ANTIOXIDANT ACTIVITY OF ROSEMARY EXTRACTS OBTAINED BY SUPERCRITICAL CARBON DIOXIDE EXTRACTION

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ABSTRACT

The main objective of this work was to apply a statistical experimental design to the components of rosemary (*Rosmarinus officinalis*) extracts responsible for its antioxidant activity in order to find the most influential variables and the favourable combination of those variables to get an extract with the highest antioxidant activity. The extraction technique was carried out with a supercritical extraction device of the type Spe-ed SFE, Applied Separations, model 701. The qualitative and quantitative analysis of the phenolic diterpenes was done through high-performance liquid chromatography (HPLC) using an equipment HP-1050, model SPD-10AVvp. The variables evaluated during the extraction process were: pressure (200-300 bar), temperature (40-60^{o}C) and static time (45-75 min). The response variables used were the proportion of carnosic acid and carnosol present in the extract. To determine the variables of influence during the extraction process a 2^3 factorial design was carried out, finding that temperature and time were the most significant for the carnosol extraction while the interaction temperature-time was the most influential for the carnosic acid extraction, but in none of the two cases these effects were very significant. Lately, the design was amplified applying a Box-Behnken response surface in order to optimize the responses, finding that the best condition for the carnosic acid extraction was low levels of pressure and temperature and high level of time. For carnosol extraction the best conditions were middle level of time and pressure and low level of temperature. The antioxidant activity of the extract obtained with supercritical carbon dioxide having the highest proportion of carnosol and carnosic acid was compared with BHT and with another extract obtained with acetone. The best antioxidant activity was showed by the extract obtained with supercritical carbon dioxide followed by the extract with solvent and ending with BHT.

INTRODUCTION

Rosemary (*Rosemarinus officinalis*) was selected because according to bibliography (1,2,3) is one of the plants with higher antioxidant activity, it is easily grown in Venezuela and can be used to produce antioxidants which could substitute some of the synthetic antioxidants currently available

Antioxidants from rosemary have been extracted and de-aromatized by supercritical CO_2 for use in natural supplements and several studies have been published. Reverchon (4) reported modelling of the extraction by Sovova in 1994, a study by Lopez-Sebastian who removed the aroma and another by Ibanez who fractionated rosemary essential oil.

The objective of this work was to applied the technique of statistical design of experiments to the supercritical carbon dioxide extraction of the antioxidant components of the rosemary plant, to determine the most influential factors in the process in order to optimize it and therefore, find the combination of factors which would result in obtaining the extract with the highest antioxidant activity.

MATERIALES Y METODOS

Extraction with supercritical CO_2

To determine the most influential variable in the extraction process, a 2^3 experimental design was used, 2 means the levels under study (low and high) and 3 means the evaluated factors: pressure (200 and 300 bar), temperature (40,60°C) and estatic extraction time (45 and 75 min.). The response variable was the percentage of extracted carnosic acid and carnosol. This factorial design gives 8 combinations of treatments and to be able to estimate the associated experimental error the experiments were duplicated to reach 16.

Table 1 presents the matrix of experiments used.

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Table 1. Structure of the factorial design 2^3 used.

	Level Codified Variables			Natural Variables		
Experiment	Pressure	Temperature	Time	Pressure (bar)	Temperature (°C)	Time (min)
1	-	-	-	200	40	45
2	+	-	-	300	40	45
3	-	+	-	200	60	45
4	+	+	-	300	60	45
5	-	-	+	200	40	75
6	+	-	+	300	40	75
7	-	+	+	200	60	75
8	+	+	+	300	60	75

Once known the more influential variables in the process, a new experimental design was carried out to determine the combination of variables to achieve maximum production of the phenolic diterpenes of interest, to do that, a response surface design Box-Behnker(6) was used.

Solvent Extraction.

To obtain rosemary essential oil using solvent extraction, this study followed the suggested methodology in the United States Patent: 5,209,870 in which acetone was the solvent used.

Analysis of the Phenolic Diterpenes

To identify carnosic acid and carnosol, Saenz (5) suggested methodology was used. He used High Performance Liquid Chromatography to identify these phenolic diterpenes in several samples of rosemary. In this study, an HP 1050 chromatograph with a nucleosil column 120 C_{18} (20cm x 0,46, 5μm) was used; the mobile phase was a mixture of solvent A (water- acetonitrile-acetic acid, 84:15:1) and solvent B (methanol), with a gradient of 0% to 100% of B during 55min. and a rate flow of 1mL/min. Chromatographic separation was registered at 250nm in an UV spectrometer. The analysis was performed simulating the previously stated conditions, in such a way, to be able to take the retention times of the two compounds considered responsable for the antioxidant activity, specifically carnosic acid and carnosol, and comparing them with the ones reported by Saenz (5).For a quantitative determination, the method of area normalization was used.

Evaluation of the antioxidant activity

The antioxidant activity of the rosemary extracts obtained by different methods was evaluated determining the peroxide index in coconut oil samples treated with the extracts and with a commercial rosemary extract.

RESULTS AND DISCUSSION

The results of analysis of variance applied to the factorial design 2^3 are shown in tables 2 and 3.

Table 2. Analysis of variance for the factorial design 2^3 for the % of extracted carnosic acid

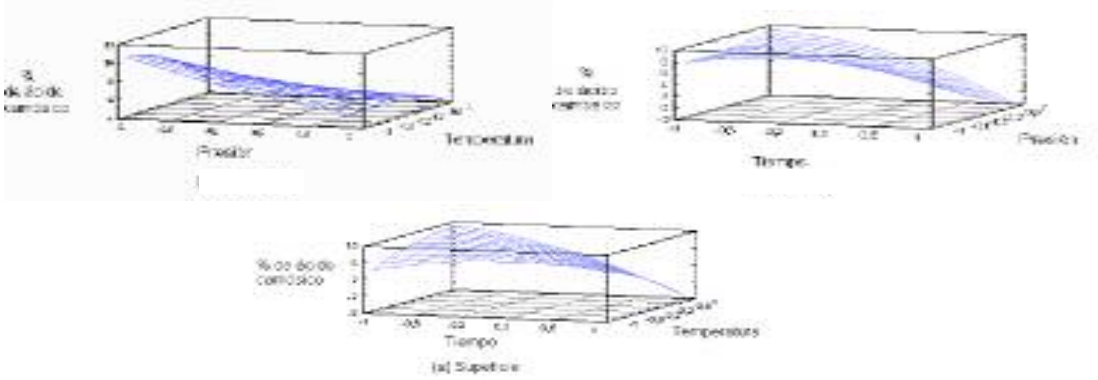
Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F_0
A: Pressure	6,7182	1	6,7182	0,59
B: Temperature	8,70634	1	8,70634	0,77
C: Time	110,055	1	110,055	9,71
AB	109,938	1	109,938	9,70
AC	13,4344	1	13,4344	1,19
BC	214,995	1	214,995	18,97
Total error	90,6837	8	11,3355	
Total	554,735	15		

Tabla 3. Analysis of variance for the factorial design 2^3 for the percentage of extracted carnosol

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F_0
A: Pressure	0,4314	1	0,4315	0,33
B: Temperature	27,4482	1	27,4482	21,25
C: Time	25,0445	1	25,0445	19,39
AB	1,9635	1	1,9635	1,52
AC	0,0437	1	0,0437	0,03
BC	0,2552	1	0,2552	0,20
Total error	10,3316	8	1,2615	
Total	554,735	15		

To consider a variable or interaction statistically significant Fo should have a higher value than the theoretic Fo, ($Fo_{0,01,\ 1,\ 1,8}$) which is 11,26. Interpreting the analysis of variance for carnosic acid it is evident that only the interaction BC (temperature, time) could be considered significant since its Fo is 18.97. For the percentage of carnosol it can be observed than B (temperature) with an Fo of 21,25 and C (time) with an Fo of 19,39 represent a statistically significant effect. Values of Fo even when they are higher than $Fo_{0,01,\ 1,\ 1,8}$ are not high enough to affirm than they are not produced by errors or noise. To clear this doubt, a surface response was build to determine how the response variable is affected when the independent variables have a set of values in an specific range of interest. So the factorial design was increased, applying a design of surface response type Box-Behnken of three variables. The surface responses for the percentages of carnosic acid and carnosol are shown on the figures 1 and 2.

.Figure 1. . Response surface for the % de extracted carnosic acid



When the surface responses from Figure 1 are examined it can be seen that at low levels of time a maximum percentage of extracted carnosic acid is reached when pressure and temperature reach their highest levels, on the opposite case at higher level of time the highest percentages of carnosic acid are reached when working at low levels of pressure and temperature; this is expected since when the sample is at highest pressure and temperatures it is susceptible to decompose.

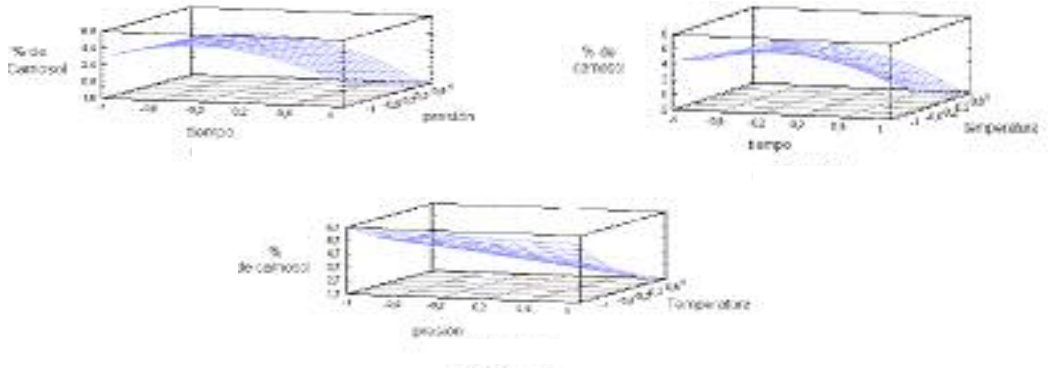


Figure 2.Response surface for the % of extracted carnosol

Analysis of figure 2 shows that the optimum extraction for carnosol is reached at low level of temperature and middle level of pressure and time. It is relevant to observe that the factor with highest influence is temperature which exerts a negative effect while time exerts a positive effect, whereas pressure doesn't have any kind of influence over the response variable. When the antioxidant activity of the extracts is evaluated and compared with BHT it was found that the lower peroxide index was obtained with the samples with the rosemary extracts obtained with supercritical CO_2 extractions and the extract obtained with solvent.

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