Application of a Statistical Mixture Design in the Pressurized Solvent Extraction From Tara Seed Coat

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Abstract

Carbon dioxide solubility capacity is greatly diminished if the target compounds to be extracted have hydroxyl, amino and nitro groups. The solubilities of such molecules may be greatly enhanced by the addition of a polar cosolvent. In this case, for practical reasons, it is desirable to avoid regions of multiple phases involving vapor, liquid and solid phases. Accordingly, a designer needs to select the conditions of pressure and temperature, as well as the mixture composition at which extraction of natural components is technically attractive. Considering this, and using a well known ternary solvent system, this work proposes the study of statistical mixture design in pressurized solvent extraction of tara (*Caesalpinia spinosa*) pods seed coat as a case study. Tara seed coat does not have a commercial application, thus representing an agro-residue of tara processing industries that could be explored as a raw material for the production of valuable phenolic- and antioxidant-rich extracts. In this work, a statistical mixture design was applied to optimize solvent mixtures for selective extraction of phenols from tara seed coat. Pressurized solvent extractions were performed at 313 K and 20 MPa, using homogeneous CO_2, ethanol and H_2O solvent mixtures. Total phenols contents and antioxidant activities of extracts were evaluated. A linear mixture model predicted total yields and it was verified that H_2O was the most effective solvent to obtain high extraction yields. A quadratic model predicted total phenols extracts´ contents and it was verified that H_2O and CO_2 had an antagonistic effect and that maximum phenolic contents can be expected for EtOH-rich mixtures. For the antioxidant activity, no model revealed to be significant (at a 95% confidence level). Nevertheless, high antioxidant activities were observed (above 80% of oxidation inhibition for most extracts). The obtained results strongly suggest that tara seed coat is a residue that has a great potential to be valorized for the production of phenolic-rich extracts presenting high antioxidant activities.

INTRODUCTION

The biggest challenge when extracting phenols or other secondary metabolites from plant matrices is to be able to isolate them with chemical and biological activities, while keeping the extraction process environmentally safe and economically feasible. The success of the extraction process is, therefore, dependent on the chosen specific extraction methodology and on the applied process conditions. Supercritical fluid extraction is a methodology that may be attractive to produce extracts for fine applications, like the pharmaceutical ones, being a possible alternative to conventional extraction. The usage of supercritical CO_2, whose physicochemical properties are easily tuned with small changes in pressure and temperature

permits to adjust its solvent power and selectivity. However, the extractability of compounds with supercritical CO_2 depends on the presence of specific functional groups in these compounds, on their molecular weights, and on their polarities.

Pressurized solvent extraction (PSE) which involves the usage of H_2O and/or organic solvents at considerable elevated temperatures and pressures may represent an attractive option to extract phenols from plant matrices. It has improved characteristics in terms of mass transfer and of solvating properties [1] that can even be improved by the utilization of gas-expanded liquids (usually obtained upon the dissolution of CO_2 in H_2O or in an organic solvent). CO_2, ethanol (EtOH) and H_2O are solvents with different polarities [2] that, when mixed in different proportions allow obtaining homogeneous solvent mixtures with polarities that go from the one of the less polar CO_2 to the one of the most polar H_2O. Moreover, EtOH is a strong proton-donor and plays an important role in the formation of hydrogen bonding associations with the diverse functional groups of phenols. The role of solvent-solvent interactions in competition for the solvation of solutes is also an important issue to consider [3]. In combination, all these factors may allow the selective extraction of phenols from plant matrices. The investigation of the effect of the composition of a ternary mixture in extraction should be addressed by techniques of experimental design for mixtures, also known by mixture design. Besides helping to minimize the number of experiments to be performed and to maximize the amount of acquired information, these designs permit the identification and characterization of synergistic and of antagonistic interaction effects between the different solvents.

Tara (*Caesalpinia spinosa*) is a native tree from Peru, whose pods have been used by the local population since the pre-Hispanic era due to their proved medicinal and nutritional benefits [4]. Tara pulp is extremely rich in hydrolysable tannins (40-60%) [5] and has diverse applications such as in leather tanning and in the manufacture of plastics and adhesives [4, 6]. Tara seeds consist of 34% of endosperm, 37.5% of germen, and 28% of coat (by weight) [4]. Both tara endosperm and tara germen find commercial applications: as a stabilizer and thickening agent, and as animal feed or as a source of protein hydrolysates, respectively [7]. Tara seed coat constitutes a residue of these industries, which could be used to recover natural compounds that could be potentially useful for pharmaceutical, cosmetic and food industries instead of being treated as a residue of environmental concern.

In this work, a mixture design has been applied to the PSE (at 313 K and 20 MPa) of phenols from tara seed coat, varying the ternary CO_2/EtOH/H_2O solvent mixture composition. Employed compositions (molar fractions) were picked taking into consideration the utilization of a homogeneous high pressure phase. Extracts´ total phenols contents and antioxidant activities were evaluated, and statistical mixture models were developed.

MATERIALS AND METHODS

Raw material – Tara seed coats were provided by Transformadora Agrícola S.A.C. from Peru, where tara seeds constitute a residue. Tara seed coats were separated from tara seed germen and used in this study. Raw material was comminuted using a knife mill and particles having a size distribution between 48–24 mesh were separated using sieves under mechanical stirring and conditioned in a plastic bag under regular atmosphere conditions.

Chemicals - Carbon dioxide (99.998%), ethanol (99.5%) and distilled water were used for extraction experiments. Analytical grade chemicals and solvents employed for extract analysis were: ethanol, Folin-Ciocalteu's phenol reagent, sodium carbonate, β-carotene,

Tween 40, linolenic acid, chloroform and bi-distilled water. Gallic acid (≥98%) was the standard employed for total phenols quantification.

Experimental procedure for enhanced solvent extractions – These assays were performed using a supercritical fluid extraction apparatus containing a ~17.8×10^{-6} m^3 stainless steel extraction cell [8]. Temperature was chosen taking into consideration the thermal sensitivity of phenols in general, and applying a solid-to-solvent ratio of 1:48 (w/w, dry basis, d.b.). Molar fractions of CO_2 and H_2O varied between 0.0 and 0.6 and the ones of EtOH varied between 0.4 and 1.0. Extracts were recovered in a glass flask placed in an ice bath. Dried extracts were obtained by vacuum evaporation and freeze drying and stored at approximately 263 K, until further analysis.

Quantification of phenols – Extracts total phenols were quantified according to the Folin-Ciocalteu's method, following the procedure proposed by Singleton and Rossi [9] with modifications [8], and were expressed as gallic acid equivalents (GAE), in percentage (w/w, d.b.).

Antioxidant Activity - Antioxidant activities of extracts were determined by a method based on the coupled oxidation of the β-carotene/linolenic acid system [10] during 6 hours, and are expressed as oxidation inhibition percentages.

Experimental design and statistical analysis - The experimental design, which is illustrated in Figure 1, included three vertices, three center of edges, three axial check blends, one interior check blend, plus one overall centroid, totalizing 14 extraction experiments (the vertices were duplicated).
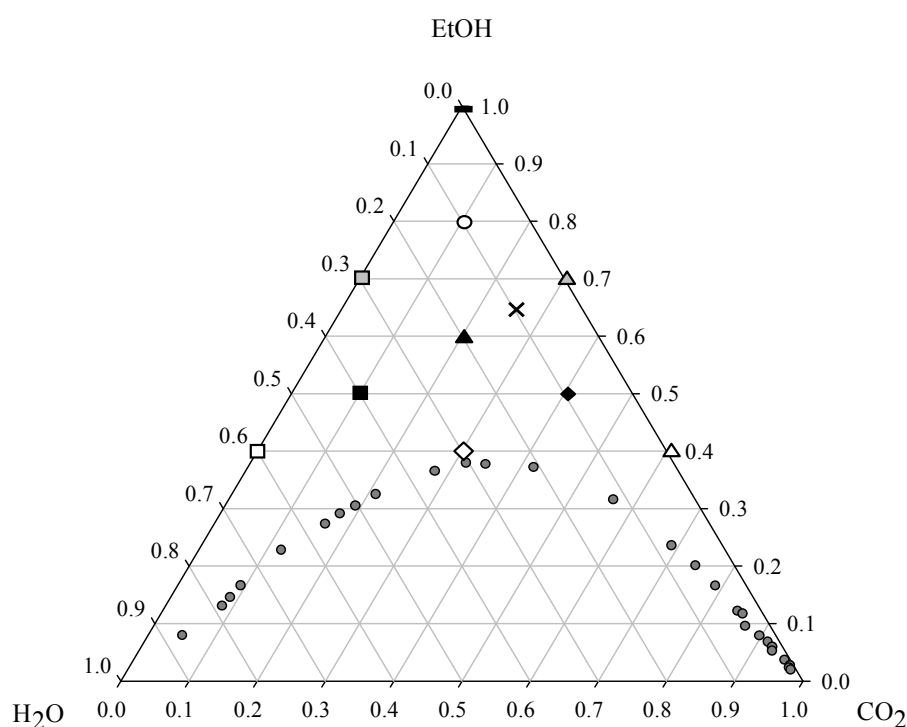
Figure 1: Representation of the assayed solvent mixtures with diverse CO_2-EtOH-H_2O molar fractions: □ (0.0-0.4-0.6), ◇ (0.3-0.4-0.3), △ (0.6-0.4-0.0); ■ (0.1-0.5-0.4), ▲ (0.2-0.6-0.2), ◆ (0.4-0.5-0.1), × (0.25-0.65-0.1), ○ (0.1-0.8-0.1); ▨ (0.0-0.7-0.3), △ (0.3-0.7-0.0), (0.0-1.0-0.0), and of experimental phase equilibrium values for the ternary system at 313 K and 20 MPa as determined by Durling et al. [11] (◉).

Linear, quadratic and special cubic mixture models were used to fit total extraction yield, total phenols and 6-hour oxidation inhibition of tara seed coat extracts. The special cubic model, which includes linear, quadratic and cubic components, is represented by Equation 1:

$$Y = \sum_{i=1}^{3} b_i x'_i + \sum_{i=1}^{3}\sum_{j\neq i}^{3} b_{ij} x'_i x'_j + \sum_{i=1}^{3}\sum_{j\neq i}^{3}\sum_{k\neq j}^{3} b_{ijk} x'_i x'_j x'_k + \varepsilon \qquad \begin{cases} x'_i \geq 0 \\ \sum_{i=1}^{3} x'_i = 1 \end{cases} \qquad \text{(Equation 1)}$$

where bi, bij and $bijk$ are regression coefficients calculated from the experimental data by multiple regression.

For each response variable, analysis of variance was used to test for model significance and lack of fit. The regression coefficients of the most significant model of each response variable were determined and the significances of all terms were judged statistically by computing their F-values at a p-value of 0.05. The regression coefficients were then used to make statistical calculations to generate contour maps from the regression models. Statistica software was used to design the experiments and to model and to analyze the results.
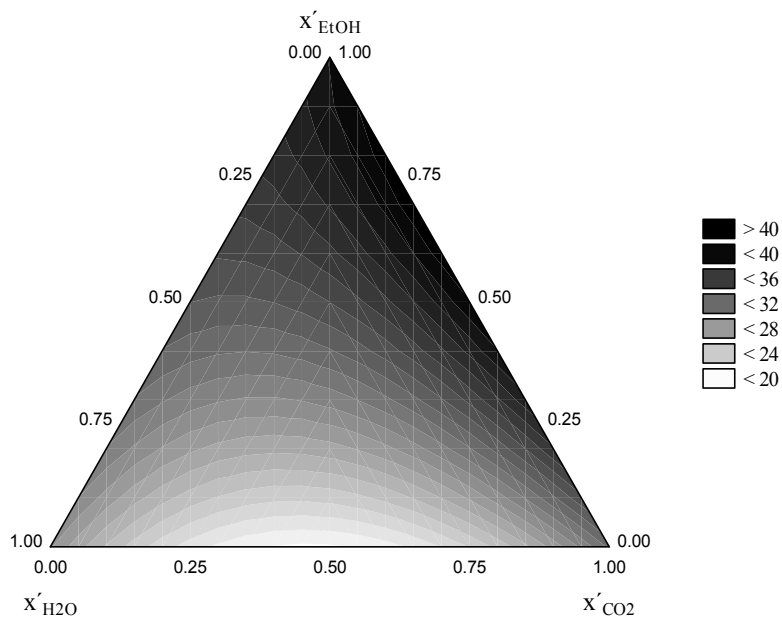

RESULTS

For the extraction yield response variable, the simultaneous test for all parameters of the linear model was statistically significant ($F(2,11)=18.88$; $p<.05$). The addition of model parameters for the quadratic and for the special cubic models did not significantly improve the fit of the surface. Figure 2.a illustrates the mixture response surface for the linear model that fitted the experimental data, in terms of pseudo-components molar fractions. Therefore, the two lower vertices stand for binary mixtures of H_2O and EtOH (0.6 and 0.4 molar fractions, respectively) and of CO_2 and EtOH (0.6 and 0.4 molar fractions, respectively), while the upper vertex stands for pure EtOH. Water was the most effective solvent in extracting compounds from tara seed coat in a high pressure extraction methodology such as the one applied in this study.

For the extracts´ total phenols response variable, though the simultaneous test for all parameters of the linear model was also statistically significant, the addition of the three quadratic model parameters significantly improved further the fit of the model ($F(3,8)=5.19$; $p<.05$). However, adding the parameters for the special cubic model did not significantly improve the fit of the surface. As can be observed (Figure 2.b), maximum total phenols contents can be expected for mixtures mostly rich in EtOH. The affinity between phenols and alcohol solvents has been widely reported in literature by several authors [12, 13], being related to the important role that these solvents play in the formation of hydrogen bonding associations with the diverse functional groups of these secondary metabolites.

In general, all extracts presented high antioxidant activities even 6 hours after the beginning of the assay: nine out of eleven extracts maintained their oxidation inhibitions above 80% after 3 hours, and above 70% after 6 hours of the beginning of the assay. However, none of the models revealed statistically significant at the 95% confidence level and therefore there was not an obvious influence of solvent mixture composition on the antioxidant activity of the extracts. There was neither a direct correlation with the total phenols contents of the extracts.
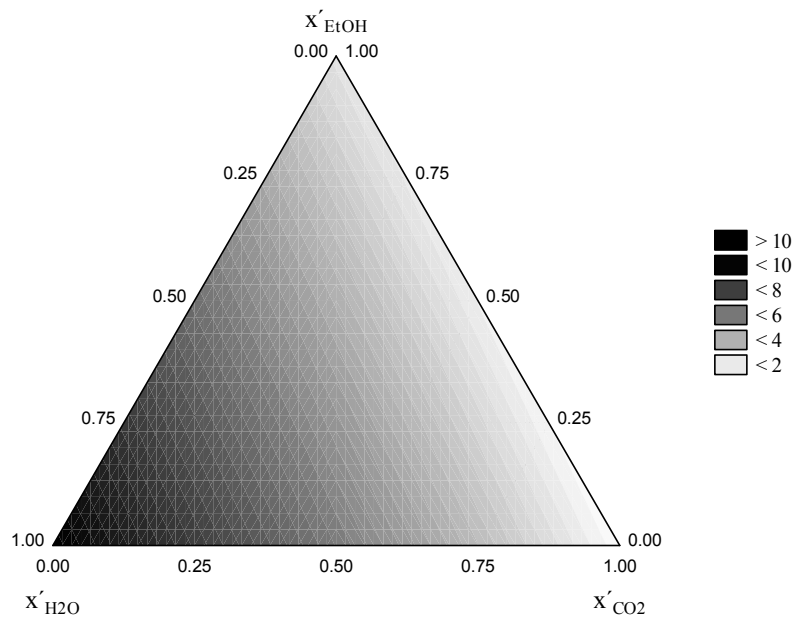
Figure 2: Response surface contour plot curve for the **(a)** extraction yield and **(b)** phenols contents of tara seed coat extracts.

CONCLUSIONS

The overall results confirmed that tara seed coat is a residue quite rich in phenols with potent antioxidant activity. Pressurized solvent extraction applying CO_2, EtOH and H_2O, at 313 K and 20 MPa, proved to be a feasible extraction methodology to achieve potentially useful extracts.

Solvent composition affected extraction yields, and composition and activity of extracts. Whereas the presence of H_2O in the extraction medium favored extraction yield, the presence

of EtOH favored the extraction of phenols with high antioxidant activity, though no direct correlation with antioxidant activity of extracts could be determined. The most remarkable features of the obtained tara seed coat extracts are their high total phenols contents and antioxidant activities. Tara seed coat is, therefore, a residue that has great potential to be valorized trough the extraction of compounds that may be applied as natural additives in food, cosmetic or pharmaceutical goods, increasing their shelf-lives and/or acting as human health promoters.

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