparagus ethanol extract can be used as an additive for food and vegetable oils, such as soybean oil, to inhibit lipid oxidation.

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فعالیت آنتی اکسیدانی عصاره اتانولی مارچوبه در برابر TBHQ و توکوفرول ها

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چکیده

مارچوبه (*Asparagus officinalis* L.) در منطقه Caborca در Sonora به لحاظ تولید زیاد و محتوای آنتی اکسیدانی آن (ترکیبات فنولی) گیاه مهمی تلقی می شود. در این پژوهش، مصرف و تاثیر آنتی اکسیدان طبیعی روی روغن سویای تصفیه شده با اندکس (نمایه) پایداری اکسیداتیو (OSI) و با استفاده از روش Rancimat ارزیابی شد. ترکیبات فنولی مارچوبه با محلول حاوی اتانول: آب به نسبت ۵:۹۵ (v:v) عصاره گیری شد. فعالیت آنتی اکسیدانی عصاره هیدروالکلی به دست آمده، با بوتیل هیدروکینون درجه سوم (TBHQ) و مخلوطی از توکوفرول ها در روغن سویای بدون آنتی اکسیدان های صنعتی (که با خواص شیمیایی و ترکیبات روغن سویای خوردنی خالص مطابقت داشت) مقایسه شد. محتوای فنول کل در عصاره مارچوبه شبیه آنچه بود که در منابع علمی برای میوه ها و مارچوبه کشورهای مختلف گزارش شده بود. روغن سویایی که دارای غلظت های بیشتر از ۳٪ (w/w) عصاره اتانولی مارچوبه بود به طورمعناداری ($p < 0.05$) زمان القای بیشتری از آنتی اکسیدانهای طبیعی (توکوفرول) و صنعتی (TBHQ) در غلظت مورد آزمون نشان داد که این امر استفاده از مارچوبه را به عنوان منبع آنتی اکسیدان طبیعی تاکید میکند.