SUPERCRITICAL ANTISOLVENT FRACTIONATION OF ANTIOXIDANT EXTRACTS OBTAINED USING PRESSURIZED LIQUID EXTRACTION

J.A. Mendiola, M. Castro-Puyana, A. Cifuentes, M. Herrero, E. Ibáñez*

Instituto de Investigación en Ciencias de la Alimentación CIAL (CSIC-UAM)
C/Nicolás Cabrera 9, Campus de Cantoblanco, 28049 Madrid, España

Email: elena@ifi.csic.es

Abstract. Rosemary (Rosmarinus officinalis, L.) is an aromatic plant widely known by its antioxidant properties. This bioactivity is correlated with the presence of two chemical families of compounds: phenolic terpenes (carnosic acid, carnosol…) and flavonoids (rosmarinic acid).

Pressurized Liquid Extraction (PLE) using ethanol and water has proved to be one of the main techniques for the isolation of those compounds, while other green extraction techniques such as SFE and PHWE (Pressurized Hot Water Extraction) provide extracts mainly enriched in one family of compounds.

The objective of the present work was to develop a continuous method for the fractionation of PLE extracts in different families of bioactive compounds. To do this, a self-designed equipment was set up. In this system the fractionation of phenolic terpenes and flavonoids took place mediated by the antisolvent effect of supercritical CO2 in the ethanol:water mixture.

The optimization of the process was carried out using a response surface methodology (RSM) based in two factors: CO_2 Pressure and ratio (CO_2:PLE extract) flow rate, using 3 levels per factor. The selected responses for the optimization were the relative amount of each compound (rosmarinic and carnosic acids and carnosol) in the vessels. Those compounds were quantified using HPLC-UV-ESI-MS.

The RSM allowed the optimization of the process by providing a mathematical model with a very high correlation with experimental data. Moreover the system developed can be used as green preparative technique for further fractionations, without the need of using tedious preparative LC steps, because it can be run in continuous mode

Keywords : Antisolvent Fractionation, Rosemary, PLE, antioxidant

1. Introduction

Rosemary (*Rosmarinus officinalis*, L.) is an herb, well known by their antioxidant properties. Those properteties are related mainly to the presence of two families of compounds. On one hand, phenolic terpenes such as carnosic acid or carnosol. On the other hand, flavonoids like rosmarinic acid [1].

In order to extract both families of compounds PLE (Pressurized Liquid Extraction) has proved one of the most effective techniques, specifically using ethanol at 150 °C, while other methods of extraction such as SFE (with CO_2 and ethanol) or SWE (with water at 200 °C) are only able to extract one of them [1].

Dietary polyphenols have been reported to be of potential therapeutic benefit in the treatment and/or prevention of several degenerative diseases including cancers and cardiovascular diseases. Carnosic acid, one of the main polyphenols in rosemary, has proven anti-inflammatory properties in neurons, antiproliferative activity in colon cancer cells, and potential to promote differentiation of leukemia cells. Carnosol, another rosemary diterpene, poses strong antioxidant and chemopreventive activities. This diterpene has demonstrated anti-inflammatory and anti-cancer activities on for example prostate, skin, or breast cancer [2]. On the other hand rosmarinic acid can have synergistic antiproliferation effect with some synthetic drugs such as cisplatin on ovarian cancer cell lines [3].

The objective of present work is the splitting of the two families of compounds present in an extract obtained by PLE. This fractionation was performed using a system designed in the CIAL, based on the precipitation mediated effect of supercritical CO_2 antisolvent (SAS).

2. Experimental

2.1 PLE

To make the starting extract a commercial ASE200 (Dionex) extractor was used. The selection of the extraction conditions was performed using a previous study in collaboration with the University Miguel Hernandez (Elche, Spain) where they studied the antioxidant and anticancer activity of the extracts [2]. In this study it was found that optimal extraction conditions were 150 °C and 103 atm, using ethanol: water (80:20) as extraction solvent.

2.2 Antisolvent Fractionation

Since commercial equipments are not available for antisolvent fractionation. The equipment used in this work (Fig. 1) was designed based on the one described by Catchpole et al [3], by modifying, a supercritical extractor Suprex Prepmaster (Suprex Corp. USA). A Jasco HPLC pump PU-2080 (Jasco Inc. Japan) was used to pump PLE extract in liquid state. The expansion vessels S1 and S2 were made in the workshop of CENQUIOR (CSIC).

The operating principles of the system consist on the next steps: hydroalcoholic PLE extract is mixed with supercritical CO_2, in this moment ethanolic fraction is dissolved in SC-CO_2 causing water salting out of the equilibrium ethanol:water; the mixture SC-CO_2 goes throught the tubbing to the next vessel where reduced pressure turns CO_2 in a gas, salting out ethanolic fraction.

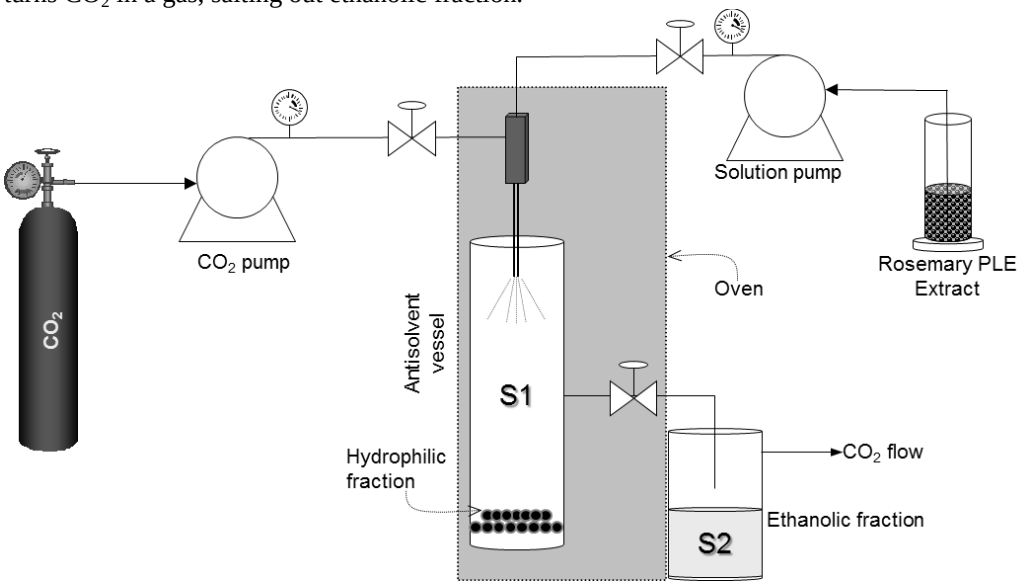

Figure 1. Scheme of antisolvent fractionation equipment used in present work.

2.3 HPLC-MS/MS

An ACCELA UHPLC system coupled to a triple quadrupole mass analyzer was used to analyze fractions recovered. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyzer via an electrospray interface .The analytical method was developed by Herrero et al. [1]. Briefly; the analytical conditions employed consisted of the use of a Hypersil Gold column (50 mm × 2.1 mm, d.p. 1.9 μm) (Thermo Scientific) using as mobile phases acetonitrile+ 0.1% formic acid (A) and water + 0.1% formic acid (B) eluted according to the following gradient: 0 min, 95% B; 0.35 min, 95% B; 3.5 min, 40% B; 6.2 min, 5% B; 6.5 min; 5% B; 7 min, 95% B; 9 min, 95% B. The flow rate was 0.4 mL/min while the injection volume was 5 μL. The diode array detector recorded the spectra from 200 to 450 nm.

To quantify carnosic acid, carnosol and rosmarinic acide, the mass spectrometer was operated in the negative ESI multiple reaction monitoring (MRM) with a Q1 and Q3 resolution of 0.7 Da FWHM using scan width 0.010 Da and scan time of 0.040 s. The values corresponding to the tube lens voltage and collision energy were optimized for each quantified compound.

2.4 Experimental design

The optimization of the process was carried out using a response surface methodology (RSM) based in two factors: Pressure and "PLE extract flow rate" at 3 levels, keeping CO_2 flow and temperature constant at 1ml/min and 40 ºC. Pressure ranged from 100 to 300 atm, while "PLE extract flow rate" ranged from 0.05 to 0.5 ml/min. The selected responses for the optimization were the relative amount of each compound (rosmarinic and carnosic acid-carnosol) in the vessels, quantified using HPLC-UV-ESI-MS.

The elected design was a Box–Behnken experimental design. The Box-Behnken design is an independent quadratic design in that it does not contain an embedded factorial design. In this design the treatment combinations are at the midpoints of edges of the process space and at the center. These designs are rotatable and require 3 levels of each factor. In our case the levels were: 100, 200 and 300 atm for pressure and 0.05, 0.265 and 0.5 ml/min for PLE extract flow (Fig. 2)

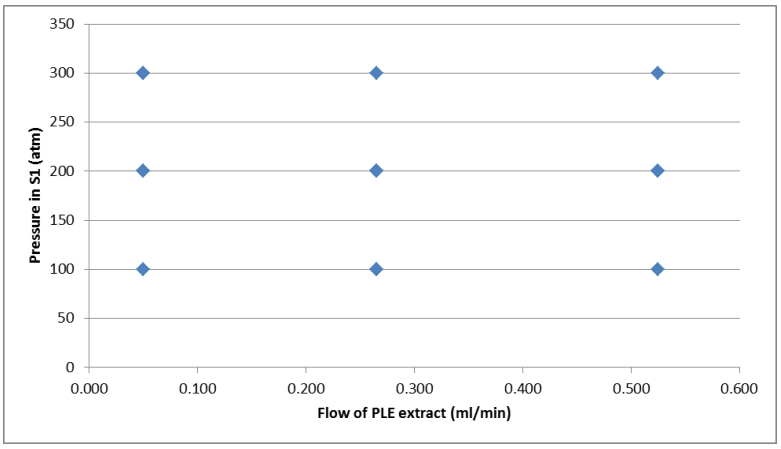

Figure 2. Experimental design used to optimize antisolvent fractionation.

3. Results

In order to optimize the fractionation of andtioxidant compounds present in rosemary extract the first step consisted in quantify the each of them in the original extract. The composition of the extract was 0.294, 0.542 and 1.706 μg/g of rosmarinic acid, carnosic acid and carnosol, respectively.

To optimize the fractionation of rosemary hydroalcoholic extract to factors were chosen, namely pressure and flow. In this sense the effect of both of them on the fractionation will be discussed.

3.1 Effect of pressure

Pressure ranged from 100 to 300 atm. In this range different flows were studied. The chemical composition of the different fractions was obtained by HPLC-MS/MS. As can be seen in Fig. 3, the relative amount of each compound was affected by pressure changes. In this graph the concentration of each compound in S1 divided by its concentration in S2 is represented. Values higher than 1 mean higher amount of the compound compared to original PLE extract, while values below 1 mean decreased amount of this compound compared to original PLE extract.

In this sense, the fraction obtained in S1 was enriched in rosmarinic acid (the most polar of the studied compounds) when experiments were performed above 100 atm. The amount of flavonoids (rosmarinic acid) was increased up to 3 times in the fraction collected in the vessel S1.

On the other hand, the less polar compounds (carnosic acid and carnosol) were obtained preferentially in the S2 fraction. This fractionation effect was increased by increasing the pressure. The last bar of the graph,

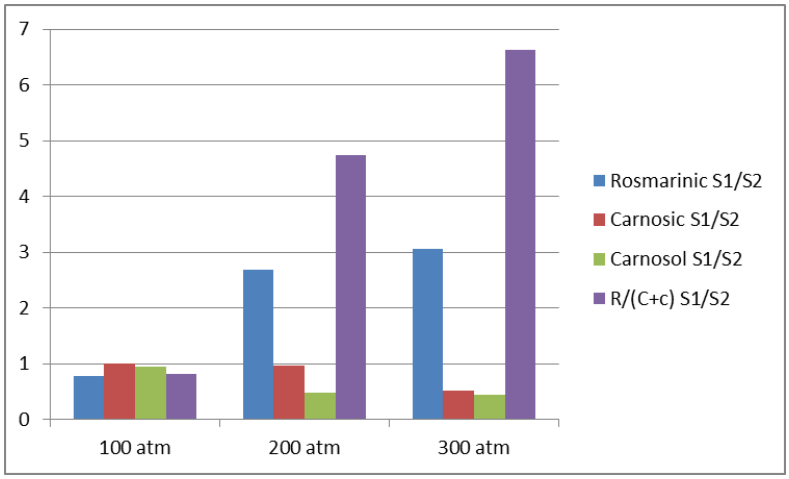

Figure 3. Effect of pressure on fractionation comparing relative amount of each compound in the different vessel (enrichment). Experiments performed using 0.05 ml/min of PLE extract

named "R/(C+c) S1/S2", indicates the relative amount of flavonoids divided by the phenolic terpenes (carnosic acid and carnosol). In this bar the effect is higher, up to 6.5 times more flavonoids in S1 than in S2.

3.2 Effect of flow

As done for pressure the effect of flow was studied. But this time the effect was opposite. As can be seen in Fig. 4 by increasing the flow the fractionation ratio decreased.

In this sense, the concentration of carnosic acid could be increased up to 3 times in the fraction collected in S2 when working with low flows.

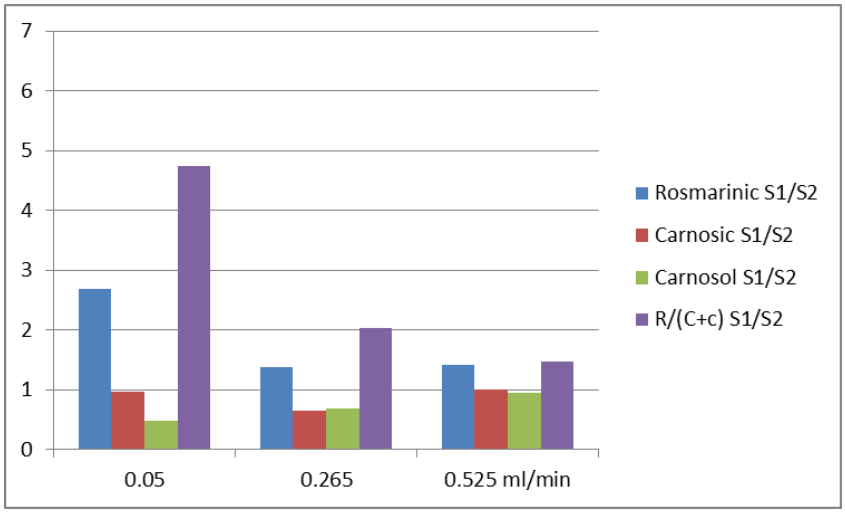

Figure 4. Effect of "PLE extract flow" on fractionation comparing relative amount of each compound in the different vessel (enrichment). Experiments performed using 200 atm.

3.3 Combined effect Optimization

One of the main advantages of working with Response Surface experimental design is the possibility of optimize all the factors at the same time in order to obtain a desired value of the response. By analyzing all the data showed in this work the result was the response surface showed in Fig. 5. The optimization provided a mathematical model with a R^2=0.9335 (93% of the experimental variability explained by the model at the 95% confidence level, $p<0.5$). The optimal fractionation was obtained using the lower flow (0.05 ml/min) and higher pressure (300 atm).

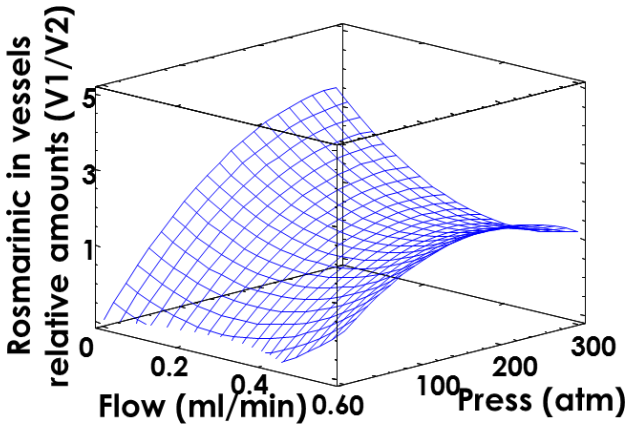

Figure 5. Response Surface obtained for the optimization of relative amount of rosmarinic acid.

This model is not only useful to get a maximum fractionation of antioxidant compounds present in rosemary extract. It's also interesting when trying to get a specific composition. Since the next step of our work consist of proving the anticancer activity of these fractions, where small amounts of some specific compound can improve greatly the bioactivity of the others present in the mixture.

4. Conclusions

The proposed method for antisolvent fractionation is a useful green method to obtain fractions of different polarity without using toxic solvents. Another advantage of this method is that it can work in continuous mode, without the need of using tedious preparative LC steps. This fact is highly useful when dealing with production of high amounts of food or pharmaceutical ingredients. Finally, the composition of the fractions could be modulated by applying the mathematical model obtained for the response surface.

Acknowledgements

This work was financed thanks to AGL2011-29857-C03-01 (Ministerio de Economía y Competitividad) project. M.C.P. thanks MICINN for her "Juan de la Cierva" contract. M.H. would like to thank MICINN for a "Ramón y Cajal" research contract.

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