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Method for the validation and uncertainty estimation of tocopherol analysis applied to soybean oil with addition of spices and TBHQ

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RESUMEN

Validación y cálculo de la incertidumbre de un método para analizar tocoferoles y su aplicación en aceite de soja suplementado con especies y TBHQ

La concentración de tocoferoles en aceite de soja refinado (muestra control), aceite de soja adicionado de romero, orégano, ajo, semilla de achiote y TBHQ fueron cuantificados durante el almacenamiento durante 12 meses a 25°C y 35°C. El método propuesto para medir tocoferoles fue validado y determinada la incertidumbre. Este método presentó linealidad y precisión adecuadas, exactitud entre 93% y 103% además de una incertidumbre expandida de 2%. Las cantidades de α-, γ- y δ-tocoferol en el aceite de soja refinado, aceite de soja adicionado de condimentos y aceite de soja adicionado con TBHQ se mantuvieron constantes durante el almacenamiento a 25°C y 35°C con excepción del β-tocoferol el cual disminuyó. El aceite de soja adicionado de condimentos (romero, orégano, ajo, y semilla de achiote) presentó mayores concentraciones de γ- y δ-tocoferol en comparación con el aceite de soja refinado utilizado como control. El aceite de soja adicionado de condimentos presentó un comportamiento semejante al aceite de soja adicionado de TBHQ.

PALABRAS CLAVES: Ajo – Almacenamiento – Orégano – Romero – Semilla de achiote – Temperatura de almacenamiento.

SUMMARY

Method for the validation and uncertainty estimation of tocopherol analysis applied to soybean oil with addition of spices and TBHQ

The tocopherol contents of refined soybean oil with the addition of rosemary, oregano, garlic, annatto seeds and TBHQ was evaluated during storage at 25 °C and 35 °C for twelve months, in comparison with a control soybean oil without the antioxidant addition. The method proposed to assess the tocopherol content was validated and the uncertainty estimation was determined. The method presented adequate linearity and precision, accuracy between 93% and 103% and expanded uncertainty of 2%. The contents of α-, γ- and δ-tocopherols of all the tested soybean oils remained constant during the storage at 25 °C and 35 °C regardless of antioxidant addition, while β-tocopherol content decreased. The addition of a mixture of rosemary, oregano, garlic and annatto seeds increased the concentration of γ- and δ-tocopherol. The oil with spices presented a similar behavior to that of the oil with the addition of TBHQ.

KEY-WORDS: Annatto seeds – Garlic – Oregano – Rosemary – Storage – Temperature of storage.

1. INTRODUCTION

The term vitamin E refers to a homologous group of the 6-hydroxy-chromanol, comprising eight structural forms, i.e. four tocopherols and four tocotrienols identified by the prefixes α-, β-, γ- and δ-. The tocopherols differ in the number and position of the methyl groups on the chromanol ring and exhibit various *in vivo* biopotencies (Sebrel and Harris, 1972; Tan, 1989; Machlin, 1991; Kamal-Elmin and Appelqvist, 1996; Eitenmiller and Lee., 2004). The antioxidant function of vitamin E is related to its ability to donate a hydrogen atom to a peroxyl radical; thus finalizing the chain reaction in the lipid oxidation processes (Sayago *et al.*, 2007). The antioxidant capacity of each vitamin E form is different, and its efficiency increases with the increase in the number of methyl substituents (Traber and Atkinson, 2007). The relative *in vivo* antioxidant capacity of the tocopherols is as follows: α > β > γ > δ; however, the reverse order is also observed (α < β < γ < δ) when the potency is compared in fats, oils and lipoproteins (Sebrell and Harris, 1972; Durán and Padilla, 1993).

In Brazil, an average of 4% per year of the world's soybean oil production (crude and refined) has been documented over the last 10 years, with the highest expression in 2006/07 and 2009/10, and a medium increase of 450 tons of oil per year (10% per year) (ABIOVE, 2011). Soybean oil has a high linoleic acid content (44 to 62%) which makes this product susceptible to oxidation during storage (MAPA, 2006; Oyewole and Olayinka, 2007). Moreover, it is also an important source of tocopherols; it contains the four tocopherols which may delay the development of oxidative changes in addition to their nutritional action as vitamin E (Ramalho *et al.*, 2006).

Several studies were conducted to evaluate the use of spices as a source of phenolic compounds to prevent oil oxidation, either alone or in combination with synthetic antioxidants (Nakatani *et al.*, 2001; Iqbal and Bhanger, 2007; Hossain *et al.*, 2008;

Ogbuagu and Nedolisa, 2010). However, these studies monitored the antioxidant capacity without measuring the tocopherol levels. The infusion of seasonings such as garlic, rosemary, oregano and annatto seeds to the oil can be beneficial to the consumer's health, since they contain phenolic compounds, carotenoids and tocopherols, as well as contributing to the product's stability.

The present study aimed to validate and to determine the expanded uncertainty assigned to the HPLC-FLU (high performance liquid chromatography with fluorescence detection) method used to determine tocopherols. In addition, the method was applied to monitor the stability of the tocopherols in refined soybean oil with the addition of rosemary, oregano, garlic, annatto seeds and TBHQ (tert-butyl hydroquinone), stored at 25 °C and 35 °C for one year.

2. MATERIALS AND METHODS

2.1. Materials

Refined soybean oil free from antioxidants was donated by Bunge Alimentos, and the spices used in this study were acquired in the local market. Dehydrated rosemary *(Rosemarinus officinalis* L.), oregano *(Origanum vulgare* L.), and annatto seeds *(Bixa orellana* L.) were added at the ratio of 1 g to 900 mL of oil. The garlic *(Allium sativum* L.) *in natura* was added at the ratio of 4 g to 900 mL of oil. The TBHQ (tert-butyl hydroquinone, Sigma) was previously diluted in ethanol and added at the ratio of 0.02% of TBHT to 900 mL of oil.

For the experiments, a control sample (refined soybean oil without antioxidant) and nine soybean oil treatments: (1) rosemary; (2) oregano; (3) garlic; (4) annatto; (5) annatto + rosemary; (6) oregano + annatto; (7) annatto + garlic; (8) mix (spice mixture of rosemary, oregano, garlic and annatto), and (9) TBHQ were prepared.

The soybean oil with the spices or the TBHQ were manually packed in 900 mL PET (polyethylene terephthalate) bottles, placed in closed cardboard boxes and stored in the dark at 25 °C and 35 °C. The tocopherol analyses were performed for seven periods (0, 90, 180, 270, 300 and 360 days) during the 12 months. For each period, one of the 900 mL bottles was used, and the analyses were performed in triplicate.

The analytical standard of the tocopherols (α-, β-, γ- and δ-tocopherol) was obtained from the Calbiochem-Merck (Art. 613424). Each ampoule was diluted in 50 mL of n-hexane, and the concentration was confirmed with the absorption coefficient of each analyte in 95% ethanol (Desai and Machlin, 1985). Only chromatographic grade solvents were used in this study.

2.2. Method

The tocopherol analysis was based on the method described by Firestone (2009). One g of oil was diluted in 10 mL of hexane, filtered through a 0.45 μm PTFE disposable filter disk (Millipore), and injected into a chromatograph equipped with a Rad Pump and a Waters fluorescence detector (excitation at 294 nm and emission at 326 nm). The mobile phase was composed of n-hexane:ethyl acetate:isopropanol (98.6:1.2:0.2, v/v/v), with a flow rate of 1.5 mL min^{-1} in an isocratic system. The separation of compounds was achieved on a normal phase column Lichrospher Si 60 (125 x 4 mm d.i., 5 μm) (Merck), using an external standard for quantification.

The results were statistically evaluated by analysis of variance (ANOVA) and by the S*tudent's t* test with a confidence level of 95%, as described by Snedecor & Cochran (1967). The degradation of the tocopherols during storage was evaluated by the regression analysis according to Neter & Wasserman (1974), with a significance level of 0.05.

2.3. Validation

The method was validated according to the parameters established by Quattrocchi *et al.* (1992) and Codex (2007), considering selectivity, sensitivity, precision and accuracy.

Selectivity: Selectivity was evaluated by the average response of the analytes, in similar concentration ranges, in samples containing the soybean oil and in samples free from the matrix (with glycerin as a vehicle for the tocopherols). The matrix effect was evaluated statistically by the *Student's t* test, as shown in Equations 1 and 2:

$$t = \left|\overline{X1} - \overline{X2}\right| / \sqrt{s^2\left(1/n1 + 1/n2\right)} \quad \textbf{(1)}$$

$$s^2 = \left[\left(n1-1\right)s^2 1 + \left(n2-1\right)s^2 2\right] / \left(n1 + n2 - 2\right) \quad \textbf{(2)}$$

where: $\overline{X1}$ and $\overline{X2}$ represent the mean of the analyte response in the samples containing the matrix and in the samples free from the matrix; s1 and s2 stand for the estimation of the standard deviation for the analyte's response and n1 and n2 are the sizes of samples 1 and 2.

Sensitivity: Sensitivity was measured by the construction of an analytical curve composed of the tocopherols in six concentrations, considering the origin. The concentrations between 0.5 and 1.8 μg mL^{-1} for α-tocol, 1.1 and 3.9 μg mL^{-1} for β-tocol, 1.15 and 3.9 μg mL^{-1} for γ-tocol and 1.1 and 3.6 μg mL^{-1} for δ-tocol were used to assess the linearity. The estimated limits of detection (LQ) and of quantification (LQ) were determined with additional curves, with three concentrations below the lowest analyte concentration, injected in triplicate. The linearity of the analytical curve, the LD and LQ were obtained by applying Equations 3, 4 and 5, respectively.

$$t_r = \frac{|r|\sqrt{(n-2)}}{\sqrt{(1-r^2)}} \quad \textbf{(3)}$$

$$Limit\ of\ detection = \frac{Y_{bl} + 3S_{bl}}{b}\frac{1}{\sqrt{n}} \quad (4)$$

$$Limit\ of\ quantification = \frac{Y_{bl} + 10S_{bl}}{b}\frac{1}{\sqrt{n}} \quad (5)$$

where: t_r, is the *Student's-t* calculated value; *r*, the linear correlation; *n*, the number of measures; Y_{bl}, the estimation of the blank's response (linear coefficient obtained in the equation of the curve concentration area^{-1}); S_{bl}, the estimated standard deviation of the blank (linear coefficient obtained in the equation of the curve concentration s^{-1}); and *b*, the angular coefficient of the analytical curve.

Precision: the system's precision was obtained from five non-consecutive injections of a point on the analytical curve. The method precision was evaluated by seven simultaneous and independent replicates. The coefficient of variation (CV) was evaluated according to Horwitz (1982), as shown in Equation 6:

$$CV\ (\%) = 2^{(1-*0,5\log C)} \quad (6)$$

where C represents the studied concentration expressed as potency of 10 (e.g., 1 mg 1000g^{-1} (ppm) = 10^{-6}, CV = 2^4 = 16%).

Accuracy: Accuracy was evaluated by the recoveries of triplicate samples in three levels of addition. The accuracy was statistically determined by Equation 7.

$$t_{cal} = \left(\left|X - \overline{X}\right| / s\right)\sqrt{n} \quad (7)$$

where: t_{cal}, stands for the *Student's t* calculated value; X corresponds to 100%; $\overline{X}$ is the average recovery; *s*, the estimated standard deviation of the recoveries; and *n*, the number of measures.

2.4. Estimation of uncertainty

By definition, "uncertainty" is a parameter associated with the result of a measurement, which characterizes the dispersion of the values that could reasonably be attributed to the "measurand" (INMETRO, 2003). In the determination of the tocopherols by HPLC-FLU, the result, the concentration of each tocopherol in mg 100 g^{-1}, was provided by Equation 8. Thus, the combined uncertainty (Equation 9) is the square root of the sum of the second power of the uncertainties of the sample dilution, sample area, concentration, and area of the standard and mass of the sample.

$$C_{toc} = \left(\left(Dil_{am} \times A_{am} \times C_P\right) / \left(A_P \times m_{am}\right)\right) \times 100 \quad (8)$$

$$\mu C_{toc} = C_{toc}\sqrt{\left(\mu Dil_{am} / Dil_{am}\right)^2 + \left(\mu A_{am} / A_{am}\right)^2 + \left(\mu C_P / C_P\right)^2 + \left(\mu A_P / A_P\right)^2 + \left(\mu m_{am} / m_{am}\right)^2} \quad (9)$$

where C_{toc} corresponds to the concentration of tocopherol in the sample; *Dil* the sample dilution; A_{am}, the sample area; C_p, the concentration of the standard; A_P, the area of the standard; and *m,* the mass of the sample.

The vitamin E content was calculated as the sum of each tocopherol form divided by the biological conversion factor (Equation 10), and the combined uncertainty was evaluated by Equation 11.

$$C_{Vit\ E} = \left(C_{\alpha\text{-}toc}/0,909\right) + \left(C_{\beta\text{-}toc}/3,333\right) + \left(C_{\gamma\text{-}to}c/6,666\right) + \left(C_{\delta\text{-}toc}/100\right) \quad (10)$$

$$\mu C_{toc} = C_{toc}\sqrt{\left(\mu Dil_{am} / Dil_{am}\right)^2 + \left(\mu A_{am} / A_{am}\right)^2 + \left(\mu C_P / C_P\right)^2 + \left(\mu A_P / A_P\right)^2 + \left(\mu m_{am} / m_{am}\right)^2} \quad (11)$$

where $C_{\alpha\text{-toc}}$, represents the concentration of α-tocopherol; $C_{\beta\text{-toc}}$, the concentration of β-tocopherol; $C_{\gamma\text{-toc}}$, the concentration of γ-tocopherol; and $C_{\delta\text{-toc}}$, the concentration of δ-tocopherol.

3. RESULTS AND DISCUSSION

3.1. Validation

Selectivity: Table 1 shows the data used in the evaluation of the matrix effect. The calculated *t*-values were compared with the *Student's-t* value tabulated with (n1 + n2 – 2) degrees of freedom for a probability of 95% *(t*-tabulated $_{(95\%,\ 4)}$ is equal to 2.78). The results indicate that the matrix does not interfere with the analysis, and that the method provides adequate selectivity.

Sensitivity: Table 2 shows the results obtained in the sensitivity tests. The *Student's-t* value for n – 2 degrees of freedom with 95% probability is 2.78, and the calculated *t*-values for linearity were 40.78 for α-tocol, 49.96 for β-tocol, 55.43 forγ-tocol and 55.43 for δ-tocol, respectively. Based

Table 1
Values used in the assessment of selectivity, matrix effect

α-tocol		β-tocol		γ-tocol		δ-tocol	
n1=3	**n2=3**	**n1=3**	**n2=3**	**n1=3**	**n2=3**	**n1=3**	**n2=3**
$\overline{X1}$ = 2.03	$\overline{X2}$ = 2.22	$\overline{X1}$ = 0.24	$\overline{X2}$ = 0.29	$\overline{X1}$ = 9.66	$\overline{X2}$ = 8.10	$\overline{X1}$ = 3.54	$\overline{X2}$ = 3.30
s1 = 0.21	s2 = 0.12	s1 = 0.04	s2 = 0.03	s1 = 0.77	s2 = 0.35	s1 = 0.22	s2 = 0.15
t_{cal} = 1.01		t_{cal} = 1.29		t_{cal} = 2.38		t_{cal} = 1.16	

n1, sample with the soybean oil matrix, with the addition of tocopherols; n2, sample without the soybean oil matrix and with glycerin; with the addition of tocopherols; n1 and n2, size of the samples 1 and 2; $X1$ and $X2$, analyte average response; s1 and s2, estimated standard deviations of the analyte response; t_{cal}, calculated *Student's t* value.

Table 2
Parameters of linearity, limits of detection (LD) and quantification (LQ) and calculated values of LD and LQ

Data	α-tocol	β-tocol	γ-tocol	δ-tocol
RL: Analytical Curve (μg area^{-1})	Area = 1222872c + 17918	Area = 804317c + 47075	Area= 953366c + 23775	Area = 1039431c + 27057
Correlation coefficient (r^2)	0.9976	0.9984	0.9987	0.9987
RL: LD and LQ curve (μg area^{-1})	Area = 760082c – 70347	Area = 697422c – 89628	Area = 809511c – 106456	Area = 79990c – 110842
RL: LD and LQ curve (μg s^{-1})	Area = –14000c + 9869	Area = 21600c – 6606	Area = 20627c – 3848	Area = 16988c + 95.5
LD (μg mL^{-1})	0.05	0.08	0.2	0.06
LD (mg 100 g^{-1})	0.94	0.08	8.6	1.23
LQ (μg mL^{-1})	0.08	0.11	0.26	0.06
LQ (mg 100 g^{-1})	1.6	0.11	10.5	1.24

RL, linear regression equation; c, concentration; s, estimated standard deviation of the areas obtained from three injections at each concentration.

on these results, the correlation was significant for the evaluated range. Similar values of correlation (0.999), but for different concentration ranges, from 0.04 to 40 μg mL^{-1} and from 1.0 to 20 μg mL^{-1}, were also found by Amaral *et al.* (2005) and Nielsen and Hansen (2008). The detection limits for α- and δ-tocol were lower than the value found by Bonvehi *et al.* (2000) (0.15 μg mL^{-1}). The quantification limits for the same tocopherols were lower than that found by Bonvehi *et al.*, (2000) (0.5 μg mL^{-1}).

Precision: the coefficient of variation found in the tests (Table 3) was lower than that obtained with the Horwitz equation (1982); therefore, both the system and the method were precise. Bonvehi *et al*. (2000) reported a lower coefficient of variation (4%) than that of the present study.

Accuracy: with values obtained in the test (Table 4), the *Student's-t* values were calculated and compared with the *t*-tabulated value with (n – 1) degrees of freedom for a probability of 95% (*t*-tabulated $_{(95\%,\ 8)}$ is equal to 2.31). The calculated *t*-values were smaller to the *t*-tabulated values; therefore, the method presented adequate accuracy with 95% of confidence. The recovery range was similar to those reported by Bonvehi *et al*. (2000) (93%-110%) and Amaral *et al*. (2005) (93%-105%).

3.2. Estimation of the uncertainty

The cause and effect diagram (Figure 1) was used to identify the sources of uncertainty, where the main sources of uncertainty associated with each step of the analytical procedure were related (INMETRO, 2003).

The least squares method was employed for the calculation of the uncertainty of linearity, and taking into consideration $A_j = c_i B_1 + B_0$ as the equation of line and coverage factor $K = 2$ was used in the determination of the expanded uncertainty (INMETRO, 2003).

The calculated expanded uncertainty was 0.29 mg 100g^{-1} for α-tocol, 0.05 mg 100g^{-1} for β-tocol, 1.151 mg 100g^{-1} for γ-tocol, and 0.28 mg 100g^{-1} for δ-tocol. The expanded uncertainty of vitamin E, considering a concentration of 24 IU 100g^{-1}, was 2%.

Table 3
Values found for the system precision and method for determining the tocopherols

Tocol	Precision	Average	s	CV (%)	Max CV (%)
α-tocol	System (n, 5; area)	990158	60186	6	8
	Method (n, 7; mg 100 g^{-1})	9.9	0.6	6	
β-tocol	System (n,5; area)	2010400	173367	9	10
	Method (n, 7; mg 100 g^{-1})	2.11	0.03	1	
γ-tocol	System (n,5; area)	2229550	142274	6	6
	Method (n, 7; mg 100 g^{-1})	59.72	1.34	2	
δ-tocol	System (n,5; area)	2464446	101624	4	8
	Method (n, 7; mg 100 g^{-1})	15.12	0.50	3	

n, number of replicates; s, estimated standard deviation; CV, coefficient of variation; CV Max, coefficient of variation obtained with the Horwitz equation.

Table 4
Values used in the evaluation of the accuracy

Tocol	Added (mg 100 g^{-1})	Recovered (mg 100 g^{-1})	s	Recovery (%)	s	CV (%)	*t*-calculated
α-tocol	2.2	1.95	0.11	93	6	7	0.33
	4.5	4.1	0.4				
	6.6	6.6	0.1				
β-tocol	0.22	0.24	0.04	103	8	8	1.17
	0.45	0.42	0.02				
	0.7	0.741	0.5				
γ-tocol	8.5	9.7	0.8	102	14	14	0.52
	16.8	17.9	0.4				
	25.2	21.8	0.5				
δ-tocol	3.43	3.54	0.23	97	10	10	0.95
	6.9	7.04	0.64				
	10.2	8.7	0.9				

s, estimated standard deviation; *t, Student's-t value.*

3.3. Stability of the tocopherols

Figures 2 and 3 show the values of tocopherols in the soybean oil with and without the addition of spices and TBHQ stored at 25 °C and 35 °C for 12 months, respectively. The tocopherol contents converted to the International Unit (IU) ranged from 20 to 30 IU 100g^{-1} at 25 °C and from 18 to 30 IU 100g^{-1} at 35 °C.

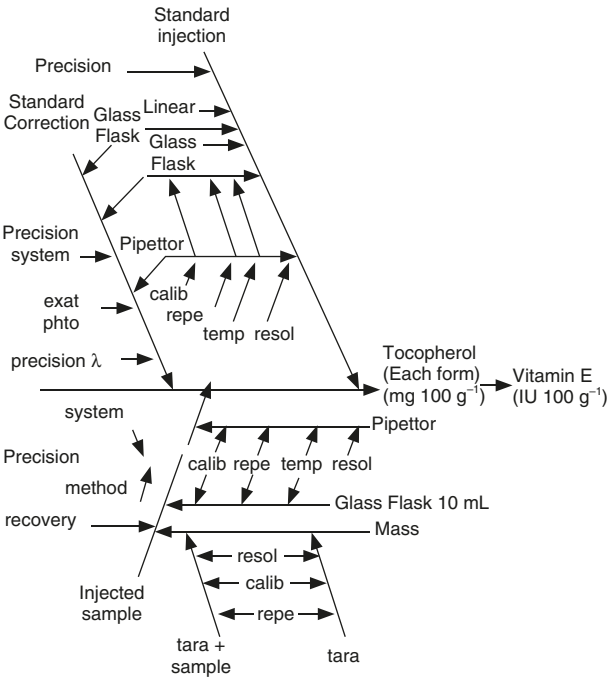

Figure 1
Cause and effect diagram of the data used for the calculation of uncertainty in the determination of tocopherols by HPLC-FLU in soybean oil. Linear, linearity; calib, calibration; repe, repetitivity; temp, temperature; resol, resolution; exat photom, photometric accuracy.

The relative concentration of the tocopherols found in soybean oil was 15% of α-tocol, 2% of β-tocol, 66% of γ-tocol, and 17% of δ-tocol. According to Evans *et al*. (2002), the tocopherol concentration in commercial oil depends on the steps and conditions used to convert the crude oil into an edible product; however, the individual relative concentration is not significantly changed. The relative concentration of the individual tocopherol is from 2 to 13% of α-tocol, from 1 to 5% of β-tocol, from 54 to 71% of γ-tocol and from 21 to 41% of δ-tocol (Evans *et al.,* 2002; El-Mallah *et al.*, 2006; Carrão-Panizzi and Erhan, 2007). The individual relative value found for the α-tocol (15%) and for the δ-tocol (17%) remained within the range reported by Schimidt and Pokorný (2005) (from 0.9 to 35% of α-tocol and 15 to 93% of δ-tocol).

The γ- and δ-tocopherols were the most stable tocopherols during storage and among the evaluated treatments (Table 5). The same behavior was observed by Yang *et al.* (2005) for the δ-tocol with and without the addition of TBHQ.

The analysis of variance of the tocopherol concentrations in the treatments throughout the study, both at 25 °C and 35 °C, presented no significant differences at the 95% confidence level. Yang *et al.* (2005) reported similar results for the α-tocol stored at 25 °C. Over time, a greater variance was observed within the groups, especially in the control oil, for the α-tocol (17.93 ± 0.35 mg 100g^{-1}) and the γ-tocol (71.69 ± 1.25 mg 100g^{-1}) during 180 days (Figures 2 and 3).

The *Student's t* test identified significant differences with a 95% confidence level in the rosemary and garlic treatments stored at 25 °C for

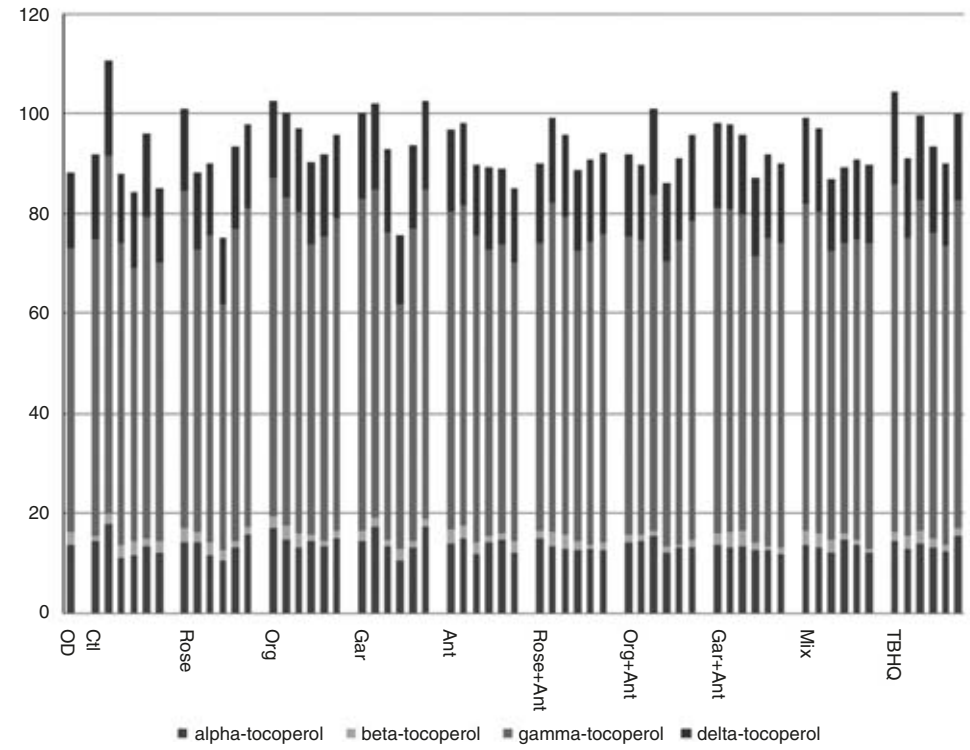

Figure 2
Average results (mg 100 g^{-1}) obtained in the evaluate treatments and periods during the storage at 25 °C. Each column corresponds to an evaluated period (90, 180, 270, 300, 330 and 360 days). Day zero, corresponds to the tocopherol contents in the soybean oil used in this study. 0 D, day zero; Ctl, control; Rose, Rosemary; Org, Oregano, Gar, Garlic; Ant, Annatto; Mix, spice mixture of rosemary, oregano, garlic and annatto; TBHQ, tert-butyl hydroquinone .

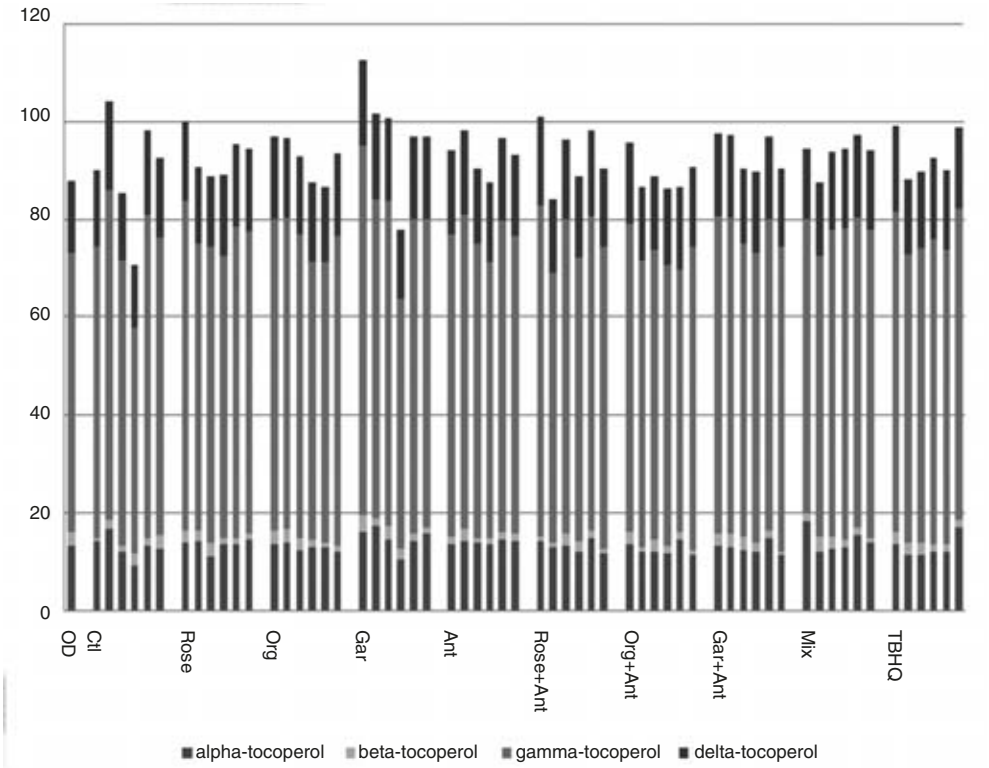

Figure 3
Average results (mg 100 g^{-1}) obtained in the evaluate treatments and periods during the storage at 35 °C. Each column corresponds to an evaluated period (90, 180, 270, 300, 330 and 360 days). Day zero, corresponds to the tocopherol contents in the soybean oil used in this study. 0 D, day zero; Ctl, control; Rose, Rosemary; Org, Oregano, Gar, Garlic; Ant, Annatto; Mix, spice mixture of rosemary, oregano, garlic and annatto; TBHQ, tert-butyl hydroquinone .

Table 5
Regressions obtained for all the treatments according to storage

Treatments		Storage at 25 °C				Storage at 35 °C			
		A	B	r^2	F	A	B	r^2	F
Control	α-tocol	–8E-03	15.09	0.1879	1.16	–7E-03	14.80	0.1848	1.13
	β-tocol	9E-04	1.94	0.0302	0.16	9E-04	1.75	0.021	0.11
	γ-tocol	–4E-04	60.96	0.0	3.55E-04	0.0	59.33	0.0	1.03E-07
	δ-tocol	–2.5E-03	16.51	0.0357	0.18	0.0	15.53	0.0	5.44E-05
Rosemary	α-tocol	–1.7E-03	13.63	0.0174	0.09	–1E-04	13.71	0.0002	9.05E-04
	β-tocol	–3.4E-03	2.94	0.5089	5.18	–4E-03	2.87	0.6269	8.40
	γ-tocol	–1.9E-03	60.10	0.0018	8.85E-03	2.4E03	60.35	0.0077	0.04
	δ-tocol	7E04	15.29	0.0049	0.02	3.2E-03	15.2	0.1884	1.16
Oregano	α-tocol	–2.3E-03	14.96	0.0546	0.29	–3.2E-03	13.92	0.5012	5.02
	β-tocol	–3.9E-03	2.96	04201	3.62	–4.8E-03	3.12	0.6275	8.42
	γ-tocol	1E-04	62.37	0.0	2.09E-05	3E-04	60.43	0.0002	0.03
	δ-tocol	3.6E-03	15.42	0.3641	2.86	1.6E-03	15.77	0.0863	0.47
Garlic	α-tocol	–2E-04	14.27	0.0002	8.51E-04	–2.1E-03	15.21	0.0173	0.09
	β-tocol	–3.3E-03	2.57	0.6128	7.91	–3.9E-03	3.09	0.4434	3.98
	γ-tocol	6E-05	61.22	0.0	8.77E-06	–9.9E-03	65.37	0.0301	0.16
	δ-tocol	1.8E-03	16.01	0.0259	0.13	1.1E-03	16.11	0.013	0.07
Annatto	α-tocol	–2.2E-03	14.08	0.0534	0.28	–5E-04	13.96	0.0071	0.04
	β-tocol	–2.9E-03	2.84	0.3659	2.89	–3.3E-03	2.41	0.5304	5.65
	γ-tocol	–1E-02	61.37	0.1696	1.02	5E-03	59.51	0.0494	0.26
	δ-tocol	–1.7E-03	15.97	0.0518	0.27	1.7E-03	15.99	0.0694	0.37
Rosemary + Annatto	α-tocol	–4.6E-03	14.22	0.5835	7.01	–2.4E-03	13.93	0.0841	0.46
	β-tocol	–2.7E-03	2.63	0.2483	1.65	–1.4E-03	1.96	0.0536	0.28
	γ-tocol	1E-02	58.45	0.1708	1.03	6.2E-03	59.86	0.0328	0.17
	δ-tocol	3E-03	15.61	0.3680	2.91	1.8E-03	15.865	0.0429	0.22
Oregano + Annatto	α-tocol	–2.4E-03	14.22	0.0833	0.45	–3.1E-03	13.81	0.1301	0.75
	β-tocol	–3.6E-03	2.26	0.6321	8.59	–3.6E-03	2.61	0.4153	3.55
	γ-tocol	1.4E-02	57.52	0.2595	1.75	6.8E-03	58.57	0.1108	0.62
	δ-tocol	2.1E-03	15.30	0.1128	2,90	2.1E-03	15.30	0.1128	0.64
Garlic + Annatto	α-tocol	–4.5E-03	13.89	0.6580	9.62	–2.1E-03	13.54	0.0608	0.32
	β-tocol	–4.2E-03	3.01	0.3780	3.04	–4.5E-03	3.11	0.5825	6.97
	γ-tocol	1.3E-03	61.24	0.0027	0.01	4.9E-03	60.56	0.0474	0.25
	δ-tocol	8E-04	15.98	0.0235	0.12	1.5E-03	15.89	0.0597	0.32
Mix	α-tocol	–2E-03	13.67	0.0848	0.46	–2E-03	13.67	0.0848	0.19
	β-tocol	–5E-03	3.151	0.6095	7.80	–3.2E-03	2.66	0.3000	2.14
	γ-tocol	–2.9E-03	61.22	0.0129	0.07	1.8E-02	57.04	0.7579	15.65
	δ-tocol	–9E-04	16.02	0.0166	0.08	6.3E-03	14.4	0.7601	15.84
TBHQ	α-tocol	–3.4E-03	13.99	0.4082	3.45	–4.5E-03	13.42	0.4995	4.99
	β-tocol	–3.1E-03	2.71	0.4492	4.08	–3.6E-03	2.93	0.7260	13.24
	γ-tocol	6.7E-03	61.37	0.0412	0.21	7E-03	59.59	0.1024	0.57
	δ-tocol	–9E-04	16.52	0.0048	0.02	2.3E-03	15.72	0.1319	0.76

A, angular coefficient; B, linear coefficient; r^2, correlation coefficient; F, Snedecor's F distribution; critical F (5%, 1.5) = 6.61; Mix, mixture of rosemary, oregano, garlic and annatto; TBHQ, tert-butyl hydroquinone.

300 days (Figure 2), for the α-tocol (10.49 ± 0.16 mg 100 g^{-1} for rosemary and 10.59 ± 0.13 mg 100 g^{-1} for garlic) and γ-tocol (49.17 ± 0.72 mg 100g^{-1} for rosemary and 49.07 ± 0.57 mg 100g^{-1} for garlic), and for the same tocopherols (9.53 ± 0.13 mg 100 g^{-1} for α-tocol and 46.20 ± 0.12 mg 100 g^{-1} for γ-tocol) in the control oil stored at 35 °C for 300 days (Figure 3).

The levels of of γ- and δ-tocol increased during the storage at 35 °C in the oil containing the spice mixture (rosemary, oregano, garlic and annatto) (Table 5). According to Kadoma *et al.* (2006), flavonoids can regenerate tocopherols depending on the chemical structure (monomethyl, dimethyl, or trimethyl tocol), favoring the least sterically hindered monomethyl tocol). The spice mixture added to the oil contains considerable levels of flavonoids (Shan *et al.,* 2005; Suhaj, 2006; Wojdylo *et al.,* 2007), which could have migrated to the oil during the storage at 35 °C.

The α-tocol content decreased during the storage at 25 °C in the oils containing rosemary plus annatto and garlic plus annatto (Table 5), and the β-tocol content decreased during the storage at 25 °C the oils containing the oregano plus annatto and the spice mixture (rosemary, oregano, garlic and annatto), and at 35 °C in the oil containing garlic plus annatto. The decrease in contents of α- and β-tocol could have been favored by the steric effect of the tocopherol structures (Kadoma *et al.*, 2006), and by the protection and regeneration of the carotenoids from annatto (Haila, 1999).

The β-tocol showed the greatest instability and degradation for both the treatments and the storage temperatures evaluated (Table 5).

4. CONCLUSIONS

The validated analytical method used to assess the tocopherol contents in soybean oil showed an expanded uncertainty of 2%, for the four tocopherols. The greatest variability in the contents of tocopherols in the oils, with or without spices and TBHQ, was observed within each treatment. β-tocopherol showed the highest instability and reduction, between the initial and final levels, in the treatments containing garlic, oregano plus annatto, and in the spice mixture (rosemary, oregano, garlic and annatto seed) stored at 25 °C, and in the ones containing rosemary, oregano, and garlic plus annatto, stored at 35 °C. The addition of the spice mixture increased the concentrations of γ- and δ-tocopherol in the oil during the storage at 35 °C.

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