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Optimization of Microwave-Assisted Extraction of a Bioherbicide from *Canavalia ensiformis* Leaves

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Abstract: The objective of this study was to optimize the operational conditions of the Microwave-Assisted Extraction (MAE) of *Canavalia ensiformis* leaves by Response Surface Methodology (RSM), based on analysis of phenolic compounds obtained from the aqueous extracts. Optimal conditions for MAE were temperature of 100°C, extraction time of 10 min and ramp of temperature of 5 min, using approximately 2 g of dry plant material and 30 mL of water. The extracts obtained were determined by HPLC-UV. Among the phenolic compounds present in plant leaves, ferulic acid was selected as reference in all analyzes of the extracts. The results showed that the developed model was able to describe the effects and the relationship between them in the performance of the extraction, taking full advantage of all the limitations of the equipment.

Keywords: Bioherbicide, Microwave-Assisted Extraction, Optimization, *Canavalia ensiformis*, Response Surface Methodology

Introduction

Phenolic compounds are a group of aromatic secondary metabolites present in plants, widely distributed in several parts of the plants, with known biological effects such as: Antioxidant capacity (Proestos *et al*., 2005), antimicrobial activity and fungal and pathogenic bacteria inhibition (Rauha *et al*., 2000).

The improvement of techniques and methodologies that minimize the use of synthetic pesticides, solvents and the generation of toxic wastes is of fundamental importance when one aims at working in compliance with green chemistry principles. The use of Microwave-Assisted Extraction (MAE), in comparison to the currently employed thermal methods, allows for the use less reagents and organic solvents, reducing the use of toxic chemicals.

Currently extraction processes for polyphenols are time- and solvent-consuming, as well as inefficient. Many hours of exposure to heat in an extraction process critically increase the degradation level of phenolic compounds and decrease the antioxidant activity of extracts (Lapornik *et al*., 2005). Nowadays, MAE is much faster and fully automated than conventional techniques such as Soxhlet extraction (Liazid *et al*., 2007).

MAE is a process that uses the microwaves energy for rapidly and efficiently heat solvents (Jain *et al*., 2009). The plant matrix already contains a significant amount of water that strongly absorbs the microwaves energy (overheating), causing disruption of cells and making the extraction process easier. In addition, the migration of dissolved ions increases solvent penetration into the matrix and, therefore, the extraction yield (Wang and Weller, 2006). The main advantages of MAE over conventional extraction techniques are reduced solvent use, shorter extraction time, moderate recoveries, reproducible (CV = 0.54%) and minimal sample manipulation (Armenta *et al*., 2008). The application of microwaves for extracting phenolic compounds present in plants requires considerable optimization of the operational parameters such as temperature, time and microwave power.

In this study, an optimized procedure for the microwave-assisted extraction of phenolic compounds from jack-bean leaves (*Canavalia ensiformis*) is proposed, in order to produce an economically viable bioherbicide.

Materials and Methods

Chemicals and Equipment

Leaves of *Canavalia ensiformis* were collected from mature plants, dried at 50°C, crushed manually and sieved (2 mm) before use in the extraction tests.

Ferulic and glacial acetic acid were purchased from Sigma-Aldrich (Germany) and methanol (HPLC grade) from PANREAC. Stock solutions of all reference materials were prepared at a concentration of 50 mg L^{-1}. Working solutions were prepared by diluting the stock solutions with methanol and were stored at (4±2) °C.

For the microwave-assisted extraction, a Microwave Digestion System (SPEEDWAVE FOUR®, with Built-in Non-Contact, Temperature and Pressure Measurement, BERGHOF, Germany) with a capacity of 12 bottles of 60 mL and 1450 W maximum power.

Microwave-Assisted Extraction

Two grams of each dried and sieved samples was extracted with 30 mL (0.067 g mL^{-1}) of deionized water. The extraction temperature ranged from 60 to 100°C, the tested irradiation times were from 10 to 40 min, using a power of 870 W (60%).

After the extraction steps, the vials were cooled until room temperature and then opened. Then, the material was filtered in 45 μm filter paper and an aliquot of 1 mL was diluted in 6 mL of methanol prior to chromatography analyses. Experimental runs were defined according to preliminary experiments and to equipment and programming conditions.

Experimental Design

In this study, a 2^3 factorial design was randomly performed comprising 16 experiments in duplicate plus 7 additional experiments, also in duplicate. Factors were: (1) Extraction temperature, (2) irradiation time and (3) ramp (time required for the equipment to reach the set extraction temperature). The response-variable was the area of ferulic acid peak. A two-way interaction linear model was adjusted to the experimental data (Equation 1), where $\hat{y}$ is the predicted value (the yield of the extracted compound, ferulic acid), β's are the model coefficients and x_1,x_2,x_3 the encoded factors. The Statistica 12 and Origin 8.0 software were used for analyzing the experimental data:

$$\hat{y} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (1)$$

HPLC Analyses

The extracted ferulic acid from *Canavalia ensiformis* leaves was determined in a HPLC system (Prominence LC-10AT, Shimadzu) equipped with a UV/Vis detector (SPD-Prominence 20 A) fixed at 282 nm and a degasser (DGU-20A5 Prominence). A reverse phase column (150×4.6 mm ×5 μm) Agilent XDB-C18 was used for separation purposes. The mobile phase was 2% glacial acetic acid in water + methanol (75:25 in volume) pumped at 1.2 mL min^{-1}. The Lab Solutions software (Shimadzu) was used for system control and data processing. The injection volume was 20 μL.

Ferulic acid standard solutions were prepared in methanol (100 mg L^{-1}) and diluted in various concentrations from 1.0 to 25 mg L^{-1}. All determinations were made in triplicate for each concentration level. The amount of compound present in the extracts was calculated in mg g^{-1}, based on the peak areas and by using the calibration curve constructed with the reference material (R^2 = 0.999).

Results and Discussion

Factorial Design Performed

Many factors affect the efficiency of MAE. Some of these are: Microwaves power, extraction temperature, extraction time, solvent type and solvent ratio (Hayat *et al*., 2009).

Before the extraction optimization, preliminary experiments were performed to assess the behavior of the microwaves power, ramp and extraction time, in accordance with the limitations on the equipment. The minimum microwaves power capable of reaching the preset maximum temperature was 60%. Water was the sole extractant, due to safety and (eco) toxicological issues.

The actual (and encoded) levels of the factors (temperature, time and ramp) for each experiment as well as the response-variable (concentration mg g^{-1} of ferulic acid) obtained are shown in Table 1.

Estimated Model

A polynomial model was adjusted to the experimental data (Equation 2) with an R^2 = 0.9992 (R^2_{adj} = 0.9987). The corresponding Analysis of Variance (ANOVA) data are shown in Table 2:

$$\hat{y} = 42.526 + 14.389x_1 - 6.582x_2 + 9.647x_3 - 11.155x_1x_2 - 559x_1x_2 - 1.281x_2x_3 \quad (2)$$

Where:

$$x_1 = \frac{temperature - 80}{20}; x_2 = \frac{time - 25}{15}; ex_3 = \frac{ramp - 3.5}{1,5}$$

When the F-value is greater than the critical F-value, the estimated model is statistically significant. Moreover, if the F-value is ten or more times greater than the critical F-value, the model is predictive (Bruns *et al*., 2006). As the calculated F-value is more than 550 times greater than the critical F-value (3.374, 95% confidence interval), this is indeed the case here.

Table 1. Experimental design: Actual (and encoded) levels of the factors and observed responses

Factors			
(1) Temperature (°C)	(2) Time (min)	(3) Ramp (min)	Concentration (mg g^{-1}) *
60 (-1)	10 (-1)	2 (-1)	14.56
60 (-1)	10 (-1)	5 (+1)	51.90
60 (-1)	40 (+1)	2 (-1)	39.51
60 (-1)	40 (+1)	5 (+1)	60.48
100 (+1)	10 (-1)	2 (-1)	84.10
100 (+1)	10 (-1)	5 (+1)	108.25
100 (+1)	40 (+1)	2 (-1)	49.17
100 (+1)	40 (+1)	5 (+1)	65.03

*Mean values (duplicate).

Table 2. Analysis of Variance (ANOVA) for the experimental results

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F-value
Regression	7,517,006,285	6	1,252,834,381	1876
Residues	6,008,909	9	667,657	
Total	7,523,015,194	15		

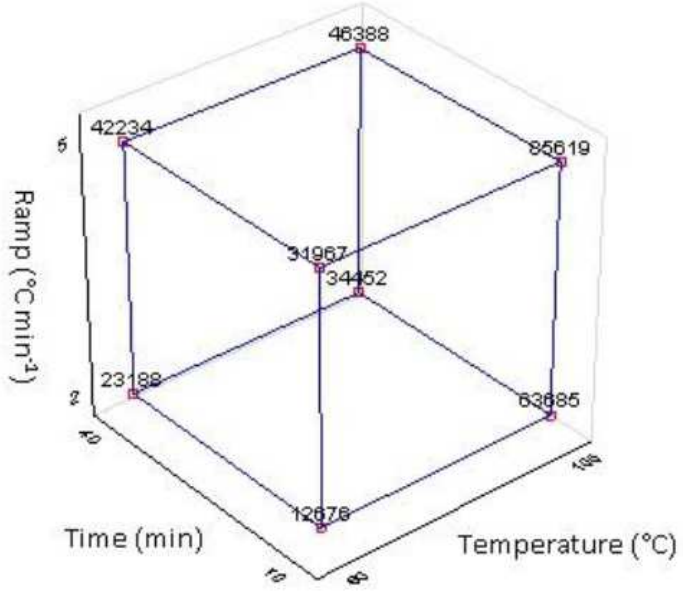


Fig. 1. Cube of responses (mean values)

According to Fig. 1, which presents the cube of obtained responses, the extraction yield is improved by increasing the temperature and ramp (the time in which the microwave oven reaches the set extraction temperature) and decreasing the extraction temperature.

Response Surface Plot Analysis

The effects of the independent variables (factors) and their mutual interactions on the extraction yield can also be observed in three dimensional response surface plots (Hayat *et al*., 2009). Figure 2a to 2c show the main interactions of the process: Time × ramp, temperature × ramp and temperature × time.

Response surface plots are very useful when one wants to preview the effects of two factors on the response, as well as the identification of optimal values, for maximum response (Karabegović *et al*., 2013). Graphs were plotted using the z-axis (response) against two of the independent variables, while keeping the third one at a fixed value. Figure 2a shows how the interaction between ramp and time affects the peak area obtained for ferulic acid. Increasing the ramp from 3 to 5 min and decreasing the extraction time from 30 to 10 min, increased the extraction yield. Figure 2b shows the interaction between temperature and ramp. Increasing the ramp time from 3 to 5 min and the

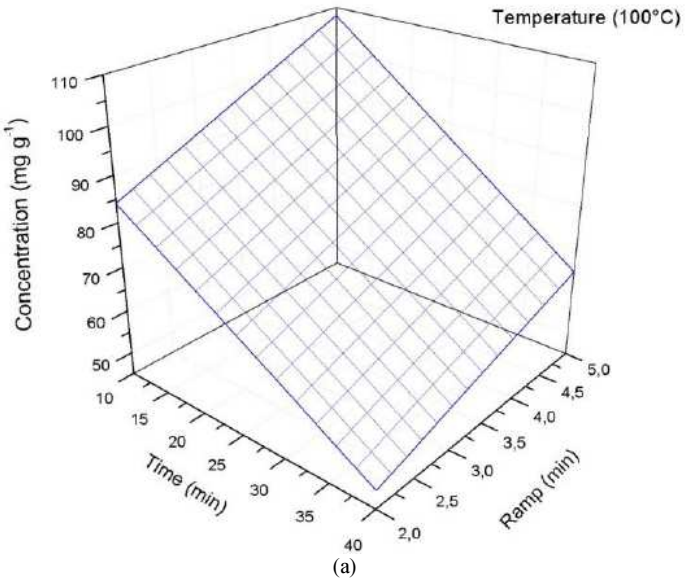
extraction temperature from 85 to 100°C, the extraction yield also increases. Finally, Fig. 2c shows that the best yields are achieved by increasing temperature and decreasing the extraction time.

Path of Steepest Ascent

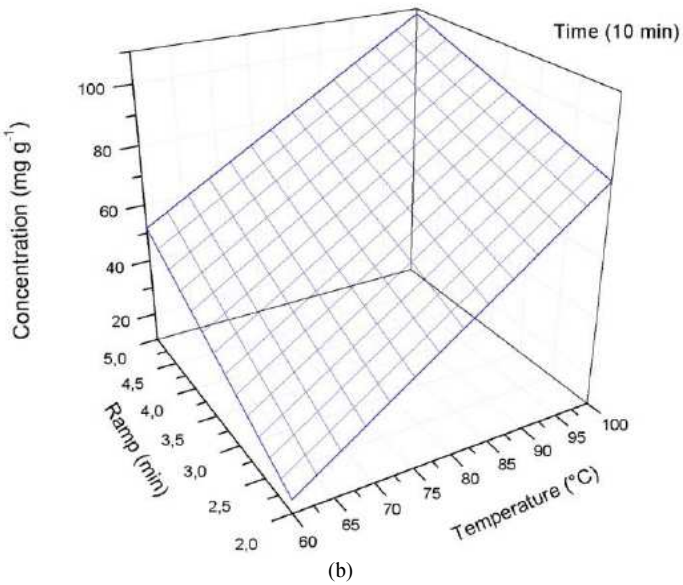
In order to determine the optimum extraction conditions, some additional experiments were performed following the path of steepest ascent. The (coded) factor with the greatest absolute estimated parameter within the model (x_i) was increased 0.3 units. The displacements of the other two factors were calculated by Equation 3. This procedure was repeated four more times. The five additional experiments, their respective decoded levels, along with the obtained ferulic acid peak area, are shown in Table 3:

$$\Delta x_j = \frac{b_j}{b_i}\Delta x_i \tag{3}$$

The results indicated that temperatures above 100°C were not feasible: Water was completely vaporized and the leaves inside the extraction vials were burnt. Therefore, temperature was set at 100°C. A few more experiments were performed, aiming at decreasing the extraction time while increasing the ramp. Table 4 shows those new experiments along with the obtained results.



(a)



(b)

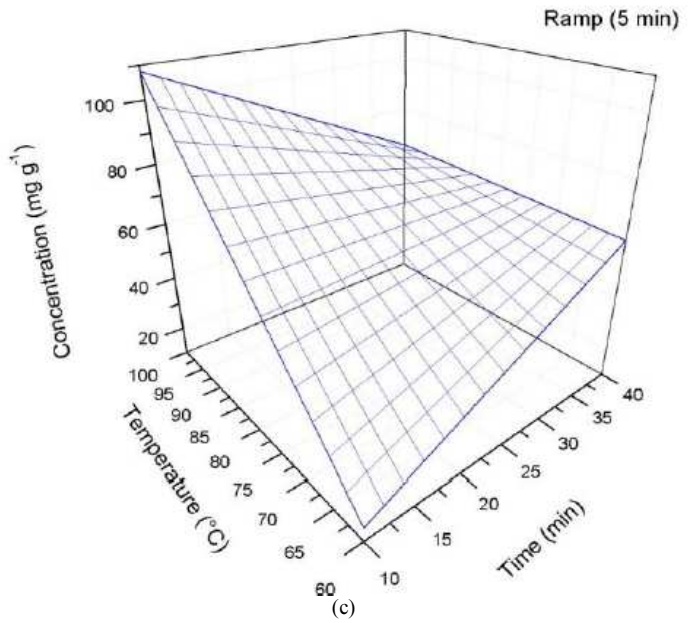


Fig. 2. Response surface plots showing the effects of the variables on concentration of ferulic acid (mg g^{-1}) in 2 g of dry material: (a) time × ramp; (b) temperature × ramp; (c) temperature × time

Table 3. Experiments along the maximum gradient patch

Factors			
(1) Temperature (°C)	(2) Time (min)	(3) Ramp (min)	Area*
100	10	5	85619
106	8	5	37408
112	6	6	35835
118	4	6	31154
124	2	6	20343

*Mean values

Table 4. Experiments along the maximum gradient patch (Temperature = 100°C)

Factors		
(2) Time (min)	(3) Ramp (min)	Area*
10	5	85619
10	8	51778
5	8	31314

*Mean values

One can observe that no improvement was achieved. So, the optimal extraction condition were: (a) Extraction temperature: 100°C, (b) Ramp: 5 min and (c) Extraction time: 10 min.

The optimal extraction conditions for the other three phenolic compounds contained in the aqueous extracts from *Canavalia ensiformis* (naringenin, chlorogenic acid and rutin) were the same. These compounds, along with ferulic acid, have allelophatic properties and can be used as bioherbicides, as pointed out by other work in the group (Mendes and Rezende, 2014).

Conclusion

In this study, the microwave-assisted extraction was fast and suitable for extracting phenolic compounds present in the leaves of *Canavalia ensiformis*. The output power (870 W) and the ramp time (5 min) were adequate to achieve the programmed maximum temperature.

The temperature was the main parameter affecting the extraction, but it cannot exceed 100°C, as water was used as the extractant.

The response surface methodology was adequate to estimate the effect of three independent variables: Temperature, time and ramp on the obtained response. The experimental results showed that linear terms and interactions have significant effect on the response obtained. Based on the analysis of variance, one can say that the estimated model was statistically significant; moreover, it was a predictive one. The optimized conditions were: Temperature 100°C, extraction time 10 min, ramp 5 min.

Other phenolic compounds present in the aqueous extracts of *Canavalia ensiformis* (naringenin, chlorogenic acid and rutin) could be determined by the same conditions; in this study, however, we highlighted the ferulic acid, a leading-character allelopathic compound.

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Authors' Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of other authors have read and approved the manuscript and no ethical issues involved.

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