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Analytical Methods

Optimization conditions of samples saponification for tocopherol analysis

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ABSTRACT

A full factorial design 2^2 (two factors at two levels) with duplicates was performed to investigate the influence of the factors agitation time (2 and 4 h) and the percentage of KOH (60% and 80% w/v) in the saponification of samples for the determination of α, β and γ + δ-tocopherols. The study used samples of peanuts (cultivar armadillo), produced and marketed in Maringá, PR. The factors % KOH and agitation time were significant, and an increase in their values contributed negatively to the responses. The interaction effect was not significant for the response δ-tocopherol, and the contribution of this effect to the other responses was positive, but less than 10%. The ANOVA and response surfaces analysis showed that the most efficient saponification procedure was obtained using a 60% (w/v) solution of KOH and with an agitation time of 2 h.

1. Introduction

Vitamins are organic substances found in many foods in low amounts and they are essential for functioning of the body. Most animal organisms do not synthesize vitamins, therefore the acquisition of these nutrients is required through the diet, in micro quantities, depending on age, gender, physiological state and the physical activity level of the individual (da Paixão & Stamford, 2004). The lack of vitamin intake may result in deficiencies in growth and development and other organic disorders (Delgado-Zamarreño, Sanchez-Perez, Sanchez-Rodriguez, Gomes-Perez, & Hernadez-Mendez, 1996; da Paixão & Stamford, 2004; Kienen, Costa, Visentainer, Souza, & Oliveira, 2008).

Vitamin E belongs to the group of fat-soluble vitamins, and comprises eight basic components existing in nature: four tocopherols and four tocotrienols, which are identified by the prefixes α, β, γ and δ. These compounds have different vitamin E activities and are found only in plants. α-Tocopherol is the most biologically active form of vitamin E (Yada, Lapsley, & Huang, 2011).

Due to its lipophilic characteristic, vitamin E is closely associated with lipids in foods (Delgado-Zamarreño, Bustamante-Rangel, Sánchez-Pérez, & Carabias-Martínez, 2004), and protects unsaturated lipids from oxidation, preserving them in biological systems and in foods (Delgado-Zamarreño, Bustamante-Rangel, Sánchez-Pérez, & Hernández-Méndez, 2001; Lavedrine, Ravel, Poupard, & Alary, 1997; Taipina, Lamardo, Rodas, & Del Mastro, 2009). Lipid oxidation is related to the appearance of an unpleasant taste (rancidity) in food (Lavedrine et al., 1997).

Another important factor is that, being potent antioxidants, tocopherols may reduce the risk of heart disease by inhibiting the oxidation of LDL cholesterol (Yang, 2009), and help to reduce the risk of certain chronic diseases such as type 2 diabetes and cancer (Köksal, Artik, Simsek, & Günes, 2006). Protection against tumors in different parts of the body can occur via inhibition of cell proliferation (Yang, 2009). The consumption of vitamin E may also combat some of the negative effects associated with aging and protect against cognitive decline and Alzheimer's disease (Köksal et al., 2006).

The main problem in the determination of vitamin E in complex samples such as food is the low concentration of this analyte. Furthermore, it is necessary to perform isolation of the vitamin before analysis (Delgado-Zamarreño et al., 2001). This procedure generally involves alkaline hydrolysis of lipid material (saponification), followed by extraction of vitamin E from the unsaponifiable material using an effective organic solvent (Delgado-Zamarreño et al., 2001, 2004). The extract is injected into a chromatograph for

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separation and the determination of analytes (Delgado-Zamarreño et al., 2001).

Additionally, there are some variations in the methodology of saponification in the literature. Taking as an example agitation time and the percentage of KOH, Delgado-Zamarreño et al. (2004) used 2 h and 80% (w/v), respectively, while Kienen et al. (2008) used 60% (w/v) KOH and agitation overnight (about 12 h). The aim of this work was to investigate the influence of two factors, agitation time and percentage of KOH, in the saponification procedure for the determination of tocopherols in foods, using chemometrics to optimize the analytical methodology.

2. Materials and methods

2.1. Sampling

The peanut samples (cultivar armadillo) used in this study were produced in the region of Maringá, PR and purchased at the local market.

Approximately 100 g of the raw samples (with peels) were ground in a food processor (Philips–Walita) until complete homogenization. The samples were vacuum packaged, protected from light and frozen until analysis.

2.2. Vitamin E analysis

Samples were saponified and the isomers of vitamin E were extracted according to the methods described by Delgado-Zamarreño et al. (2001). Two factors were tested: the agitation time (2 or 4 h) and the percentage of KOH (60% or 80% w/v) in the saponification procedure. Under agitation, 50.0 mL of ethanol, 5.0 mL of an aqueous solution of ascorbic acid (10%, w/v), 10 mL of aqueous potassium hydroxide (variable percentage (w/v) according to the factorial design) and 25 mL of water were added to 2.000 g of the ground sample and protected from light.

Then, the analytes were extracted in a separatory funnel with hexane (2 × 25 mL) and the extracts were washed with water (2 × 10 mL). The organic phase was removed by evaporation in a rotary evaporator under vacuum at 50 °C and the residue was dissolved in methanol.

The tocopherols were determined by high efficiency liquid chromatography (Varian) using a C18 column (Microsorb, 250 mm × 4.6 mm, 5 μm particles). The mobile phase used was methanol/dichloromethane in a 85:15 (v/v) ratio; the flow rate was 0.8 mL min^{-1} (Kornsteiner, Wagner, & Elmadfa, 2006). The tocopherols were quantified using the external standard method, according to Instituto Adolfo Lutz. Normas analíticas do Instituto Adolfo Lutz. IV-Métodos químicos e físicos para análise de alimentos (2005). The sum of β-tocopherol and γ-tocopherol isomers is given since the separation of these is not possible by this methodology (Kornsteiner et al., 2006).

Table 1
Factors investigated and the levels used for the development of the 2^2 full factorial design with duplicates.

Factors	Unit	Symbol	Type	Levels	
				−1	+1
KOH	%	A	Numeric	60	80
Agitation time	Hours	B	Numeric	2	4

Table 3
2^2 Full factorial planning (in duplicate) and the responses obtained in the assays (mg 100 g^{-1} of sample).

Assays	Independent variables Levels		Responses		
	KOH (%)	Time (h)	α-Tocopherol	(β + γ)-Tocopherol	δ-Tocopherol
1	60	2	1.99	1.61	0.76
2	60	2	2.03	1.57	0.75
3	80	2	1.33	1.20	0.71
4	80	2	1.32	1.18	0.70
5	60	4	1.17	1.12	0.71
6	60	4	1.15	1.11	0.71
7	80	4	0.94	0.97	0.67
8	80	4	0.95	0.97	0.66

Table 4
Main and interaction effects, calculated for the 2^2 factorial design shown in Table 3, and the three responses studied.

Response	Effects		
	A = KOH	*B* = time	*A* × *B*
α-Tocopherol	−0.45	−0.62	0.24
(β + γ)-Tocopherol	−0.27	−0.35	0.13
δ-Tocopherol	−0.048	−0.043	2.500×10^{-3}

Table 5
ANOVA results for the responses studied in the 2^2 factorial model.

Source	Degree of freedom	Sum of square	Mean square	*F* test	*P*
Response 1 = α-Tocopherol					
Regression	3	1.27	0.42	1541.70	<0.0001
A = KOH	1	0.41	0.41	1472.73	<0.0001
B = time	1	0.76	0.76	2750.73	<0.0001
A × *B*	1	0.11	0.11	401.64	<0.0001
Pure error	4	1.100×10^{-3}	2.750×10^{-3}	–	–
Total	7	1.27	–	–	–
Response 2 = (β + γ)-Tocopherol					
Regression	3	0.42	0.14	536.56	<0.0001
A = KOH	1	0.15	0.15	565.76	<0.0001
B = time	1	0.24	0.24	920.05	<0.0001
A × *B*	1	0.033	0.033	123.86	0.0004
Pure error	4	1.05×10^{-3}	2.625×10^{-4}	–	–
Total	7	0.42	–	–	–
Response 3 = δ-Tocopherol					
Regression	3	8.137×10^{-3}	2.712×10^{-3}	72.33	0.0006
A = KOH	1	4.513×10^{-3}	4.513×10^{-3}	120.33	0.0004
B = time	1	3.613×10^{-3}	3.613×10^{-3}	96.33	0.0006
A × *B*	1	1.250×10^{-5}	1.250×10^{-5}	0.33	0.5946
Pure error	4	1.500×10^{-4}	3.750×10^{-5}	–	–
Total	7	8.287×10^{-3}	–	–	–

Table 2
Mathematical equations for all the responses by applying the response surface model.

Response	Equation	R^2
α-Tocopherol	α-Tocopherol = 1.36–0.23 * KOH − 0.31 * *T* + 0.12 * KOH * *T*	0.9991
(β + γ)-Tocopherol	(β + γ)-Tocopherol = 1.22–0.14 * KOH − 0.17 * *T* + 0.064 * KOH * *T*	0.9975
δ-Tocopherol	δ-Tocopherol = 0.71–0.024 * KOH − 0.021 * *T* + $1.250.10^{-3}$ * KOH * *T*	0.9819

% of KOH = x_1; *T*, agitation time = x_2.

2.3. Experimental design

A 2^2 full factorial design (two factors at two levels) with duplicates was performed to investigate the influence of two factors on the saponification of samples for the determination of tocopherols. The factors were agitation time in the saponification procedure (2 and 4 h) and percentage of KOH (60% and 80%), as shown in Table 1. The responses used were the content of α, β and γ + δ-tocopherols.

2.4. Statistical analysis

Initially, the values of the main effects, interaction and analysis of variance (ANOVA) were obtained. Thereafter, all variables had their normality and homogeneity of variance assessed by residual plots. Then, analysis of variance (two-way ANOVA between groups) was performed for all the responses. To evaluate the effect of independent variables on the responses, response surface methodology (RSM) was applied. The basic model equation used to fit the data was:

$$E(y) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \quad (1)$$

where $E(y)$ is the expected response, β_0 is a constant, β_1, β_2, β_{11}, β_{22} and β_{12} are the regression coefficients and x_1, x_2 are the levels of independent variables (Granato, Bigaski, Castro, & Masson, 2010). The equations for each model along with their coefficients of correlation (R^2) are listed in Table 2.

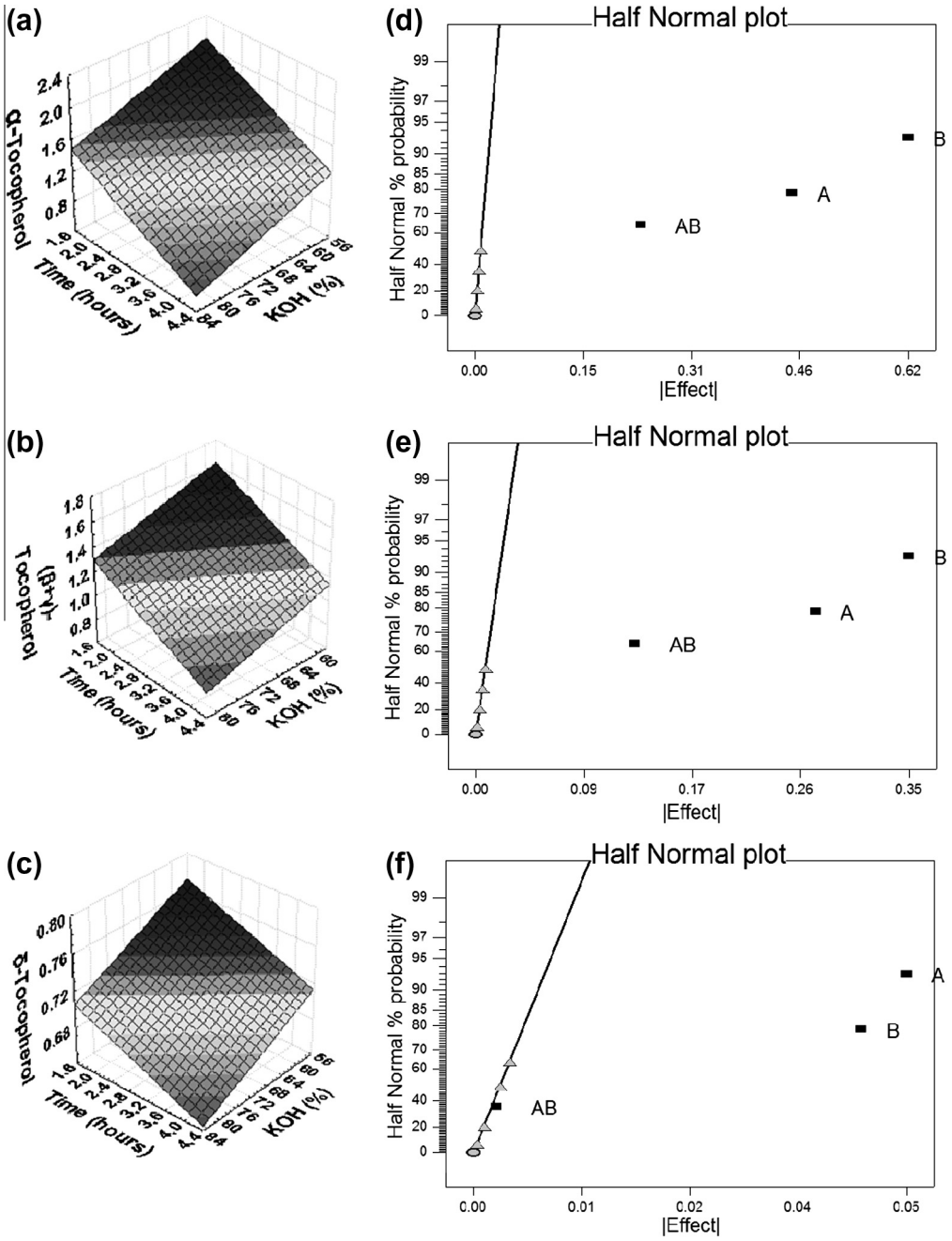

Fig. 1. Surface response to the amount of (a) α-tocopherol, (b) (β + γ)-tocopherol and (c) δ-tocopherol depending on the percentage of KOH and the agitation time. Half normal probability for the effects of the 2^2 complete factorial design variables presented in Tables 1 and 3 for the responses (d) α-tocopherol, (e) (β + γ)-tocopherol and (f) δ-tocopherol.

3. Results and discussion

Table 3 shows the conditions of the factorial model 2^2 design applied to the experiments, in duplicate, and the values obtained for all the responses studied: α, (β + γ) and δ-tocopherols (mg 100 g^{-1} of sample).

The graphs of the residuals for each response indicated that the data exhibited normality and homogeneity of variance in a very satisfactory way, showing that all models were significant, and showing no significant lack of fit. The coefficients of regression (R^2) and the *F* value for each model, obtained by ANOVA and shown in Tables 2 and 5, respectively, also indicate the positive significance of the models.

The data belonging to independent variables and the responses were analyzed to obtain the linear regression equations (Table 2), as well as the values of each main effect and interaction between these effects, as well as the percentages of the contribution of these effects to the model, using ANOVA.

Table 4 shows the values of the main effects and interactions for all the responses, and Table 5 presents the results obtained by ANOVA for the 2^2 full planning in duplicate for each of the studied responses. Fig. 1 shows the graphs of the effects for all responses analyzed using the factorial design. Response surfaces were constructed for levels and independent variables, as shown in Fig. 1.

The ANOVA results, shown in Table 5, indicate that the main factors were significant for all responses, and that the interaction between the main factors was not significant for the response δ-tocopherol. This result can be confirmed by Fig. 1, which shows the point of the interaction effect for the response δ-tocopherol (c), right next to the normal line, where the points have no significance.

Fig. 1(d and e) and Table 4 make it evident that the factor that most contributed to the responses α-tocopherol and (β + γ)-tocopherol was the agitation time, because the points corresponding to this main effect, for both responses, are further away from the normal line in Fig. 1(d and e), and are further from zero (Table 4).

According to the data provided by the ANOVA table (Table 5), the contribution percentage of the variable agitation time for the responses α-tocopherol and (β + γ)-tocopherol was 59.4% and 57.0%, respectively. The percentage of KOH was the second most important factor for these two responses, with a contribution of 31.8% for α-tocopherol and 35.1% for (β + γ)-tocopherol. The interaction effect had the least influence, and ANOVA table (Table 5) indicated that there was a positive but small (less than 10%) contribution of this effect to increase the values of the responses.

Fig. 1(f) and Table 4 show that the variable KOH had a greater influence on the values of δ-tocopherol, but the values of the effect for this variable were close to the values presented for the variable agitation time. According to the data provided by ANOVA (Table 5) for this response, there was a contribution of 54.5% of the factor KOH and 43.6% of the factor agitation time. Additionally, the ANOVA indicated a contribution of 0.15% of the KOH X agitation time interaction. Although this result did not significantly influence the response δ-tocopherol, this coefficient allowed improved "straightening" of the linear model; if it was removed, the *R*-squared value was affected.

The response surfaces arranged in Fig. 1(a–c) and Table 4 showed that the increase in agitation time from 2 to 4 h and the increase in the percentage (w/v) of KOH solution, from 60% to 80%, caused a reduction in the values of all responses. Thus, increasing the agitation time and KOH concentration negatively influenced the quantification of all tocopherol isomers.

Accordingly, the response surfaces (Fig. 1a–c), obtained by the 2^2 factorial design indicated that the minimum values of KOH% (w/v) and agitation time provided more efficient quantification of all tocopherol isomers.

4. Conclusion

A factorial design carried out to optimize the methodology for the saponification of samples for tocopherol analysis. We concluded that the factors % KOH and agitation time were significant, and an increase in these factors contributed negatively to all the responses. The interaction effect was not significant for the response δ-tocopherol, and the contribution of this effect to the other responses was positive, but less than 10%. The ANOVA and response surface analysis showed that the most efficient saponification procedure was obtained using a 60% (w/v) solution of KOH and an agitation time of 2 h.

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