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Effect of the addition of cosolvent on the supercritical fluid extraction of bioactive compounds from *Helianthus annuus* L.

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Abstract

An analysis of the application of supercritical carbon dioxide in the extraction of bioactive compounds from *Helianthus annuus* L. (sunflower) has been carried out. The influence of different variables, including pre-treatment of the sample, temperature, pressure and modifiers, was investigated. The samples were either dried or not dried and the extraction conditions were as follows: 35–50 °C, 100–500 bar, and addition of 5% of methanol, water or dimethyl sulphoxide (DMSO) as a modifier. The best extraction yields were achieved on a dried sample at a temperature of 50 °C and a pressure of 500 bar using a 5% water as a modifier.

The bioactivities of the extracts obtained under the different conditions were compared. The best activity profiles were obtained for the non-dried samples extracted at 50 °C and 500 bar with supercritical carbon dioxide.

Keywords: Sunflower; Natural products; Supercritical carbon dioxide; Extraction; Bioactive compounds

1. Introduction

The inappropriate use of agrochemicals may give rise to undesirable side effects. Indeed, there may be a need to develop new management systems – based on ecological manipulations – to reduce dependence on synthetic herbicides and insecticides. In agricultural practice, allelopathy has been exploited as a tool for weed management and has increased in importance. Although progress in this regard is slow, some promising results have been found and more are expected in the near future [1,2].

There are a large number of higher plants that show suppressive effects on other plants in their vicinity, but only some of these have shown effects on weeds and pathogens. Several studies have shown that *Helianthus annuus* L. (sunflower) contains chemical substances that have allelopathic properties [3]. The extracts of this plant can be used as a natural herbicide to reduce the dependence on synthetic herbicides in the control of crops.

Since active compounds in herbal plants are usually present in low concentrations, it is very important to develop more effective and selective extraction methods for the recovery of the desired compounds from the raw materials. Supercritical fluid extraction (SFE) is one alternative to supplement or replace the conventional separation systems (distillation or liquid solvent extraction) because the energy yields and separation effectiveness are both good. The SFE technique minimizes sample handling, provides fairly clean extracts, expedites sample preparation and reduces the use and disposal of environmentally aggressive solvents. Additionally, in many cases SFE provides recoveries that are as good as, or even better than, those of more conventional solvent extraction techniques [4,5]. This technique has been extensively studied for the separation of active compounds from herbs and other plants [6,7] as well as for samples for the alimentary industry [8,9].

The bioactivities of several extracts obtained with supercritical carbon dioxide have been studied recently. One example that warrants particular attention was reported by Ki-Pung [10], who studied the extraction of bioactive coumarin and its various derivatives (i.e., hydroxyl-, methyl- and methoxy-derivatives) in the range 308.15–328.15 K and 10–30 MPa. The extraction of bioactive compounds from the sunflower (*H. annuus* L.) with supercritical carbon dioxide has been studied before and the effect of pre-treatment of the raw material on the extraction

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yields and bioactivities of the extracts obtained under different conditions investigated [11].

In most of these studies carbon dioxide was used as the solvent because of its relatively low critical temperature (31.1 °C), non-toxicity, non-flammability, good solvent power, ease of removal from the product and low cost. However, quantitative extraction of polar analytes requires the addition of an organic modifier, with methanol and water being the most common choice in natural products [4].

SFE methods often involve the investigation of many variables, which may affect the efficiency of extraction. Selection of these variables and their levels is critical. Several statistical techniques, such as factorial design, were employed in the optimization of analytical methods. Factorial design has some advantages in that the global optimum can be provided, large amounts of quantitative information can be extracted and both discrete and continuous factors can be estimated. Factorial designs have been used for to determine the effect of these parameters in the process including temperature, pressure, pre-treatment of sample, extraction time, fluid flow-rate and addition of modifier [12].

The work described here involved the extraction of bioactive compounds from the sunflower (*H. annuus* L.) with supercritical carbon dioxide. The effect of cosolvent on the extraction yields and the bioactivities of the extracts obtained under different conditions (pre-treatment of sample, pressure and temperature) were evaluated.

2. Materials and methods

2.1. Samples and chemicals

Leaves of *H. annuus* L. (variety Aitana) were collected in July 2003 during the third plant development stage [3] (plants were 1.2 m tall with flowers, 1 month before harvest) and plants were provided by Rancho of Merced, Agricultural Research Station (CIFA), Junta of Andalucía, Jerez, Spain.

The sample was stored under two sets of conditions in order to evaluate the behaviour of each sample in terms of extraction yield and bioactivity of the extracts:

- sample congealed at −25 °C;
- sample dried at room temperature (25 ± 1 °C) until a constant weight was reached.

The specifications of the other chemical reagents used are given in Table 1.

Table 1
Reagents

Reagent	Purity	Company	Use
Carbon dioxide	99.995%	Carburos metalics	Extraction at high pressure
Water	Milli Q		Cosolvent
Methanol	PA	Panreac	Collection of extracts and cosolvent
Citric acid monohydrate	PA	Panreac	Preparation of buffer
Potassium phosphate di-basic 3-hydrate	PA	Panreac	Preparation of buffer
Sucrose	PA	Panreac	Preparation of buffer
Dimethyl sulfoxide	PA	Panreac	Dissolution of the extracts and cosolvent

2.2. Extraction at high pressure

The extractions were carried out in an Isco extractor (Nebraska, USA, model SFX 220). The equipment consisted of one extractor with a maximum capacity of 10 ml and 2 μm filters at the inlet and outlet to avoid haulage of the sample. The SFX extractor was also fitted with a thermostatic system that allowed the extraction to be carried out at a constant temperature. The solvent was introduced by syringe pumps (Isco model 260D and 100DX), which allowed a constant flow of solvents.

The samples leave the vessel through a restrictor, which was thermostated coaxially to avoid obstructions due to the solidification of CO_2. A schematic diagram of the SFE apparatus used in this research is shown in Fig. 1.

In order to achieve complete extraction of the substances in question, a relatively long extraction time was used (5 h) and the measured flow rate for the supercritical fluids was 7.03 mmol/min. Extreme conditions of pressure were tested, with a lower limit of 100 bar chosen because it is near to the critical pressure of CO_2 (72 bar). The upper pressure limit was dictated by operational cost and safety precautions (500 bar). Experiments were carried out at the relatively low temperatures of 35 and 50 °C due to the possible thermal degradation of substances. The restrictor was maintained at 40 °C and the pump was kept at 20 °C. The operating methodology involved loading the extraction cartridge with approximately 2.0 g of the sample, which had previously been homogenized in order to maintain a constant density in all experiments. The experiments on each sample were carried out in duplicate in order to evaluate the variability of the measurements.

In order to determine the influence of cosolvent, three different solvents were tested: methanol, water and dimethyl sulfoxide (DMSO), all at a level of 5% volume (vol.). Methanol and water are the most commonly used solvents in the SFE of natural products. Water has the advantages of a lack of toxicity, non-flammability, good solvent power and low cost. DMSO is used in the bioassay and it was therefore appropriate to study its behaviour as a cosolvent.

The extracts were collected in methanol and stored at 4 °C with the exclusion of light. The methanol was later evaporated and the resulting extract weighed.

2.3. Design experiment

In order to determine the factors that influence the process and the relationships between them, a 2^3 full factorial design was carried out. The variables selected to perform the experimental design were as follows: pre-treatment of the sample, extraction temperature and extraction pressure. The factor levels (coded values) and physical values are shown in Table 2. The experiments were performed in randomized order.

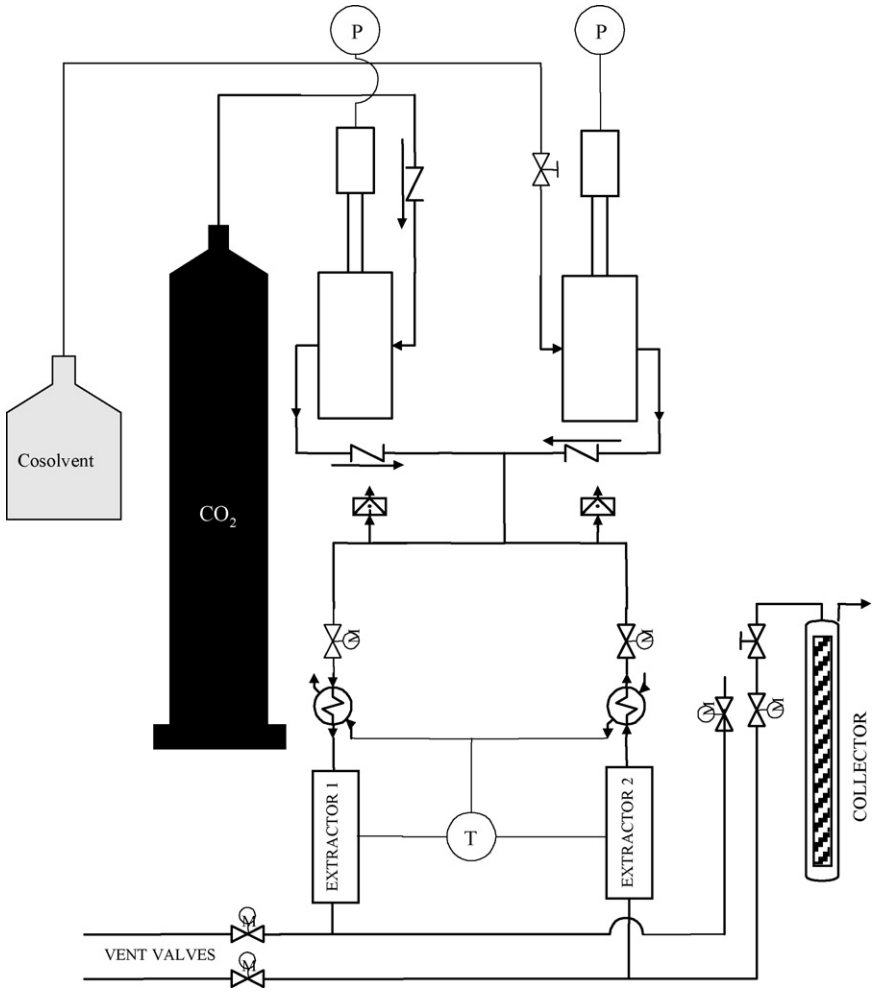

Fig. 1. Schematic diagram of the equipment used for the SFE.

Statistical calculations and analysis were performed using STATGRAPHICS Plus 4.0 (1994–1999, Statistical Graphics Corp.)

2.4. Coleoptiles bioassay

Bioassays constitute one important tool to evaluate the inhibiting or stimulating activity in terms of growth of the isolated substances according to the conditions described in the previous session.

Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in 15 cm diameter Petri dishes moistened with water and were grown in the dark at 22 ± 1 °C for 3 days. The roots and caryopsis were removed from the shoots. The latter were placed in a guillotine and the apical 2 mm were cut off and discarded. The next 4 mm of the coleoptiles were removed and used for bioassays. All manipulations were performed under a green safelight. Compounds were dissolved in DMSO and diluted to the final bioassay concentration. Parallel controls were also run [3].

A sample (16 mg) of each extract obtained under the conditions described in Section 2.2 was weighed out. The extracts to be assayed for biological activity were added to test tubes and were dissolved in 16 ml of an aqueous solution of phosphate/citrate buffer (pH 5.6) containing 2% sucrose. The extracts were insoluble in water and so DMSO (5 μl/ml of plug) was added to ensure total dissolution. Solutions of 500, 250 and 125 ppm were prepared in a similar way for each extract. Five coleoptiles were placed in each test tube and the samples were rotated at 6 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptile lengths were measured by digitalization of their images. Data are presented as percentage differences from the control.

Each assay was performed four times and on two different days.

Table 2
Factor levels and physical values in the design experiment

Factor	Experimental variable	Levels	
		Low level (−1)	High level (1)
A	Pre-treatment	Not-dried	Dry
T	Temperature (°C)	35	50
P	Pressure (bar)	100	500

2.5. Cluster analysis

Statistical treatments were performed using the SPSS 10.0 program (Statistical Package for Social Sciences). Association

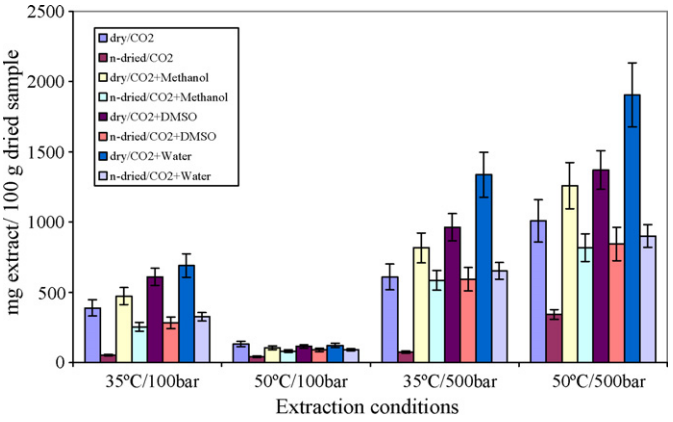

Fig. 2. Extraction yields.

analysis of the data based on the bioactivity profile was performed for each of the different sets of extraction conditions.

To further clarify the relationships between the clusters and those individuals forming the clusters, a dendrogram was generated by hierarchical cluster analysis; the squared Euclidean distance between normalised data was used to measure the similarity between samples.

3. Experimental results

The extraction yields expressed as milligrams of extract/100 g of dry leaves are shown in Fig. 2 for an extraction time of 5 h under different conditions of pressure, temperature and pretreatment of the sample for each solvent system employed.

A Pareto chart for the standardized effects in the design experiments is shown in Fig. 3. The vertical line indicates the statistical significance for the effects.

The results of the bioactivity assays for the extracts with the best extraction yields for each of the four systems solvents tested (500 bar of pressure at the two studied temperatures) are shown in Fig. 4. The data are expressed as percentage differences from the control, which means that a value of zero represents an identical value to the control. On the other hand, a positive value represents stimulation of the parameter in question and a negative value represents inhibition of the growth of the wheat coleoptiles under the given experimental conditions.

The statistical result of the cluster analysis applied to the activity data for each of the sets of conditions employed in the bioactivity study is shown in Fig. 5.

4. Discussion of the results

4.1. Extraction yield

According to our experimental data (Fig. 2), the best extraction yields were obtained from the dried samples. The moisture from the congealed samples seems to be a factor that diminishes the extraction yield, with the water acting as a solvent that competes with supercritical CO_2. If excess water remains in the extraction vessel, highly water-soluble solutes prefer to partition into the aqueous phase and, consequently, the SFE recovery will be low. A freshly extracted sample has a high moisture content (76%) and this can cause mechanical difficulties such as restrictor clogging due to ice formation.

Depending on the type of sample matrix and the affinity of the analyte for the matrix, the modifier may influence the extraction in three different ways: (1) increase the analyte's solubility in the supercritical fluid as a result of analyte–modifier interactions in the fluid phase; (2) facilitate analyte desorption—the molecules of polar modifiers are able to interact with the matrix and compete efficiently with the analyte for the active sites in the matrix; (3) distort the matrix–analyte diffusion process and favour penetration of the supercritical fluid inside the matrix when the modifier swells the matrix.

Although the extraction yield increases on adding small amounts of polar modifiers to the congealed samples, differences were not detected in the extraction yields obtained using

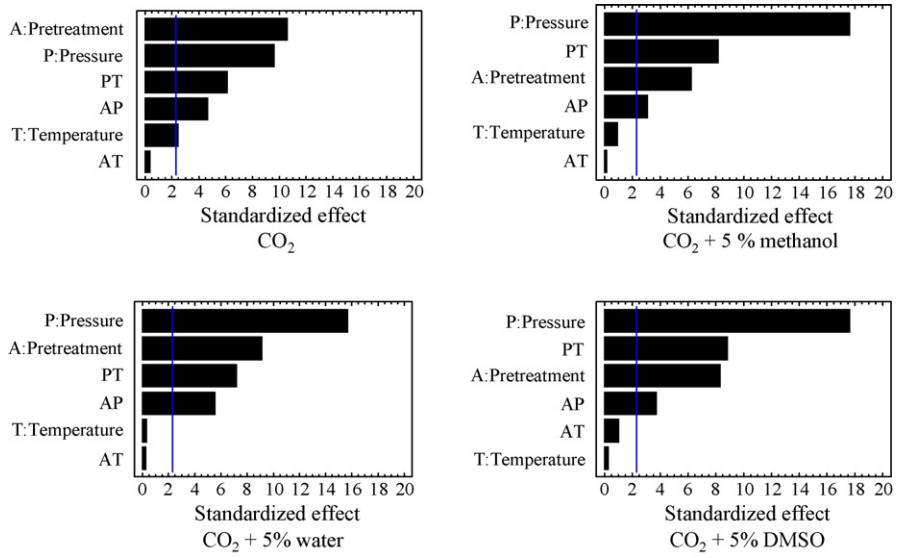

Fig. 3. Pareto diagram for the four solvent systems considered in the study.

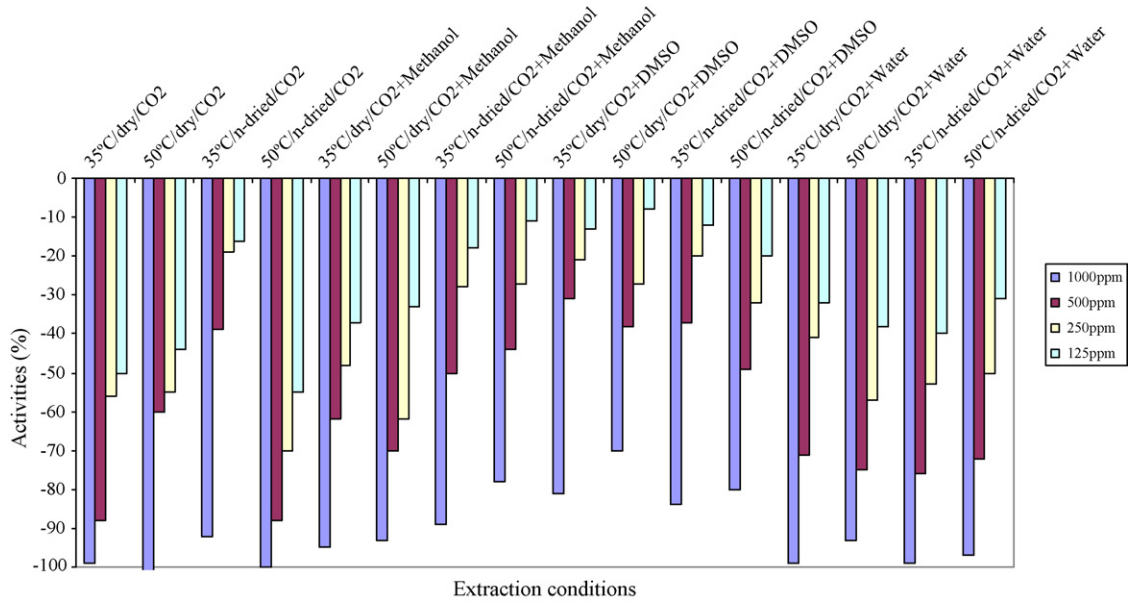

Fig. 4. Bioactivities of extracts obtained at 500 bar.

the three solvent–CO_2 mixtures: CO_2 + 5% methanol, CO_2 + 5% water and CO_2 + 5% DMSO. This fact supports the observation that highly water-soluble solutes prefer to partition into the aqueous phase and remain in the extractor.

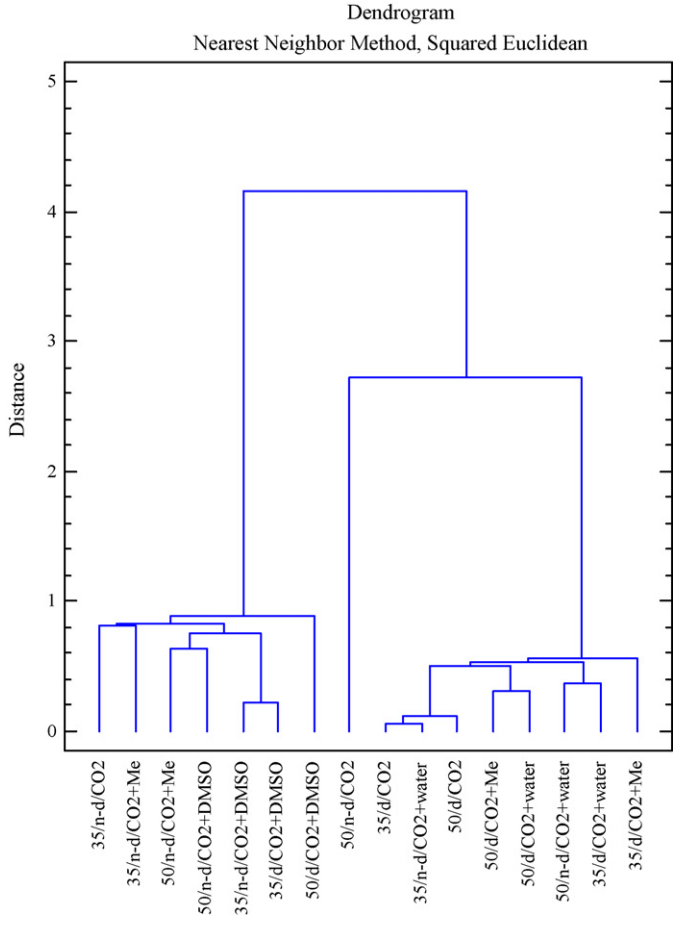

Fig. 5. Cluster analysis.

The total yields of extracts obtained with pure CO_2 and various solvent–CO_2 mixtures is shown in Fig. 2. The best extraction yields were achieved using 5% water as a modifier and drying the sample under different conditions of pressure and temperature. The advantages of using water as a modifier are evident. DMSO is more polar than methanol but the extraction yields obtained on using DMSO were lower. In this case the most important factor is mass transfer. The viscosity of DMSO is higher than that of methanol and, for this reason, the CO_2 + 5% DMSO system is detrimental in the impregnation process and the transfer of material from the solvent.

In SFE, the solvating power of the fluids can be manipulated by changing pressure (P) and/or temperature (T) and, in this way, a remarkably high selectivity can be achieved. This tuneable solvating power of SFE is particularly useful for the extraction of complex samples such as plant materials. It can be seen from Fig. 2 that, at a constant temperature, raising the pressure increases the density of the SCF, i.e., its solvating power becomes greater and more substances are transferred to the supercritical CO_2—meaning that the extraction process is favoured. For this reason, it appears advantageous to carry out the extraction at elevated pressure. An increase in temperature, at constant pressure (100 bar), proved detrimental to the extraction process. For example, increasing the temperature at a pressure of 100 bar caused a decrease in the extraction yield. This phenomenon is attributed to the decrease in the density of the supercritical fluid and, therefore, it is dissolving power. On the basis of these results, it is not advisable to carry out the extraction at 50 °C and 100 bar since the yields are very low. Nevertheless, at higher pressure (500 bar) an increase in the temperature benefits the extraction process due to the increase in the vapour pressure of the substances extracted, a change that more than compensates for the decrease in the density of supercritical CO_2. The SFE was not performed at temperatures above 50 °C in order to avoid thermal degradation of the com-

pounds. The discussion presented is based on reference results [7,8,11].

The extraction conditions of 50 °C and 100 bar of pressure are so unfavourable to the process that an increase in the polarity of the extraction system on adding 5 vol.% of cosolvent does not cause marked changes in the extraction yield.

4.2. Experimental design

The results of the experimental design analysis are gathered in Fig. 3. When the process was performed with pure CO_2, pre-treatment, temperature, pressure and the crossed interactions pressure–temperature and pre-treatment-pressure have a significant influence (95% confidence level) on the process and that influence is positive. Nevertheless, when the process was performed with various solvent–CO_2 mixtures the number of significant variables was reduced. On using cosolvent the variations of density with changes of temperature were less accentuated than when pure CO_2 was used.

The behaviour of the system when we add a cosolvent to the carbon dioxide is different. This addition decreases the number of variables with influence in the supercritical process attributed to a lower change in density with temperature in the process with cosolvent regarding to the process without it.

4.3. Bioassay

It was necessary to perform a general bioassay in order to select the conditions that provide the extracts with the best bioactivity because, in general, the more bioactive the extract, the greater its allelopathic potential [3].

The aim of this study was not to determine specific values, but to attempt to obtain activity profiles on the basis that an extract will be more bioactive when its activity levels persist as the sample is diluted.

The activity profiles, with respect to the control, determined for the extracts obtained in the highest yields (to 500 bar) from samples extracted in the four different solvent systems are shown in Fig. 4. The experimental error was 9.2% approximately. The behaviour of the extracts is very different, making it necessary to perform a cluster analysis and to group the cases.

The results obtained in the cluster analysis are shown in Fig. 5 and it can be seen that three groups are formed. A single criterion does not exist to choose the number of clusters, but most investigators agree that the process should be stopped when a marked change in the distances is observed, i.e. when the bars become larger in the dendrogram [11].

The first group consists of seven elements: all of the extractions with DMSO as a modifier, congealed samples extracted with $CO_2 + 5\%$ methanol, and the congealed sample extracted with pure CO_2 at 35 °C. All of these sets of conditions gave rise to a marked decrease in activity as the dilution was increased; at 1000 ppm samples showed inhibition levels of −92% and this rapidly decreased to −15% at 125 ppm. These conditions led to the worst activity profile in this study.

The congealed sample extracted with pure CO_2 at 50 °C formed a conglomerate that gives rise to a small decrease in the activity on increasing the dilution; 1000 ppm shows 100% activity and 125 ppm shows 55% activity. These conditions represent the best activity profile.

Under the investigated extraction conditions, the density of water is higher than the density of CO_2 except for the pressure of 500 bar and temperature of 35 °C when carbon dioxide is heavier. Those highly water-soluble solutes prefer to partition into the aqueous phase and, if most of water remains in the extraction vessel, the SFE recovery is low. Thus, as the solvent flow was to the bottom of the extractor and as the bioactive substance was water-soluble, it could explain why there is a large difference in the bioactivity of extracts obtained at 500 bar from not-dried leaves at different temperatures. At 35 °C, the lighter water phase would remain in the extractor and the recovery of the bioactive substance would be low, while at 50 °C the water phase would be heavier than CO_2 and it would preferably flow out of the extractor with bioactive substance dissolved in it.

The last group is formed by the extractions carried out with water as cosolvent, dry samples extracted with pure CO_2 and $CO_2 + 5\%$ methanol. In these cases the levels at 1000 ppm are 100%, but an inhibitory effect of around −37% is shown for the 125 ppm dilution. The elements in this cluster show intermediate results in the bioassay.

The best yields were obtained using 5% water as a modifier and the activity profiles for these samples are satisfactory.

5. Conclusions

The study described above allows the following conclusions to be drawn concerning the yield and activity of the extracts:

1. The use of methanol, water and DMSO as modifiers increased the efficiency of extraction of the process study. The best yields were obtained using 5% water as a modifier.
2. At 500 bar the best yields were obtained at a temperature of 50 °C, but at 100 bar the best yields were obtained at 35 °C.
3. All of the extracts obtained are bioactive, but samples congealed extracted with supercritical carbon dioxide at 50 °C and 500 bar show the best activity profile.
4. The dry sample extracted at 500 bar with pure $CO_2 + 5\%$ water gave the best yields and a satisfactory activity profile.

To obtain the best bioassay profile it will be interesting to continue the develop of the process using carbon dioxide as solvent. Nevertheless the addition of a 5% of water increased the extraction yields with good bioactivity levels. These levels are better than those obtained with the other cosolvents studied.

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