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MAXIMIZATION OF ESSENTIAL OIL ANTIOXIDANT CAPACITY VIA STAR ANISE HYDRODISTILLATION

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Abstract - Chinese star anise essential oil (EO) is prized for its pleasant aroma and mainly due to its antioxidant capacity (AC), which can combat cancer and treat neurodegenerative diseases. This study shows the innovative approach of optimizing not only EO yield, but also to therapeutic activity. The product obtained follows the principle of the Pure Food and Drug Act and of eco-friendly technology. Also, the best analytical control methodology of the active principle during industrial production was also defined. The AC was found to be dependent on the extraction conditions, and the best antioxidant performance was reported after 3h of hydrodistillation time, using 500 mL distilled water and 8% dry fruits whose granulometry was inferior to 425 μm. Although all four analytical methodologies used for assessing AC are precise, they cannot be correlated. The antioxidant potential was 59.6±1.1 mg GAE/g EO for TP; 14.3±0.5 mmol TEAC/g EO for FRAP; 25.7±0.8 mmol TEAC/g EO for ABTS and 23.5±0.3 mmol TEAC/g EO for DPPH. The EO exhibited better AC than the trans-anethole and D-limonene standards, suggesting a positive synergistic effect. The DPPH method exhibited a good coefficient of determination (R^2=0.9311) and has the advantage of using a solvent compatible with the EO.
Keywords: Antioxidant capacity; DPPH; Essential oil; Hydrodistillation; *Illicium verum* Hook.

INTRODUCTION

Antioxidant substances have been described as a preventive agent for degenerative processes that evolve to chronic diseases such as cardiovascular diseases (Vieira et al., 2011) (Shahidi and Ambigaipalan, 2015). Star anise is an aromatic vegetable grown in Asian countries (Asif et al., 2016). It contains large amounts of an essential oil (EO) whose predominant component is trans-anethole (Asif et al., 2016; Bhadra et al., 2011). Recently, this EO has been receiving more attention from researchers due to its many beneficial properties and usefulness in combating cancer (Asif et al., 2016) and treating neurodegenerative diseases (Bhadra et al., 2011). Although its antioxidant properties have also been documented (Wong et al., 2014), there is very little data in the literature regarding star anise EO production and its AC. Thus, no report was found on the best analytical methodology for estimating this property, much less whether the conditions for obtaining this noble oil influenced this health protective activity.

The extraction of antioxidants of plant origin with the use of solvents has been discouraged because recovery of the solvent causes oxidative transformations (López-Sebastián et al., 1998). So hydro-distillation, steam distillation and CO_2-supercritical extraction have been described as the best alternatives (Conde-Hernández et al., 2017). Hydrodistillation uses only water and has low energy demand, low-cost equipment, and moderate operating conditions. It is known that the yield and trans-anethole content in the hydrodistilled aromatic oil (HD) is dependent on the parameters of granulometry (G), dried fruit mass (m), extraction time (t), and water volume (V): the highest oil yield was obtained by the use of smaller particles

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(<425 μm), high particle ratio to water (16%), and longer hydrodistillation times (3h), but highest trans-anethole content (96.6% TA) was verified by using shorter time (1h) and lower particle ratio to water (8%) than previously (80.4% TA) (Destro et al., 2019). However, the individual or interaction effects of grain size, fruit mass, extraction time and water volume on the AC of the essential oil obtained were not known. The condition with the highest volume of essential oil is not necessarily the condition of highest AC per unit volume. Furthermore, oxidative capacity evaluation can be performed by various techniques and it is not conclusive which method is best for star anise EO.

One of the methods used to evaluate AC is the Folin-Ciocalteau method (Huang et al., 2005; Shahidi and Zhong, 2015), also called total phenolic compounds (TP) method. It is based on the observation that phenolic compounds may present antioxidant characteristics. The Folin-Ciocalteau reagent is composed of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, which are reduced by oxidation of the phenolic compounds under alkaline conditions, producing a blue chromophore, probably $(PMoW_{11}O_{40})^{4-}$, with an absorbance at 720 to 765 nm (Huang et al., 2005; Shahidi and Zhong, 2015). Other methods that may be used to evaluate *in vitro* AC include the Ferric Reducing Antioxidant Power (FRAP) method (Huang et al., 2005), the ABTS radical method [2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)] (Huang et al., 2005) and the free radical sequestration method with 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Huang et al., 2005; Shahidi and Zhong, 2015; Singleton et al., 1999).

In the FRAP method, the ferric salt $Fe(III)(TPTZ)_2Cl_3$ is used as an oxidant (TPTZ = 2,4,6-tripyridyl-s-triazina) and the reaction occurs in acidic conditions (pH 3.6). The oxidant, called the "FRAP reagent", is prepared by mixing TPTZ, acetate buffer and ferric chloride (Huang et al., 2005). The AC is based on the ability of the antioxidant to reduce the Fe^{3+} ion of the $Fe(III)(TPTZ)_2$ complex to Fe^{2+}, resulting in a strong blue color whose spectrophotometric wavelength is 593 nm (Shahidi and Zhong, 2015). The results may be expressed as micromoles of Fe^{2+} or with reference to a standard antioxidant such as trolox (Pulido et al., 2000).

The ABTS radical method uses a blue-green ABTS radical cation chromophore ($ABTS^{\bullet+}$) produced from its precursor, 2,2-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid. The aqueous ammonium ABTS solution is dissolved in water, potassium persulfate is added, and the solution is left to rest at room temperature for 12-16 h, resulting in a dark blue solution (Huang et al., 2005). In the reaction medium, the antioxidant again reduces the monocation radical of ABTS to ABTS, resulting in a loss of coloration in the reaction medium (Re et al., 1999). The results of the test are expressed with reference to trolox (Prior et al., 2005).

Finally, the DPPH assay method consists of using a stable organic nitrogen radical having a violet color and whose absorption ranges from 515 to 520 nm (Noipa et al., 2011). When antioxidant compounds bind to the nitrogen of DPPH•, the solution turns from purple (Prior et al., 2005; Shahidi and Zhong, 2015) to yellow (Wong et al., 2014). These results may also be expressed with reference to trolox (Shahidi and Zhong, 2015).

Thus, the objective of this study was to evaluate the effect of hydrodistillation parameters on the *in vitro* AC of star anise essential oil (EO), as determined comparatively using four different methodologies, in order to verify if the AC is due exclusively to TA, also determine which one is best for use with this essential oil, in view of the scarce amount of data available in the literature for the fruit, its EO, or for their test correlations.

MATERIALS AND METHODS

Hydrodistillations of star anise (*Illicium verum* Hook) essential oils (EO) were conducted under nine different sets of conditions (Table 1) , according to a fractional factorial experimental design 2^{4-1} with three center points added, as reported by Destro et al. (2019) for dried fruits (moisture on a dry basis of 12.98 ± 0.14%, w/w). The effects of the granulometry (G), fruit mass (m), extraction time (t), and water volume (V) were evaluated initially to determine which hydrodistillation parameters produce the highest yield and highest purity of EO (in terms of trans-anethole) (Destro et al., 2019). For the present study, the EOs obtained under the same conditions were also analyzed for AC.

The extraction was conducted for 180 min in a Clevenger apparatus using a QUIMIS® heating mantle (Q-321A25, 315 W). After being cooled and separated by decantation in the apparatus itself, the oil was added to microtubes (1.5 mL) and centrifuged in a THERMO SCIENTIFIC® Centrifuge (Heraeus Fresco 21) at 10,000 rpm/5 min (14,257 xg at 5 min). The

Table 1. Hydrodistillation conditions for star anise essential oil extraction.

Tests	G (mm)	m (%)	t (h)	V (mL)
HD1	< 0.43 (-1)	8 (-1)	1 (-1)	200 (-1)
HD2	> 0.85 (+1)	8 (-1)	1 (-1)	500 (+1)
HD3	< 0.43 (-1)	16 (+1)	1 (-1)	500 (+1)
HD4	> 0.85 (+1)	16 (+1)	1 (-1)	200 (-1)
HD5	< 0.43 (-1)	8 (-1)	3 (+1)	500 (+1)
HD6	> 0.85 (+1)	8 (-1)	3 (+1)	200 (-1)
HD7	< 0.43 (-1)	16 (+1)	3 (+1)	200 (-1)
HD8	> 0.85 (+1)	16 (+1)	3 (+1)	500 (+1)
HD C (i)[1]	0.43 < G < 0.85 (0)	12 (0)	2 (0)	350 (0)

[1]Center points in triplicate, i = 1-3. Source: Destro et al. (2019).

lipid phase was transferred to another set of microtubes containing approximately 7% (m/v) anhydrous sodium sulfate (CAS 7757-82-6, ANALYTICALS®), commonly used as an oil drying agent (Zhai et al., 2009). The dehydrated oils were subsequently stored under refrigeration (4 ± 1°C) (Bhadra et al., 2011; Chempakam & Balaji, 2008; Wang et al., 2007), and after stabilization (overnight), they were again centrifuged under the same conditions. The dried essential oil was hermetically conditioned in amber vials and stored under refrigeration.

The following reagents used for the analyses were purchased from Sigma-Aldrich (St. Louis, MO., EUA): ABTS (CAS number 30931-67-0, 2,2′-azinobis [3-ethylbenzothiazoline-6-sulphonic acid]), gallic acid (CAS number 149-91-7, 97.5%), DPPH (CAS number 1898-66-4, 2,2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteau reagent, trolox (CAS number 53188-07-1, 6-hydroxy-2,5,7,8-tetramethylchromo-2-carboxylic acid) and TPTZ (CAS number 3682-35-7, [2,4,6-tri (2-pyridyl) -1,3,5-triazine]).

The oil was previously diluted using a methanol-water solution to eliminate possible interferents, as recommended by Bail et al. (2008), Parry et al. (2005) and Taghvaei et al. (2014). Next, 0.25 g of essential oil was transferred using a micropipette to 2 mL microtubes (duplicates). A 90:10 methanol-water solution (750 μL) was then added, and the mixture was vortexed until complete homogenization was achieved. The supernatant produced by centrifugation at 3000 g for 5 min was reserved for dosing. The precipitate received more methanol-water solution (90:10), after which it was again homogenized and centrifuged. This procedure was performed 3 times and the supernatants were then extracted for evaluation, being kept under refrigeration until analysis.

An evaluation of the antioxidant compounds of the trans-anethole standard (TA) (CAS 4180-23-8, Eastman Chemical Company®) and the EO samples obtained by hydrodistillation (HD) was conducted in 6 replicates for each sample using each of the above methodologies: TP, DPPH, FRAP and ABTS (details below). All antioxidant analyses were performed on a 96-well flat bottom polystyrene microplate (Corning®) and the spectrophotometric readings were taken using a Multi-Detection Microplate Reader Synergy HT (Biotek®) device. A 2^{4-1} fractional factorial design (FFD), analysis of variance (ANOVA, $\alpha = 0.05$) and determination factor for the corresponding design (R_{model}^2) were applied to each methodology using STATISTICA 7® (StatSoft, Inc.) software. A Tukey test ($\alpha = 0.05$) was conducted using the Action Stat supplement (Estat Camp) for Excel 2013® (Microsoft Corporation). In order to verify whether any correlation existed between the methods used, the results from the samples containing trans-anethole, including the standard TA and all the EOs obtained from the extraction optimization were used. The data were then plotted using Excel 2013® (Microsoft Corporation). For each comparison between two individual methods, a linear regression analysis was applied, with an equation for the line and determination coefficient (R^2_{pairs}).

The TP evaluation was based on Singleton and Rossi (1965). Distilled water (144 μL) was added to the wells of the microplate, followed by the sample (12 μL). The Folin-Ciocalteau reagent (15 μL) was then added and, after 3 min, 20% (w/v) Na_2CO_3 (36 μL) solution was added, the plate being agitated slightly in order to achieve homogenization. The set of samples was allowed to stand for 60 min in the absence of light, and a further reading was taken at 720 nm. The concentration of antioxidants as equivalent mg gallic acid per g EO - or per g of the standard analyzed (mg GAE/g) - was calculated using data interpolation from the calibration curve as being between 0.3 and 2.5 mg gallic acid/mL ethanolic solution.

For the FRAP method (Benzie and Strain, 1996), the standard spectrophotometric curve at 593 nm used an aqueous trolox solution of 0.0 to 1.0 mmol/L. For this purpose, a fresh FRAP solution (100 mL of sodium acetate buffer, 10 mL of $FeCl_3$ solution and 10 mL of TPTZ solution in 0.05M HCl solution) was prepared and added to the microwells (300 μL), followed by distilled water (30 μL) and the sample (10 μL). The set was allowed to stand in the absence of light for 30 min, and the readings were subsequently taken. The results were expressed in mmol of trolox equivalent AC per g of EO - or per g of analyzed standard (mmol TEAC/g).

The ABTS radical (Arnao et al., 2001; Re et al., 1999) was prepared using the mixture of 5 mL stock ABTS solution and 88 μL of $K_2S_2O_8$ solution maintained in a dark environment for 16 h in order for the reaction that forms the solution to take place. At the end of this period, 1 mL of the solution was diluted in ethyl alcohol to a final absorbance of 0.700 ± 0.050 nm at 734 nm on the day of analysis. The ABTS reagent (300 μL) was added to the microwells, followed by the sample (3 μL). Readings were taken after the set of samples was allowed to rest for 6 min in a dark environment, in order to infer the trolox equivalent content (mmol TEAC/g EO), based on a calibration curve of 0.2 to 0.9 mmol ethanolic trolox/L.

The DPPH test is based on the methodology of Brand-Williams et al. (1995), with a reading at 515 nm using a standard curve ranging from 0 to 1.0 mmol methanolic trolox/L. For this test, a methanolic solution of DPPH 0.06 mmol/L (125 μmol/L) was added (196 μL) to the microwells, followed by the sample (5 μL), and the mixture was allowed to stand in the absence of light for 30 min, after which a further reading was taken. The

concentration of antioxidants (mmol TEAC/g EO) was calculated based on the standard curve.

The TA quantification was performed using gas chromatography coupled to a mass spectrometer (GC-MS/MS; GC-2010 Plus, Shimadzu®, Japan), based on Bhadra et al. (2011). For this purpose, 1.0 µL of sample and a 1:20 split ratio were used. Helium gas (1.2 ml/min) was used in a DB5 capillary column (30 m x 0.32 mm, 0.25 µm film thickness, J&W Scientific Inc., Folsom, CA, U.S.A.) with cross-linked 5% phenylmethyl-silicone under isothermal conditions (40°C for 5 min). It was heated (5°C/ min) until 220°C, and maintained under isothermal conditions for 10 min. The mass spectrum of TA in the EOs was identified using the NIST/EPA/NIH virtual library and quantified from the areas of the standard curve.

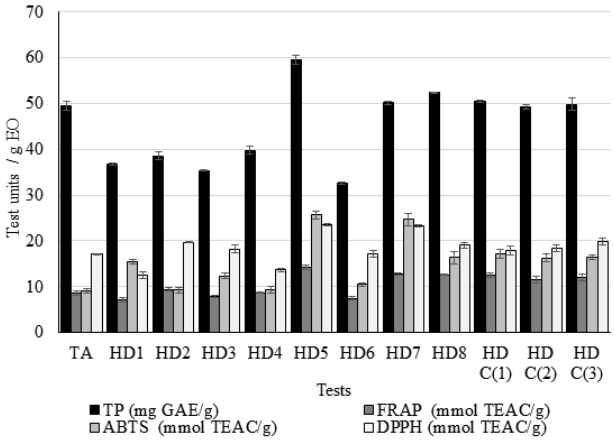

Figure 1. Different tests for evaluating the AC of TA and EO by HD.

RESULTS AND DISCUSSION

Hydrodistillation of fragmented star anise produces an essential oil that respects the principle of the Pure Food and Drug Act, because it does not use organic solvents such as ethyl ether which may increase the amount of oil produced, but would require a complementary purification step (Destro et al, 2019). This also reveals an eco-friendly technology due to a solvent-free extraction. The obtained essential oil (EO) has antioxidant capacity (AC). The influence of the extraction conditions (HD) on the antioxidant activity (Table 2) of the obtained oil was evaluated using four different analytical techniques (TP, DPPH, FRAP and ABTS). All techniques were employed in order to also estimate their respective influence (Figure 1). The essential oil obtained under the condition set HD5 (G < 425 µm, m 8%, 3h, 500 mL) exhibited the highest AC (Table 2), surpassing even that of the TA used as a reference. This pure substance presented similar values for both the ABTS (only 5.8% higher, Table 3) and FRAP methodologies. The value obtained using the DPPH method was almost double (98.8% higher) that of the first two methods, while the value for TP was almost 5 times higher (475.6%) than FRAP and ABTS. These disparate results reveal that the values used to evaluate antioxidant activity cannot be used to describe the activity directly, thus logically preventing the comparison of data obtained using different analytical techniques.

Based on the product of the test values for the TA standard and the observed TA content in HD5 oil of 82.2% (Destro et al., 2019), the hypothetical results should be 40.7 mg GAE/g EO for TP, 7.1 for FRAP, 7.5 for ABTS, and 14.1 mmol TEAC/g EO for DPPH (Table 3). However, the actual values obtained in analyses were 59.6 mg GAE /g EO, 14.3, 25.7 and 23.5 mmol TEAC/g EO, respectively. This fact suggests that the AC of the essential oil may not be ascribed exclusively to trans-anethole, as described by some authors (Cai et al., 2013; Wong et al., 2014), but also to other compounds present in the essential oil. It is even possible that these other components may be even more effective anti-oxidants than the TA itself, and that the extraction of these subcomponents may be more

Table 2. AC for EO extracted under different sets of conditions.

Exp.	G (mm); m (%); t (h); V (mL)	TP (mg GAE/g EO)	FRAP (mmol TEAC/g EO)	ABTS (mmol TEAC/g EO)	DPPH (mmol TEAC/g EO)
TA[1]	-	49.5 ± 0.9^{a}	8.6 ± 0.6^{ac}	9.1 ± 0.4^{a}	17.1 ± 0.1^{a}
HD1	< 0.43; 8; 1; 200	36.7 ± 0.3^{b}	7.2 ± 0.4^{b}	15.5 ± 0.6^{b}	12.5 ± 0.8^{b}
HD2	> 0.85; 8; 1; 500	38.6 ± 0.9^{c}	9.4 ± 0.4^{c}	9.2 ± 0.5^{ae}	19.7 ± 0.1^{c}
HD3	< 0.43; 16; 1; 500	35.4 ± 0.1^{b}	7.8 ± 0.2^{abd}	12.4 ± 0.6^{c}	18.2 ± 0.9^{ac}
HD4	> 0.85; 16; 1; 200	39.7 ± 0.8^{c}	8.7 ± 0.2^{acde}	9.4 ± 0.7^{ae}	13.6 ± 0.3^{b}
HD5	< 0.43; 8; 3; 500	59.6 ± 1.1^{d}	14.3 ± 0.5^{f}	25.7 ± 0.8^{d}	23.5 ± 0.3^{d}
HD6	> 0.85; 8; 3; 200	32.6 ± 0.3^{e}	7.5 ± 0.4^{ab}	10.5 ± 0.3^{ace}	17.1 ± 0.8^{a}
HD7	< 0.43; 16; 3; 200	50.1 ± 0.4^{a}	12.7 ± 0.2^{g}	24.6 ± 1.3^{d}	23.3 ± 0.3^{d}
HD8	> 0.85; 16; 3; 500	52.3 ± 0.2^{f}	12.6 ± 0.2^{g}	16.3 ± 1.4^{b}	19.0 ± 0.5^{c}
HD C (i)[2]	0.43 < G < 0.85; 12; 2; 350	49.9 ± 0.7^{a}	12.0 ± 0.7^{g}	16.6 ± 0.8^{b}	18.7 ± 0.8^{ac}
HD C (1)[3]		50.6 ± 0,3	12.5 ± 0.5	17.2 ± 1.0	17.9 ± 0.9
HD C (2)[3]		49.3 ± 0.4	11.5 ± 0.5	16.3 ± 0.9	18.4 ± 0.8
HD C (3)[3]		49.8 ± 1.3	12.0 ± 0.8	16.4 ± 0.4	19.8 ± 0.8
	R_{model}[2] [4]	0.8586	0.8166	0.9533	0.9311

[1] Trans-anethole standard. [2] Center points media. [3] Center points in triplicate, i = 1-3. [4] 2^{4-1} Fractional factorial design correction factor. * The same letter does not differ significantly according to the Tukey test ($p \geq 0.05$).

Table 3. Observed and hypothetical values for HD5, based on TA content and comparison with D-limonene.

	TA			HD5				D-lim
	Observed value	Times greater than FRAP	Greater than FRAP (%)	Hypothetical value	Observed value	Times greater than FRAP	Greater than FRAP (%)	Observed value
TP (mg GAE/g)	49.5	5.756	475.6	40.7	59.6	4.168	316.8	26.7
FRAP (mmol TEAC/g)	8.6	1.000	0.0	7.1	14.3	1.000	0.0	2.5
ABTS (mmol TEAC/g)	9.1	1.058	5.8	7.5	25.7	1.797	79.7	3.0
DPPH (mmol TEAC/g)	17.1	1.988	98.8	14.1	23.5	1.643	64.3	10.1

sensitive to the extraction conditions. This hypothesis is reinforced by the difference in the estimated AC of HD5 oil for the various analytical techniques based on the present TA content of 82.2%.

In addition to confirming that the AC is not directly related to the TA (%) content of the EOs, the values obtained by Destro et al. (2019) were used to compare the four different methodologies, revealing that there was no actual direct relationship between the bioactive compound and AC in the tests applied in the present study (Figure 2), as becomes evident when the trend lines of the values are compared.

The GCMS identified traces of D-limonene and estragole in HD1, HD3, HD5, HD8, HD C (i) and only traces of estragole in HD2, HD4 and HD6, suggesting that this compound may have contributed to the antioxidant activity. In HD7, traces of feniculin were also identified, in addition to traces of D-limonene and estragole. Estragole (Chouksey et al., 2013; Howes et al., 2009; Wang et al., 2011; Wong et al., 2014), D-limonene (Chouksey et al., 2013; Padmashree et al., 2007; Zhai et al., 2009) and feniculin (Chouksey et al., 2013; Scopel et al., 2016; Singh et al., 2006) are naturally occurring compounds present in the edible star anise essential oil.

Thus, the AC of D-limonene (occurring in a more significant amount than estragol in the HD5 sample, which exhibited the greatest AC) was also verified (Table 3) as being 26.7 mg GAE/g (± 1.3) for TP, 2.5 (± 0.2) mmol TEAC/g for FRAP, 3.0 (± 0.3) mmol

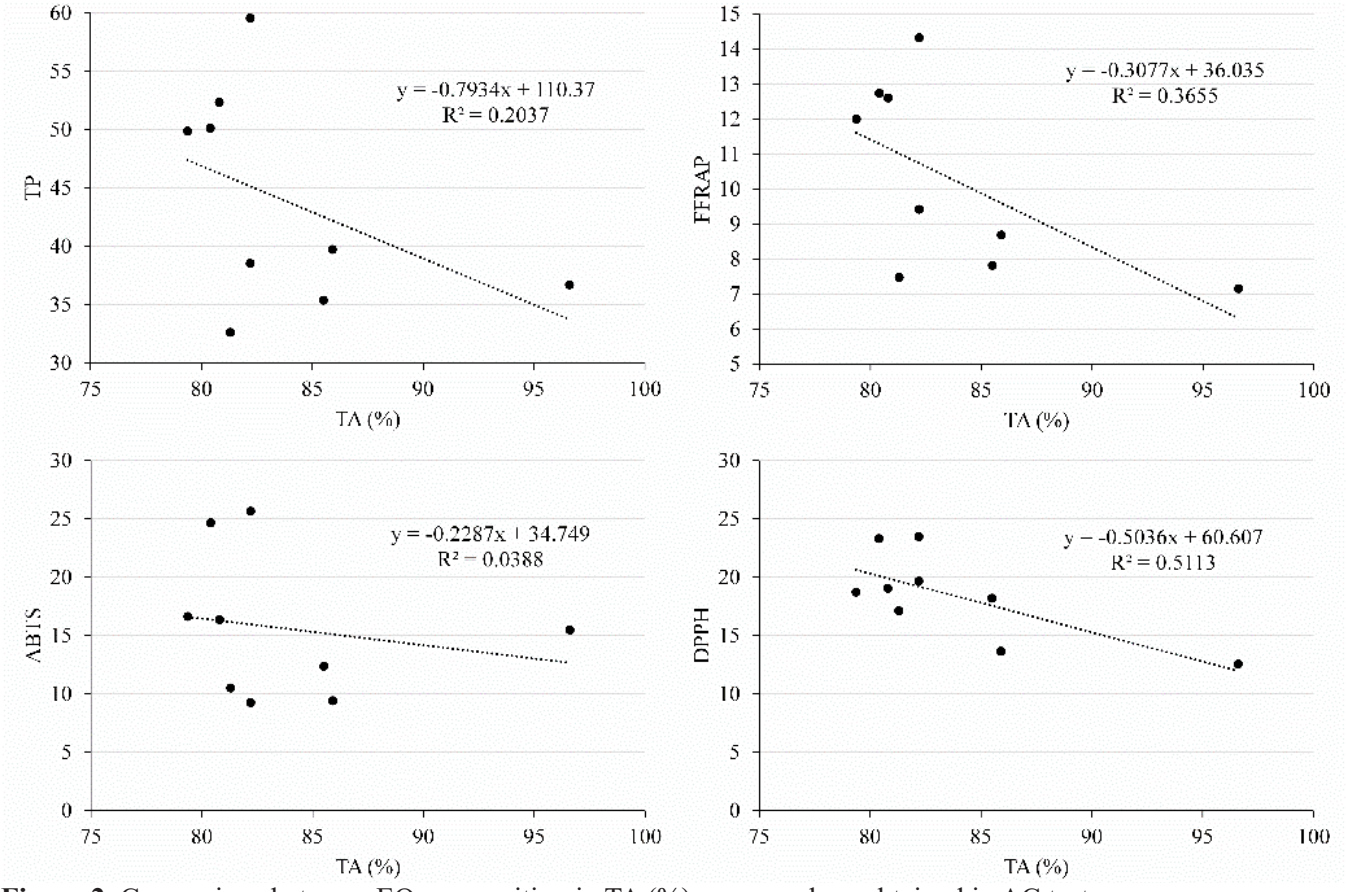

Figure 2. Comparison between EO composition in TA (%) versus values obtained in AC tests.

TEAC/g for ABTS and 10.1 (± 0.2) mmol TEAC/g for DPPH. Since these values were lower than those observed for TA, the exceptional antioxidant activity of HD5 should be attributed to another trace element, or else to a possible synergistic effect among the components of the EO, as reported previously (Scopel et al., 2016; Singh et al., 2006). According to this hypothesis, the antioxidant potential predicted by ABTS (25.7) for HD5 oil was 79.7% higher than for FRAP (14.3 mmol TEAC/g EO), the previous behavior being only 5.8% (Table 3). The DPPH value (23.5 mmol TEAC/g EO) surpassed the FRAP value by 64.3%. Thus, these atypical behaviors can only be explained by the presence of other antioxidant compounds that are selectively extracted according to hydrodistillation conditions and whose activity is extremely varied.

A variance analysis (ANOVA) for TP (Table 4, TP column) was applied using STATISTICA 7® (StatSoft, Inc.) software, as described in the methodology, showing significant differences ($p < 0.05$) for t, V, G and m, and the Gm, Gt and GV interactions. The regression model (also obtained using STATISTICA 7®, StatSoft, Inc.) showed that the oil that exhibits the best AC is the one obtained using the longest extraction time (3h), smallest particle size (<425 μm), greatest water volume (500 mL) and smallest mass ratio 8%). This is confirmed by the HD5 expression, which according to the Tukey test ($p < 0.05$) was statistically superior to the others (Table 2).

The highest AC according to the FRAP was also observed for the HD5 condition set (Table 2), the p-values revealing a statistical significance for the factors (Table 4, FRAP column) quite similar to that of the TP test: t, Gt interaction, V, Gm interaction, G, GV interaction and m (Table 4, FRAP column).

According to the ABTS and DPPH methods, m did not significantly affect AC. The Tukey test (Table 2) revealed for both methods that HD5 and HD7 did not differ from each other and exhibited a higher AC than did the other sets of conditions.

In general, a longer extraction time, smaller particle size and greater volume of distilled water are the parameters that positively affect the AC of the oils produced. The mass of the sample mass added to the system was not significant. Thus, although the TP test is an excellent tool for evaluating phenolic compounds, interference from reducing sugars, amino acids and other components (Shahidi and Zhong, 2015) limit its usefulness for evaluating AC. AC may be much better described by the FRAP, ABTS and DPPH methods, although differences in values and model adjustment from one method to another are a result of the specific reaction conditions peculiar to each methodology, such as acidic application (FRAP), aqueous solutions (ABTS) and the use of methanol as a solvent (DPPH).

Table 4. Summary of p-values for effects of the significant conditions generated in the hydrodistillation ANOVAs on AC.

	p-values			
	TP	**FRAP**	**ABTS**	**DPPH**
G	0.000	0.004	0.000	0.000
m	0.011	0.010	0.187 [1]	0.199 [1]
t	0.000	0.000	0.000	0.000
V	0.000	0.000	0.014	0.000
Gm	0.000	0.000	0.000	0.000
Gt	0.000	0.000	0.000	0.000
GV	0.009	0.007	0.000	0.000
R_{model}^2	0.859	0.817	0.953	0.931

[1] Not significant ($p > 0.050$). R^2 = Determination coefficient of 2^{4-1} Fractional factorial design.

The determination coefficients of the response surfaces varied greatly according to the analytical methodology, a fact that can be attributed to the specific reaction conditions. This divergence is confirmed by the response determination coefficients, FRAP and ABTS ($R_{FRAP\text{-}ABTS}^2 = 0.6403$) (Figure 3) being the best, slightly higher than FRAP and DPPH ($R_{FRAP\text{-}DPPH}^2 = 0.6364$). The divergence between ABTS and DPPH ($R_{ABTS\text{-}DPPH}^2 = 0.4714$) may be related to the type of aqueous reaction medium for ABTS. Despite the lack of selectivity of TP, which could explain the low adjustments ($R_{OthersPairs\text{-}TP}^2$) of TP with DPPH and with ABTS, the correlation coefficient was the best of all for FRAP (0.8066), conducted in aqueous-acid medium. This lack of correlation between the analytical techniques was unexpected, since all methods presented a high coefficient of determination value for calibration curves ($R^2_{TP} = 0.9941$, $R^2_{FRAP} = 0.9971$, $R^2_{ABTS} = 0.9974$, $R^2_{DPPH} = 0.9960$). This is compatible with a high precision in results, but which lack accuracy.

With regard to predictability of behavior by response surface methodology, although the ABTS methodology presented a better fit to the factorial planning ($R_{modelABTS}^2 = 0.9533$, Table 4), a reaction of the compounds was observed in the microwells analyzed, resulting in their irreversible turbidity. Thus, in order to avoid interference, the authors suggest that the DPPH methodology be applied for determining AC, since this method also exhibited a good fit ($R_{modelDPPH}^2 = 0.9311$, Table 4). Thus, AC can be predicted from Equation 1, from which it is possible to obtain the values predicted by the experimental model and compare them to those observed empirically (Table 5), with a low standard deviation between the data. Moreover, the solvent used for DPPH (methanol) is commonly applied in the study of *Illicium verum* Hook extracts due to its affinity to the oily fraction, as has been observed in the literature (Asif et al., 2016; Bhadra et al., 2011; Li et al., 2010; Padmashree et al., 2007; Wang et al., 2007; Wang et al., 2011; Wong et al., 2014).

$$\mathrm{DPPH}\left(\frac{\mathrm{TEAC}}{\mathrm{g}}\mathrm{EO}\right) = 0.0078\,\mathrm{G} + 10.0944\,\mathrm{t} + 0.0221\,\mathrm{V} - 0.0078\,\mathrm{Gt} + \\ + 8.39.10^{-3}\,\mathrm{GV} - 0.0080\,\mathrm{tV} - 0.8486 \quad (1)$$

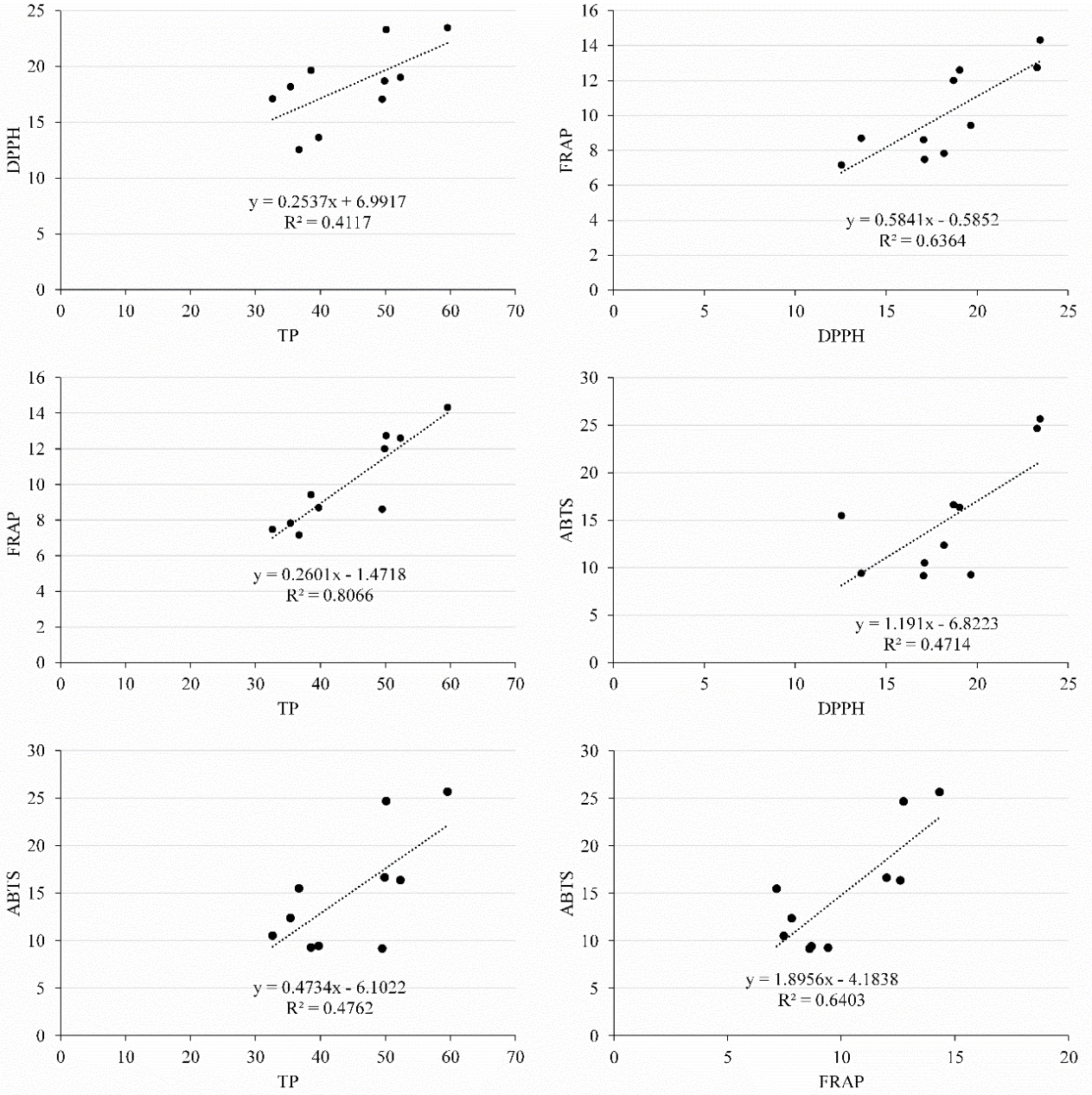

Figure 3. Correlations between tests for AC of TA and EO by HD.

Table 5. Predicted and observed values for the DPPH test.

Exp.	Predicted values[1]	Observed values	Standard deviation
HD1	12.81	12.54	0.35
HD2	19.92	19.65	0.13
HD3	18.10	18.18	0.44
HD4	13.55	13.63	0.16
HD5	23.73	23.47	0.21
HD6	17.38	17.11	0.40
HD7	23.22	23.30	0.16
HD8	18.96	19.04	0.27
HDC (i)	18.46	18.71	0.52
HDC (1)	18.46	17.87	0.48
HDC (2)	18.46	18.43	0.41
HDC (3)	18.46	19.82	0.68

[1]Predicted values by the equation of the 2^{4-1} Fractional factorial design.

The methodologies used to evaluate antioxidant potential could not be correlated (Figure 3). Thus, although the DPPH test showed better compatibility with EO, all four techniques can be used simultaneously, since there is no specific standardization for their application; each one uses a different reaction mechanism, obtaining results that reflect different types of physiological activity (Prior et al., 2005).

This application of several techniques simultaneously is often reported in the literature, as in the case of asam gelugur (*Garcinia atroviridis*) (Al-Mansoub et al., 2014), several Brazilian fruits (Rufino et al., 2010), blackberry (*Rubus fruticosus* L.) residues (Paula et al., 2015), guabiroba fruit (*Campomanesia*

xanthocarpa O. Berg) (Pereira et al., 2015), pitanga seed (*Eugenia uniflora* L.) (Santos et al., 2015), red pitaya (*Hylocereus polyrhizus*) (Wu et al., 2006) and tea plant (*Camellia sinensis* L) (Wang et al., 2012).

For all the analyses, HD5 tended to possess a higher AC, followed by HD7, both of which which used EO extracted from fractions smaller than 425 μm and with a processing time of 3h. It is possible that this extraction time recovers not only trans-anethole, but also other antioxidant compounds, as confirmed by GCMS. The results obtained for HD5 as compared to the values reported in the literature were as follows: for the TP method, the value 59.56 ± 1.08 mg GAE/g EO can be considered the same as that obtained by Asif et al. (2016) for star anise essential oil (63.51 ± 3.24 mg GAE/g EO); for ABTS analysis, the value obtained in the present study, 25.65 ± 0.84 mmol TEAC/g EO, was higher than the value of 0.203 mmol TEAC/g for dehydrated star anise fruits (Shan et al., 2005; Wang et al., 2011). Obviously, this is because hydrodistillation yields an oil that is purer and has a higher concentration of the compounds than in the fruits themselves. . In addition, this unit operation uses only water, low energy demand, low cost equipment and moderate operating conditions. Therefore, it follows the principle of the Pure Food and Drug Act and of eco-friendly technology.

Despite the presence in the EOs of compounds other than TA, their proportional influence on AC was not conclusive; however, it is assumed that a synergistic effect occurs among them, as suggested by Scopel et al. (2016) e Singh et al. (2006). The results of this study are also essential information for other human health laboratory studies. For example, the HD5 laboratory condition should be used to obtain EO to be applied in pharmacological studies to combat cancer, treat neurodegenerative and cardiovascular diseases.

CONCLUSIONS

The highest overall antioxidant power for star anise essential oil (HD5) was obtained using a particle size inferior to 425 μm, 8% crushed dehydrated fruit, 3h hydrodistillation time and 500 mL distilled water (for the production of trailing steam). This condition should be adopted as the exploratory condition at the industrial level due to the scale-up. The oil exhibited a higher AC than that of the main component (82.2% trans-anethole in HD5), as well as those of D-limonene and estragole (traces). This suggests either a positive synergistic effect for this property or an extremely high AC for estragole, since this capacity cannot be analyzed. For example, EO obtained in the HD5 laboratory condition, with high antioxidant content, should be used in future studies of protective action against some degenerative processes, such as cardiovascular diseases or even tumors. The methodologies used to evaluate antioxidant potential are unquestionable, since they could not be perfectly correlated among them. However, among these methods, the DPPH test exhibited the best response curve for the experimental design, a general equation with good prediction of the data and possessed the advantage of using a solvent that has a high chemical affinity for the oil. The EO antioxidant capacity can be adequately determined by the DPPH methodology, avoiding reagent expense and also saving analysis time by the other methodologies. In the future, this methodology should also be applied to an *in vitro* study of the release of oil components from an excipient matrix suitable for targeted release into the gastrointestinal system.

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NOMENCLATURE

ABTS	Analytical method of antioxidant activity based on 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)
AC	Antioxidant capacity
DPPH	Analytical method of antioxidant activity based on 2,2-diphenyl-1-picrylhydrazyl
EO	Star anise (*Illicium verum* Hook) essential oil
FRAP	Analytical method of antioxidant activity based on Ferric Reducing Antioxidant Power
G	Star anise granulometry (mm)
GAE	Gallic acid equivalent (mg) for EO or standard (g), in dry basis
GC-MS	Gas Chromatography-Mass Spectrometry
HD (i)	Hydrodistillation condition from fractional factorial design
m	Star anise hydrodistillation mass (%)
R_{pairs}^2	Determination coefficient of linear regression analysis
R_{model}^2	Determination coefficient for 2^{4-1} fractional factorial design
t	Hydrodistillation time (h)
TA	Trans-anethole

TEAC	Trolox equivalent antioxidant capacity (mmol) for EO or standard (g), in dry basis
TP	Analytical method of antioxidant activity based on total phenolics
TPTZ	2,4,6-tri(2-pyridyl)-1,3,5-triazine
V	Star anise hydrodistillation volume (mL)

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