Trans-resveratrol Extraction in Four Brazilian *Arachis hypogea* L. Cultivars with Microwave-Assisted Extraction: Optimization with Response Surface Methodology and Comparison with Conventional Maceration

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Authors' contributions

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ABSTRACT

Peanut (*Arachis hypogaea* L.) is the fourth most consumed oleaginous in the world, producing highly energetic seeds, rich in lipids, proteins, vitamins and carbohydrates. Several bioactive constituents and pharmacological activities have already been observed in extracts of roots, leaves and seeds, including resveratrol. In this work, we report a study of two extraction methodologies,

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conventional maceration and microwave-assisted extraction (MAE) for the extraction of *trans*-resveratrol. Parameters that affect the efficiency of microwave-assisted extraction of *trans*-resveratrol from aerial parts of *A. hypogaea* were optimized adopting the response surface methodology mathematical experimental designs. The contents of different organs of four Brazilian important cultivars (IAC 886, IAC Caiapó, Tatu ST IAC, and IAC 8112) were evaluated by HPLC. Microwave-assisted extraction, with the use of 37 mL of solvent per gram of dry tissue, 1200 g agitation for 15 minutes at 37°C, proved to be more efficient than conventional maceration extraction. Root extracts from IAC Tatu prepared with this methodology showed the highest *trans*-resveratrol content (1.371 ± 0.07 mg/g extract) among the cultivars tested.

1. INTRODUCTION

Groundnut (*Arachis hypogaea* L., Leguminosae) is the fourth most planted and consumed oilseed worldwide, and also a great option for crop rotation [1]. Several parts of the peanut plant, such as seeds [2], skin [3], roots [4], and leaves [5] have already been studied in terms of their chemical constituents.

The most widely investigated secondary metabolite in peanut extracts is *trans*-resveratrol (3,4',5-trihydroxystilbene), a polyphenolic compound initially classified as a stilbene phytoalexin [6], since it was recognized as an active compound against plant pathogens. *Trans*-resveratrol can be found in grapes [7], eucalyptus [8], pine [9], and in a vast number of other plant species. It is associated with many health benefits, such as reducing the risk of atherosclerosis [10], as well as antioxidant [11], anti-inflammatory [12], and cancer preventive activities [13].

Previous studies focusing on the quantification of *trans*-resveratrol from different peanut cultivars have applied distinct extraction techniques with different results according to methodology, type of tissue and genotype (Table 1).

In the conventional maceration extraction (CME), plant material is placed in contact with the extractor solvent, preferably in a closed vessel. Although it is a widely used method because it is easy and cheap, spending on organic solvents is higher than in other methods [28]. In addition, the total extraction time depends on the periods determined for incubation with the solvent, centrifugation and evaporation [29].

An alternative to CME is the microwave-assisted extraction methodology (MAE). Microwaves are located between the radio-frequency range at the lower frequency and infrared at the higher frequency in the electromagnetic spectrum, between the range of 300 MHz to 300 GHz. The extraction is based on the interaction of the energy of electrical field with compounds of the material [30]. The main advantages of microwave-assisted extraction over the conventional maceration are reduced solvent consumption, shorter operational times, high recoveries, good reproducibility and minimal sample manipulations [31].

In the present study, the influence of variables that influence the efficiency of microwave-assisted extraction of *trans*-resveratrol from aerial parts of *A. hypogaea* was evaluated, adopting the response surface methodology design (RSM). RSM is a statistical tool for developing and optimizing processes with one or more responses influenced by several independent variables. An advantage of RSM is that it allows the user to collect large amounts of information from a small number of experiments. It also enables the observation of effects of individual variables and their interactions on the response. A comparative study of the *trans*-resveratrol content in four Brazilian cultivars of extreme importance due to their nutritional characteristics and resistance to climatic factors, was then performed using both MAE and CME.

2. MATERIALS AND METHODS

2.1 Plant Material

Aerial parts, roots, and seeds of four Brazilian peanut cultivars were used in this study: IAC 886 (Virginia Runner), IAC Caiapó (Virginia Runner), IAC Tatu ST (Valência) and IAC 8112 (Spanish). Plants were grown from seeds in a greenhouse of the Rio de Janeiro State University, Brazil (latitude of -22.91, longitude of -43, 23 and altitude of 20 m) at spring, with averages

Table 1. Extraction of resveratrol from peanut using different techniques

Cultivar	Material	Extraction technique	Maximum resveratrol amount	Reference
Twenty accessions	Seeds	Maceration	1.626 µg/g of fresh material	[2]
Tainan 9, Tainan 11, Tainan 12	Roots	Maceration	0.905 ± 0.311 mg/g of extract	[4]
(non-specified)	Leaves, pods, seeds, seed coats, shells and roots	Maceration	2.8 µg/g of fresh roots	[5]
Georgia green medium runner	Sliced seeds	Sonication	4.73 ± 1.20 µg/g of dry weight	[13]
NC-9 (Virginia), Forunner (Runner), SunOleic 95R (Runner), Starr (Spanish)	Seeds	Maceration	1.792 ± 0.616 µg/g of fresh material	[14]
(non-specified)	Leaves, roots, shell, seed coat and seeds	Maceration	2.6 µg/g of fresh shells	[15]
Tainan 14	Callus	Maceration	11.97 ± 0.64 µg/g of fresh material	[16]
Georgia green medium runners	Seeds	Maceration	3.96 ± 0.96 µg/g of fresh material	[17]
Tainan Selected 9, Tainan 11, Tainan 14	Seeds	Maceration	47.1 ± 8.9 µg/g of dry material	[18]
Tainan 14 (Spanish)	Seeds	Maceration	147.3 ± 14.0 µg/g of extract	[19]
Georgia Green (Runner), Virugard (Runner), GT-101 (Valencia)	Cotyledons, embryo, epicotyl/plumule, hypocotyl, testa, root, root tip and mucilage	Maceration	1.93 ± 0.78 µg/g of dry material	[20]
Runners	Seeds	Stirring, Sonication, Soxtec, Microwave, Sonication	0.1998 mg/g of fresh seeds	[21]
Georgia green medium runner	Seeds	Maceration	4.40 µg/g of dry material	[22]
(non-specified)	Roots	Maceration	1.7 mg/g of dry weight	[23]
C34 24 (Runner)	Seeds	Sonication	0.640 µg/g of dry weight	[24]
Tainan 14 (Spanish)	Callus	Maceration	16.35 µg/g of fresh weight	[25]
Twenty cultivars (Runner and Valencia)	Seeds	Maceration	2.0 µg/g of fresh weight	[26]
Caiapó (Virginia Runner)	Leaves	Maceration	687.5 ± 229.0 µg/g of fresh weight	[27]

temperature and rainfall of 27°C and 90 mm, respectively. They were also grown in natural conditions, with no fertilization practices or pest and disease management. Pots containing a mixture of Plantmax® and vermiculite (v/v, 2:1) were used and light intensity in a clear day during the growing period was as high as 1600 $\mu E/(m^2/s)$. Aerial parts and roots were taken for extraction 30 days after planting.

2.2 Calibration Curve

Trans-Resveratrol was quantified at 307 nm by reference to the peak area of an external authentic standard (Sigma®) (Fig. 1). Data were expressed in mg resveratrol/g extract.

2.3 Microwave-Assisted Extraction (MAE)

The extraction procedure was carried out in a microwave reactor. A closed-vessel single-mode microwave system (Monowave™ 300; Anton Paar GmbH, Graz, Austria), in a Pyrex® vessel (10 mL capacity) was used. The reaction was performed at fixed pressure (6 bar) and time (30 min). The equipment was operated in a temperature control mode, internally measured by a ruby thermometer. Briefly, triturated dried

materials in different solvent proportions were placed in bottles inside the reactor and extracted with ethanol. Then, the material was filtered, evaporated, weighed and stored at -4°C for later HPLC-DAD analysis. The yield of the extracts was calculated according to the following formula:

Yield (%) = Amount extract obtained x 100 / fresh weight.

2.4 Experimental Design

The preliminary experiments of MAE were performed with aerial parts of cultivar IAC Caiapó. A fractional factorial design (2^{3-1} FFD) was used to analyze the response pattern, and establish the new ranges to be adopted in the next set of experiments. The three independent variables selected were temperature (30, 50 and 70°C), solvent/solid ratio (40, 50 and 60 mL/g), and stirring (600, 900 and 1200 g), while the dependent variable was the resveratrol amount. At this step, seven experiments were conducted in order to identify which variables influence the extraction conditions. After that, a central composite rotatable design (2^3 CCRD) was employed to obtain the optimum conditions for the variables. The same variables were used, but with different levels due to the necessity of axial points: 30, 34, 40, 46 and 50°C for temperature; 30, 34, 40, 46 and 50 mL/g for solvent/solid ratio, and 900, 961, 1050, 1139 and 1200 g, for stirring. The variables temperature, solvent/solid ratio, stirring and time were used for the comparison of the efficiency of resveratrol extraction from aerial parts, roots and seeds of the four cultivars selected for this study.

2.5 Conventional Maceration Extraction

Aerial parts, roots and seeds were dried at 45°C untill constant weight, crushed, weighed and macerated in ethanol (50 mL/g), stirred in the dark at room temperature. After filtration on filter paper and using a rotary evaporator at 45°C, the extract was weighed and stored at temperatures between -10°C and -4°C until used.

2.6 HPLC-DAD Analysis

Extracts were centrifuged (10000g for 1 minute), and the supernatant was subjected to HPLC analysis in a Dionex® equipment, Ultimate 3000 model, with a *Diode Array* detector and a C18 column (Dionex Bonded Silica Products, 5 μm, 120 Å, with 4,6 x 250 mm). The mobile phase consisted of (A) distilled water, (B) methanol and (C) acetonitrile. All solvents were purchased from Tedia Co. The flow rate was 1.0 mL/min with the following gradient elution protocol: 90% A, 8% B and 2% C for 2 minutes; 70% A, 8% B and 22% C for 8 minutes; and 5% A and 95% C for 8 minutes, totalizing 18 minutes. The temperature was adjusted to 30°C and the analyses were monitored with a diode array detector at 317 nm absorbance.
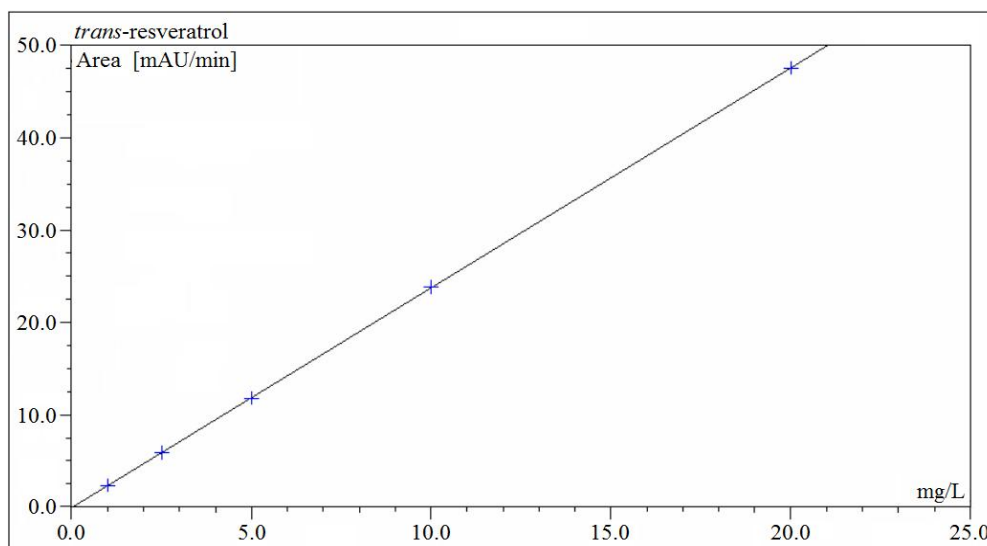

Fig. 1. Standard curve of *trans*-resveratrol

2.7 Statistical Analysis

The experimental designs and results analysis were carried out using the *software* Statistica 6.0 (Statsoft, Inc., USA), according with the significance level established to obtain the mathematical model. The significance of the regression coefficients and the associated probabilities were determined by Student's *t* test. The model equation significance was determined by Fisher's *F* test. The variance explained by the model is given by the multiple determination coefficients, R^2. For the reaction kinetics, and the comparison of the extraction efficiency among the cultivars, statistical analysis was carried out by repeated measure ANOVA followed by post hoc Tukey test performed using the same software. Mean separations were done using Tukey's test at $p \leq 0.05$.

3. RESULTS

3.1 Microwave-assisted Extraction (MAE)

3.1.1 Initial evaluation of MAE extraction parameters using fractional factorial design (FFD)

In order to analyze the effect of temperature, solvent/solid ratio and stirring, the reaction time was fixed in 30 minutes [32]. The efficiency of *trans*-resveratrol extraction was evaluated in seven experiments carried out according to a fractional factorial design 2^{3-1}. The variables with the respective levels of FFD are presented in Table 2, and the experimental design with the corresponding results is shown in Table 3.

The estimated effects of the tested variables are shown in Table 4. Temperature and solvent/solid ratio displayed negative values within the range studied. An increase in temperature from 30°C to 60°C, as well as increased volumes of solvent in relation to the amount of material, caused a significant decrease in *trans*-resveratrol

extraction (Table 3). On the other hand, increased stirring resulted in an improvement of the extraction efficiency (Tables 3 and 4). Therefore, the highest amount of *trans*-resveratrol (0.6105 mg/g) was observed on entry 1, where the lowest temperature and solvent/solid ratio, as well as the highest stirring, were adopted.

3.1.2 Central composite rotatable design (CCRD)

Considering that all studied variables had statistical significance in the process ($p < 0.05$) (Table 4), a central composite rotatable design (CCRD) was further applied in order to determine their optimal values, maximizing the extraction rates. The analysis of data from Table 3 led to the conclusion that curvature in the model by including axial points presenting $p < 0.05$ was required. The variables, along with their coded and uncoded values, are given in Table 5.

Table 2. Real and coded values (+ higher level, 0 intermediate, − lower level) for the independent variables, 2^{3-1}

Variables	-1	0	+1
Temperature (ºC)	30	50	70
Solvent/solid ratio (mL/g)	40	50	60
Stirring (g)	600	900	1200

In the CCRD, the three selected parameters were varied at five levels, resulting in 17 experiments that included eight factorial points and three central points in order to check the curvature (Table 6). To fit a second order model, six extra points with the same distance from the central point were added at the matrix for this design. The results obtained for the efficiency of extraction of *trans*-resveratrol are presented in Table 6, showing that the best results were achieved with high levels of stirring, but with intermediate levels of temperature and solvent/solid ratio.

Table 3. Experimental factorial design and results of FFD for the extraction of resveratrol

Entry	Variable levels			*Trans*-resveratrol content
	T (ºC)	*S* (mL/g)	*St* (g)	(mg/g extract)
1	-1 (30)	-1 (30)	+1 (1200)	0.6105
2	+1 (70)	-1 (30)	-1 (600)	0.1814
3	-1 (30)	+1 (70)	-1 (600)	0.0687
4	+1 (70)	+1 (70)	+1 (1200)	0.0877
5	0 (50)	0 (50)	0 (900)	0.0579
6	0 (50)	0 (50)	0 (900)	0.0577
7	0 (50)	0 (50)	0 (900)	0.0612

Entries 4 and 5 (Table 6) show a clear negative effect of temperature and solvent/solid ratio, since when decreasing the levels of these variables, the *trans*-resveratrol extraction was increased from 0.617 to 1.009 mg/g. A positive effect of stirring was also evident in the same experiment.

The experimental data were adjusted to the proposed model and adequacy was performed by the analysis of variance and parameter R^2. Statistical testing of the model was done by the Fisher's statistical test for ANOVA. The following equation represents the mathematical model of the extraction of *trans*-resveratrol in function of the variables.

$$Y = 0.802 - 0.059T - 0.086T^2 + 0.030S - 0.056S^2 + 0.196St - 0.134TSt - 0.122P.St$$

Where, *Y* is the percentage yield extraction, and *T*, *S* and *St* are the uncoded values of temperature, solvent/solid ratio, and stirring, respectively.

Table 7 represents the analysis of variance (ANOVA), which shows the validity of the model by F test and residue that represents the magnitude of experimental error. The calculated *F* (8.48) was higher than the tabulated *F* (3.29), showing the validity of the experimental model, considering the determination coefficient (R^2). The determination coefficient ($R^2 = 0.87$) implies that the sample variation of 87% for the efficiency of *trans*-resveratrol extraction is attributed to the independent variables and can accurately be explained by the model.

The analysis of the interaction between the variables is showed in Figs. 2, 3 and 4.

Table 4. Estimated effect for variables studied for resveratrol extraction

Variable	Effect	*p*-Value
Mean	0.802576	0.000269
Curvature check	- 0.356367	0.000075
Temperature (*T*)	- 0.086329	0.006157
Solvent/solid ratio (*S*)	- 0.056613	0.014145
Stirring (*St*)	0.196407	0.000990

Optimized MAE conditions (37ºC, 37 mL/g and 1200 g) were chosen for the comparative analyses between the four chosen cultivars.

Table 5. Real and coded values (+ level, 0 intermediate, - lower level) for the independent variables, 2^3

Variable	-1,68	-1	0	+1	+1,68
Temperature (ºC)	30	34	40	46	50
Solvente/solid ratio (mL/g)	30	34	40	46	50
Stirring (g)	900	961	1050	1139	1200

3.2 Conventional Maceration Extraction (CME)

The maximum *trans*-resveratrol amount obtained by CME was 1.214±0.36 mg/g extract in roots of IAC Tatu ST, while the minimum was found in defatted seed extracts of IAC Caiapó (0.125±0.09 mg/g). The highest amount in aerial parts (0.862±0.06 mg/g) was found in IAC Tatu ST (Table 8).

Table 6. Experimental factorial design and results of CCRD for resveratrol extraction

Entry	Variable levels			*Trans*-resveratrol content (mg/g extract)
	T (ºC)	*S* (mL/g)	*St* (g)	
1	-1 (34)	-1 (34)	-1 (961)	0.142
2	+1 (46)	-1 (34)	-1 (961)	0.226
3	-1 (34)	+1 (46)	-1 (961)	0.428
4	+1 (46)	+1 (46)	-1 (961)	0.617
5	-1 (34)	-1 (34)	+1 (1139)	1.009
6	+1 (46)	+1 (46)	+1 (1139)	0.721
7	-1 (34)	+1 (46)	+1 (1139)	0.970
8	+1 (46)	+1 (46)	+1 (1139)	0.456
9	-1.68 (30)	0 (40)	0 (1050)	0.745
10	+1.68 (50)	0 (40)	0 (1050)	0.581
11	0 (40)	-1.68 (30)	0 (1050)	0.735
12	0 (40)	+1.68 (50)	0 (1050)	0.760
13	0 (40)	0 (40)	-1.68 (900)	0.588
14	0 (40)	0 (40)	+1.68 (1200)	1.145
15	0 (40)	0 (40)	0 (1050)	0.791
16	0 (40)	0 (40)	0 (1050)	0.768
17	0 (40)	0 (40)	0 (1050)	0.813

Table 7. Variance analysis for validation of mathematical models (ANOVA)

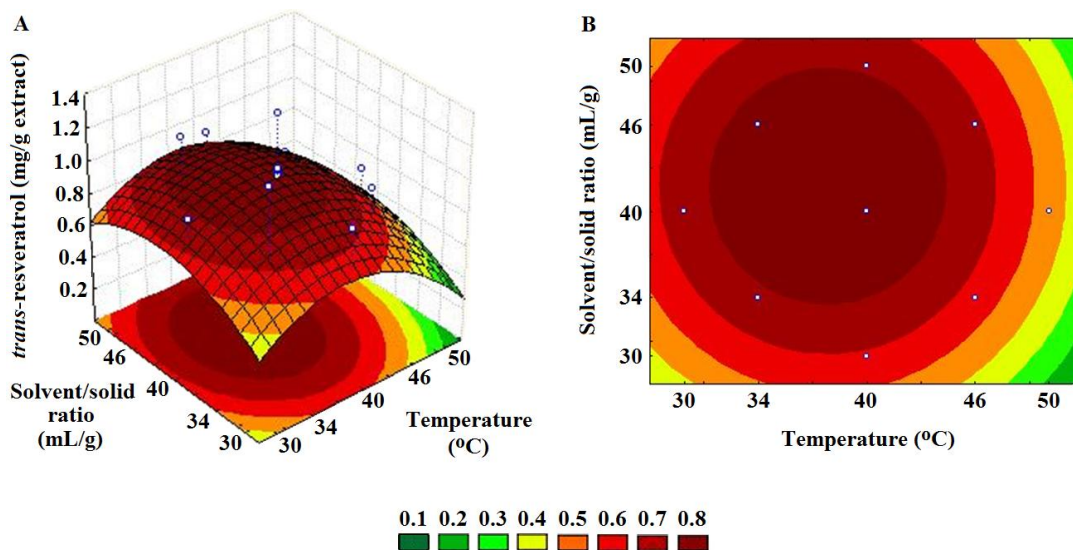| Factor	Sum of squares	Degrees of freedom	Mean square	*F* calculated	*F* tabulated	*p*-Value
Regression	0.9500	7	0.1356	8.4783	3.2927	0.0024
Residuals	0.1400	9	0.0159			
Lack of fit	0.1430	7				
Pure error	0.0010	2				
TOTAL	1.0935	16				

[a] *Confidence level 95%.*

Fig. 2. Response surface (a) and contour curves (b) for the *trans*-resveratrol extraction in function of solvent/solid ratio and temperature
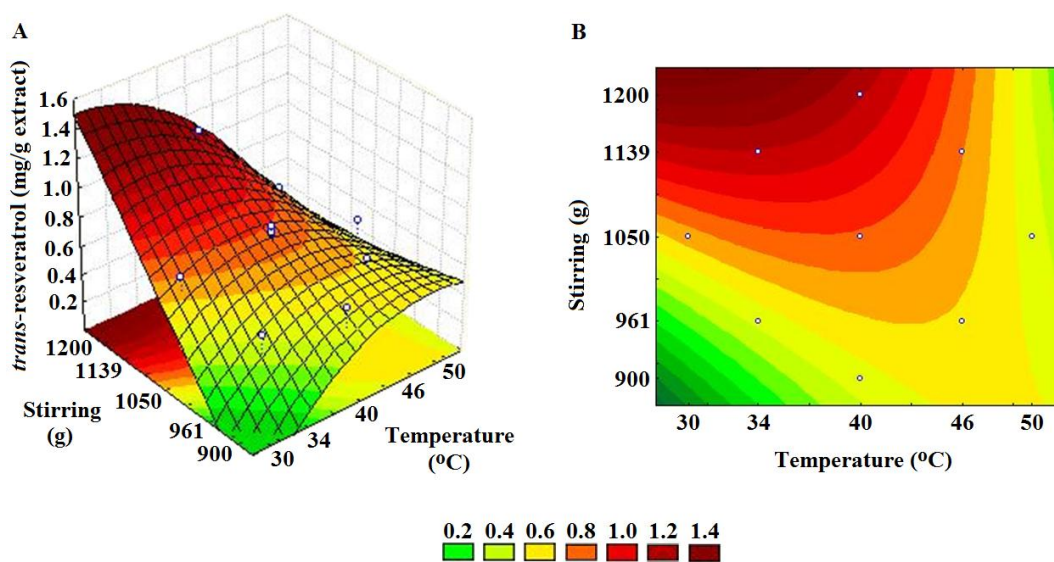

Fig. 3. Response surface (A) and contour curves (B) for the *trans*-resveratrol extraction in function of stirring and temperature
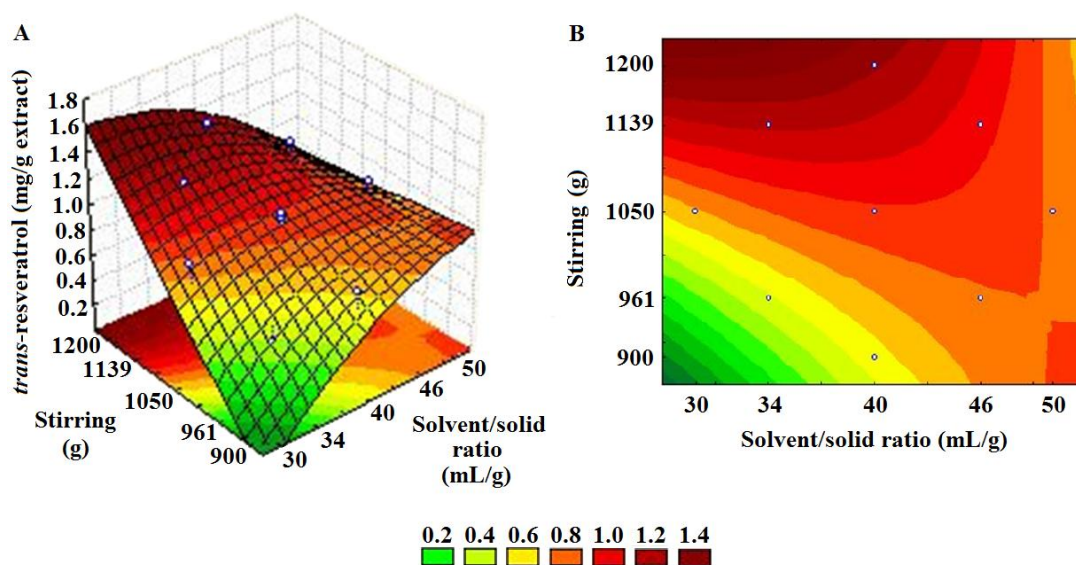
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Fig. 4. Response surface (A) and contour curves (B) for the *trans*-resveratrol extraction in function of stirring and solid/solvent ratio

3.3 Comparison of Trans-resveratrol Amounts among Cultivars

Resveratrol was detected by HPLC-DAD, adopting a calibration curve with the standard. The HPLC chromatograms of standard *trans*-resveratrol and of a root extract are shown in Fig. 5.

In the comparison between extraction methods, MAE generally showed better results than CME. MAE extracts from aerial parts and roots of IAC 8112 showed almost three times more *trans*-resveratrol than those obtained with CME. The positive effect of MAE in extract preparation was also observed in seeds of IAC Caiapó and IAC 8112, once that MAE showed three times more *trans*-resveratrol than CME. Root extracts prepared with MAE showed the highest resveratrol amounts, followed by aerial parts and seed extracts. For example, IAC 886 root extracts showed more than five times more *trans*-resveratrol than seed extracts. Considering the same material in each cultivar, IAC Tatu ST aerial part extracts showed more *trans*-resveratrol than IAC 886 and IAC 8112. IAC Tatu ST and IAC 8112 seed extracts showed more *trans*-resveratrol than IAC 886.

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4. DISCUSSION

The choice of the appropriate extraction methodology is a crucial element for the study of plant secondary metabolites. In this work, we compared the amount of *trans*-resveratrol in extracts of four peanut cultivars obtained with CME or MAE. In general, the use of MAE resulted in higher extraction efficiency from the tested materials. The extractive capacity of MAE is determined by the increased probability of intermolecular shocks due to successive changes in orientation of polar molecules, with the possibility to control factors that influence the extraction efficiency, such as agitation, temperature, pressure, and time. The efficiency of MAE process is directly related to the

operation conditions elected. Special attention should be given to solvent composition, solvent-to-solid ratio, extraction temperature and time, microwave power, and characteristics of the matrix, including its water content [33-34].

In order to optimize the reaction conditions and to produce maximum extraction of *trans*-resveratrol with MAE, a response surface methodology (RSM) was adopted in this study. The RSM was first used in 1951 [35], and currently many workers use this approach for the optimization of extraction parameters of different compounds from several species. These include, for example, polysaccharides from *Lilium davidii* [36], isoflavonoids from *Dalbergia odorifera* [37], and anthocyanins and phenolic acids from sour cherry Marasca [38].
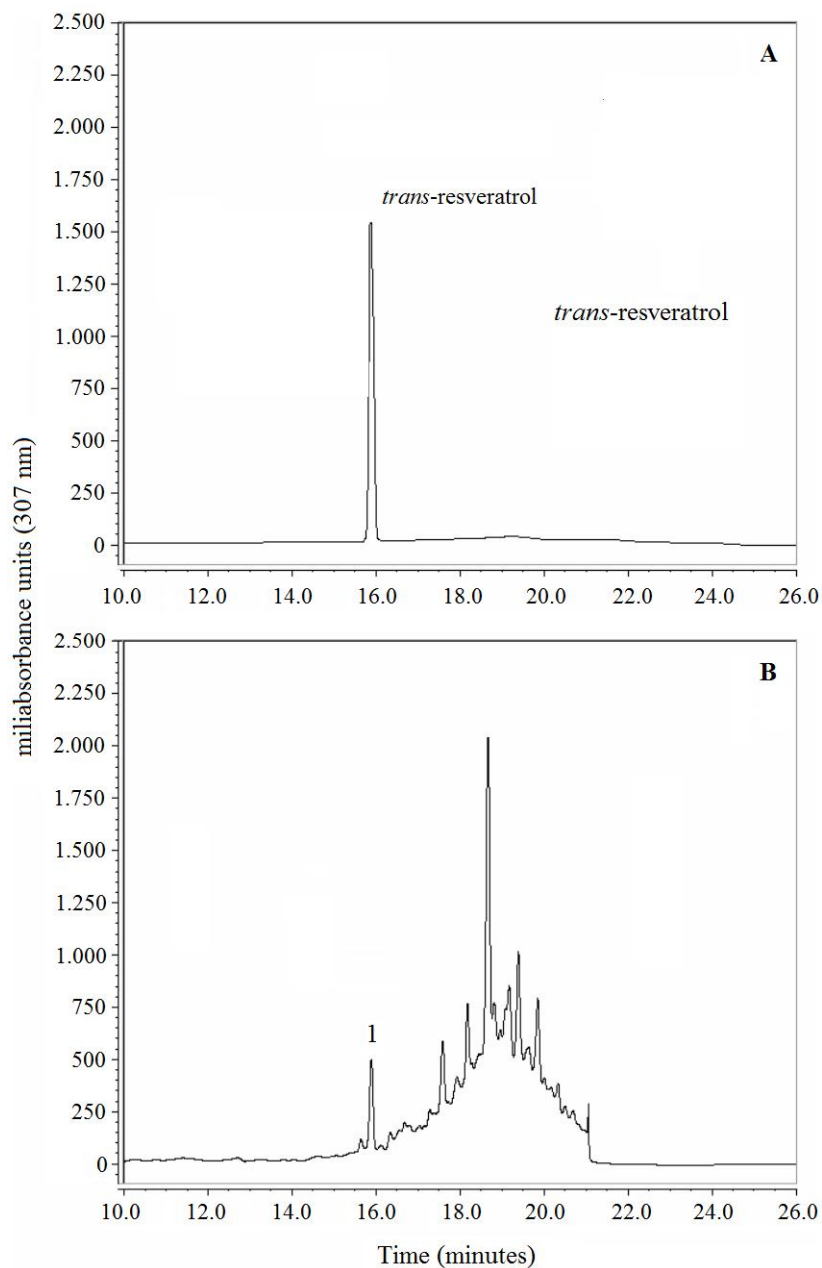

Fig. 5. HPLC chromatogram of *trans*-resveratrol standard (A) and root extract of *A. hypogaea* IAC Tatu ST (B) showing trans-resveratrol (peak 1) identified by its retention time at 307 nm.

Table 8. Comparison of concentrations of resveratrol extracted from aerial parts, roots and seeds of fours cultivars of peanut (*A. hypogaea*) using conventional maceration extraction (CME) and microwave-assisted extraction (MAE) (37ºC, 37 mL/g and 1200 g)

Cultivar	Plant material	CME		MAE	
		Yield* (%)	Resveratrol content (mg/g extract)	Yield* (%)	Resveratrol content (mg/g extract)
IAC 886	Aerial part	1,26	0,299 ± 0,05 [ef]	1,66	0,739 ± 0,03 [c]
	Root	2,09	0,536 ± 0,29 [cde]	2,98	1,228 ± 0,10 [a]
	Seed	1,03	0,212 ± 0,05 [f]	1,22	0,229 ± 0,08 [f]
IAC Caiapó	Aerial part	1,31	0,409 ± 0,16 [de]	1,72	0,929 ± 0,15 [bc]
	Root	1,84	0,418 ± 0,07 [e]	2,29	1,188 ± 0,10 [ab]
	Seed	0,97	0,125 ± 0,09 [g]	1,18	0,318 ± 0,07 [ef]
IAC Tatu ST	Aerial part	1,35	0,862 ± 0,06 [bc]	1,79	1,074 ± 0,10 [b]
	Root	2,87	1,214 ± 0,36 [ab]	3,38	1,371 ± 0,07 [a]
	Seed	1,01	0,417 ± 0,02 [e]	1,21	0,345 ± 0,04 [e]
IAC 8112	Aerial part	1,20	0,249 ± 0,06 [f]	1,69	0,739 ± 0,04 [c]
	Root	2,26	0,432 ± 0,09 [de]	2,73	1,281 ± 0,03 [a]
	Seed	0,95	0,278 ± 0,10 [fg]	1,18	0,386 ± 0,07 [e]

* Amount of extract obtained x 100 / fresh weight
Data represent mean ± standard deviation. The same letters indicate that means are not statistically different according to Tukey test.

In our work, increasing of temperature had a negative effect on the extraction efficiency, probably due to degradation of *trans*-resveratrol from the middle point. Higher values of solvent/solid ratio showed a similar variation in the amount of resveratrol extracted, probably as a result of inadequate agitation. This is an important result, which shows that just the complete immersion of the tissue in the solvent is sufficient to provide high extraction efficiency. Additionally, excessive quantities of extracting solvent also require more energy and longer extraction time [39]. High stirring values provided a better yield, probably by enhancing desorption and dissolution of active compounds bound to the sample matrix [40].

In the reaction kinetic study, the extraction efficiency increased up to 15 min, where it remained constant. Similar optimal periods of time were found in studies on the optimization of the extraction of flavonolignans [41] and anthocyanins [42].

In relation to the comparison between the different materials of the four cultivars (Table 8), root extracts IAC Tatu ST showed high amounts of *trans*-resveratrol (1.371 ± 0,07 mg/g of extract). The yields found in our work were higher than others that reported up to 0.905 ± 0.311 mg of *trans*-resveratrol per gram of root extracts of *A. hypogaea* [4]. Root extracts of cultivar IAC Tatu ST prepared with MAE presented a much higher amount of *trans*-resveratrol (46.3 µg per gram of fresh weight)

than other ones found using CME (2.8 µg/g of fresh roots) [5].

Trans-resveratrol amounts found in the present study are also comparable to some data reported for grapes and wine, two major sources of this stilbene. For example, up to 39.5 µg of *trans*-resveratrol per gram of fresh weight was found in Mexican wild grapevine leaf extracts [43], which was less than our results with MAE extracts from IAC Tatu ST roots. We can also assume that the extract of aerial parts of this cultivar, at the concentration of 6 g/mL, would contain 8.2 µg of *trans*-resveratrol/mL, a value higher than the ones found in some types of red wine [44].

5. CONCLUSION

In conclusion, we have compared CME and MAE for *trans*-resveratrol extraction from roots, aerial parts and seeds of four Brazilian peanut cultivars. MAE proved to be more effective than CME. Root extracts showed higher amounts of *trans*-resveratrol, while seed extracts displayed lower amounts. Some differences between the cultivars were also observed. For the microwave-assisted extraction, the response surface methodology allowed to establish the best conditions for efficient extraction.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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