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Extraction of natural compounds with biological activity from sunflower leaves using supercritical carbon dioxide

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Abstract

The application of supercritical carbon dioxide in the extraction of bioactive compounds from *Helianthus annuus* L. (sunflower) has been investigated. The influence of different variables, including pre-treatment of the sample, temperature and pressure, was investigated. The samples were either dried or congealed and the extraction conditions were as follows: temperatures of 35, 40, 45 and 50 °C, and pressures of 100, 200, 300, 400 and 500 bar. The best extraction yields were achieved on using a dried sample at a temperature of 50 °C and a pressure of 500 bar.

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

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

1. Introduction

The complex nature of natural products often requires maximum performance from the sample preparation, separation and identification methods used. Natural products are often obtained by conventional approaches involving extraction and separation techniques, such as the use of organic solvents to extract the material and column chromatography and high-performance liquid chromatography (HPLC) to purify it. These techniques involve the use of organic solvents, which are not friendly to our environment, and the conventional separation methods are often tedious, time consuming, require multiple steps and, worse still, the samples are adsorbed irreversibly onto the stationary phase.

In the field of natural products, the new techniques of microwave-assisted extraction, accelerated solvent extraction and supercritical fluid extraction (SFE) utilise smaller amounts of organic solvents. Supercritical carbon dioxide is an alternative that does not have any of the negative effects related to traditional organic solvents [1]. At optimal conditions, SFE offers effective, reproducible and fast extraction [2]. This technique has been proven to produce equivalent or better results than Soxhlet, sonication and accelerated solvent extractions [3].

Extraction with carbon dioxide under supercritical conditions constitutes an emerging technology in terms of environmental impact. The advantages in using carbon dioxide include its lack of toxicity, chemical inertness, low cost and ready availability [4]. Furthermore, the use of carbon dioxide is also beneficial in adding quality to the products obtained since this technique does not give rise to excessive heating, which usually has a negative effect on thermolabile compounds. SFE uses CO_2 instead of organic solvent and CO_2 has unusual properties such as high compressibility, liquid-like density, high diffusivity, low viscosity and low surface tension. As a result, the supercritical fluid shows a greater ability to diffuse into the ultrafine matrix than conventional organic solvents, thus improving the extraction yield of desired materials from complex matrices.

Compared to other extraction techniques, the optimization of a supercritical-fluid extraction procedure is a complex process due the multitude of parameters: extraction time, pressure, temperature, flow, tapping technique and supercritical fluid composition. In addition to the large number of parameters, each factor can also have a marked effect on the extraction efficiency. Therefore, the establishment of the optimal settings is both a very important and potentially

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time-consuming process. Several statistical techniques, such as factorial design and multi-lineal regression, have been employed in the optimization of analytical methods. Factorial design has some advantages in that the global optimum conditions can be provided, large amounts of quantitative information can be obtained and both discrete and continuous factors can be estimated. Factorial designs have been used to determine the effect that numerous parameters have on the process, including temperature, pressure, pre-treatment of sample, extraction time, fluid flow-rate and addition of modifier [5].

Several studies have shown that *Helianthus annuus* L. (sunflower) contains chemical substances that have bioactive properties [7–11]. The extracts of this plant can be used as a natural herbicide to reduce the dependence on synthetic herbicides in the control of crops. The sunflower is one of the most widely studied plants in terms of its bioactivity and the first reference concerning its allelopathic effects was published in 1931. However, in the vast majority of these cases the bioactivity of aqueous extracts from the leaves was studied and extraction with non-polar solvents has rarely been investigated [6,12].

In previous work, we began to address the extraction of bioactive substances from sunflower leaves. Studies were carried out on the pre-treatment of samples and the effect of the presence of a cosolvent on both the yield and the bioactivity of the resulting extracts. The experiments involved the use of low and high extremes of temperature and pressure, i.e. 100 and 500 bar pressure and 35 and 50 °C. However, it is also important to study intermediate values of pressure and temperature. It was decided to use carbon dioxide as the extraction solvent on the basis of the promising results obtained in previous work [6,12].

The pre-treatment of sunflower leaves is a crucial factor in terms of the yield and bioactivity of the extracts. When the extractions were carried out at extreme temperature and pressure values it was found that the dried samples gave the best yields and the congealed samples the best biological activity profiles. As a result, it was decided to carry out a more in-depth study on the influence of pressure and temperature for both types of pre-treatment.

In allelopathy studies, bioassays are useful tools for the screening of plant species for allelopathic potential and for following the bioactivity of crude extracts, fractionated components and pure compounds. Strategies for allelochemical discovery involve the screening of crude extracts and purified compounds for biological activity. This initial bioassay must be quick, economical and relevant to the system in question. A bioassay-directed fractionation procedure for the isolation of pure compounds is followed by bioassays; therefore the full process (extraction, isolation and purification steps) depends on the bioassay results. At this stage we are at the first level; the study of an efficient new extraction technique for a solid extract without losing bioactivity. The next level of the study will involve the characterization of the chemical structures and the biological activity profiles of the final bioactive compounds found, and comparison of these structures with those of compounds previously isolated using conventional extraction methods.

The work presented here involved taking the experimental data and carrying out a multilevel factorial design in order to analyze the effect that the temperature and the operational pressure have on the extraction yield of bioactive compounds using supercritical carbon dioxide. The program Statgraphics Plus 5.1 (Statistical Graphics Corp.) was subsequently used to develop an empirical equation to predict the yields obtained in the extraction processes.

2. Materials and methods

2.1. Samples and chemicals

Leaves of *H. annuus* L. (variety Aitana) were collected in July 2005 during the third plant development stage [7] (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 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.

2.2. Extraction at high pressure

The extractions were carried out in an Isco extractor (Nebraska, USA, model SFX 220). The equipment consisted

Table 1
Reagents

Reagent	Purity	Company	Use
Carbon dioxide	99.995%	Carburos Metallic	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

of an extractor, an SFX 200 controller, a restrictor and a syringe pump. A schematic representation of the equipment and further details can be found in a previous publication [12].

The operating methodology involved loading the extraction cartridge with approximately 2 g of sample, which had previously been homogenized to maintain a constant apparent density in all experiments. The cartridge was then introduced into the extractor and left for 15 min to reach the operating temperature. The pump was loaded with carbon dioxide until the operating pressure was reached in the pump. The automatic decompression valves of the extractor were closed, the valve connecting the pump was opened and the extractor was opened. The extractor was then pressurized with CO_2. A period of 15 min of static extraction was allowed to elapse.

When a balanced state had been attained, the micrometric valve was opened up from the thermostatically controlled restrictor (at 40 °C) until a constant flow of 7.03 mmol/min was achieved. An extraction was then carried out for 5 h. The experiments on each sample were carried out in duplicate in order to evaluate the variability of the measurements.

The extracts were collected in glass tubes containing methanol and were stored at 4 °C with the exclusion of light until subsequent analysis. After the extraction process was complete, the solvent was removed with a nitrogen stream at a temperature of 40 °C.

Intermediate 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 set at the limit of the equipment used (500 bar). Experiments were carried out at the relatively low temperatures of 35, 40, 45 and 50 °C due to the possible thermal degradation of the substances.

2.3. Design experiment

A multilevel factorial design was carried out in order to determine the factors that influence the process and the relationships between them. The variables selected to perform the experimental design were as follows: pre-treatment of the sample, extraction temperature and extraction pressure. The physical values are shown in Table 2. On the basis of this design, a total of 40 experiments were carried out in a random way in order to minimize errors.

The experimental data were used in conjunction with the program Statgraphics Plus 5.1 (1994–2001, Statistical Graphics Corp.) to develop an empirical correlation that predicts the yields obtained in the extraction.

Table 2
Physical values in the experimental design

Factor	Experimental variable	Levels				
A	Pre-treatment	Congealed			Dry	
T	Temperature (°C)	35		40	45	50
P	Pressure (bar)	100	200	300	400	500

2.4. Coleoptile bioassay

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

Wheat (*Triticum aestivum* L. cv. Duro) seeds 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 caryopses were removed from the shoots. The latter were placed in a guillotine and the apical 2 mm sections were cut off and discarded. The next 4 mm lengths 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 [7].

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 DMSO (5 μl/ml of plug) was therefore 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.

2.5. Cluster analysis

Statistical treatments were performed using the SPSS 10.0 program (Statistical Package for Social Sciences). Association 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 mg of extract/100 g of dry leaves are shown in Fig. 1 for an extraction time of 5 h under different conditions of pressure, temperature and pre-treatment of the sample.

The results from the analysis of the experimental design are shown in Table 3. The equation obtained is represented graphically in Fig. 2 for the dry sample under different operating conditions.

The results of the bioactivity assays for the extracts obtained at 300 and 500 bar of pressure are shown in Fig. 3. The data

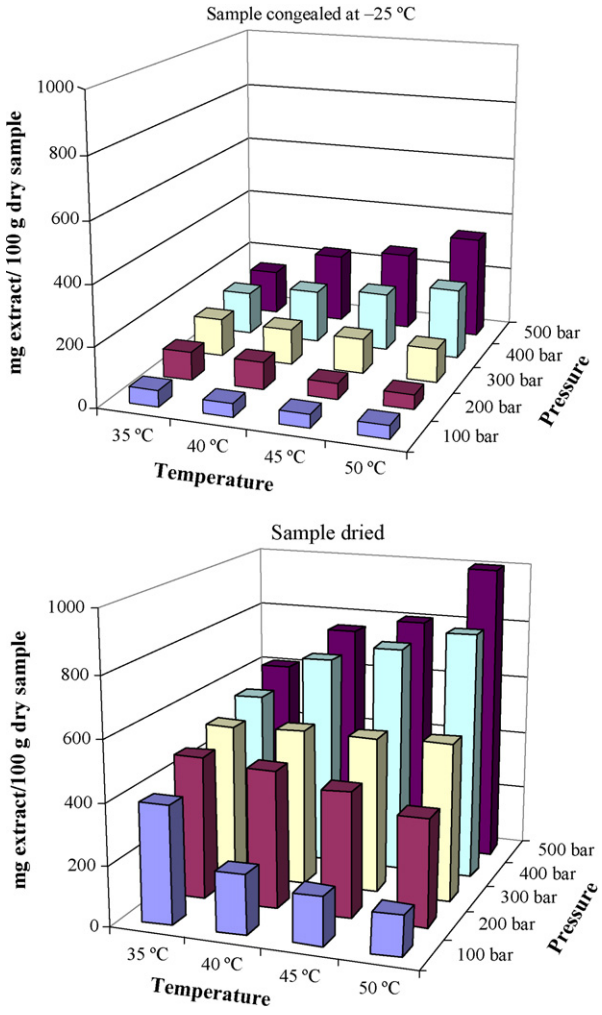

Fig. 1. Extraction yields.

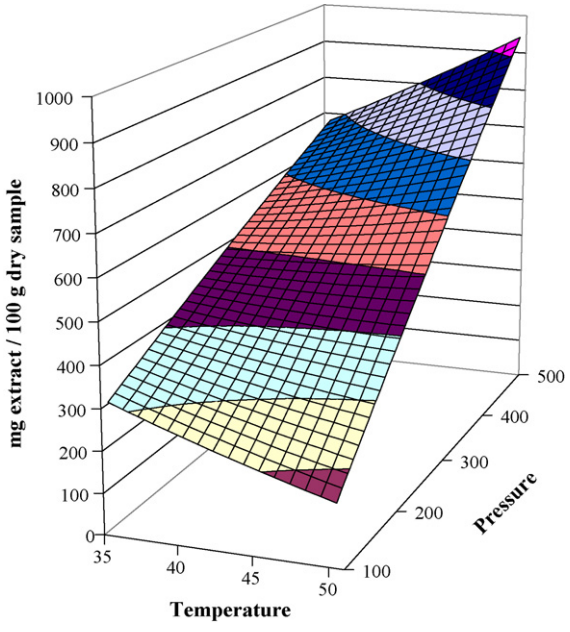

Fig. 2. Estimated yields of bioactive compounds with supercritical carbon dioxide using the empirical correlation.

Table 3
Estimated effects and analysis of variance for the process with supercritical carbon dioxide

Variable	Effects	*p*-value
Pressure (*P*)	390.69	0.0000
Temperature (*T*)	47.29	0.0010
Pre-treatment (*A*)	399.28	0.0000
PP	–16.38	0.5049
PT	212.95	0.0000
PA	184.08	0.0000
TT	12.99	0.5739
TA	8.42	0.5413

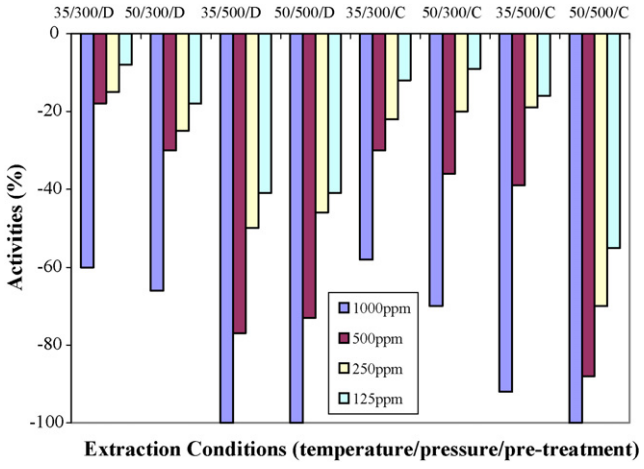

Fig. 3. Bioactivities of extracts obtained at 300 and 500 bar.

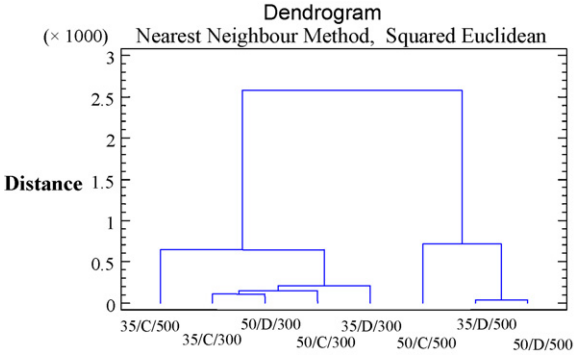

Fig. 4. Cluster analysis.

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 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. 4.

4. Discussion of the results

4.1. Extraction yields

According to our experimental data (Fig. 1), the best extraction yields were obtained from the dried samples. The storage of the raw material once the leaves had been cut is a fundamental factor, since it is crucial to know how the extraction yield and bioactivity of substances are influenced by the treatment that they undergo. Furthermore, two simultaneously studied variables that significantly influence the selectivity of the extraction process are the pressure and the temperature.

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.

It can be seen from Fig. 1 that, at a constant temperature, raising the pressure increases the density of the supercritical fluid, 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 and 200 bar), proved detrimental to the extraction process. For example, increasing the temperature at a pressure of 100 and 200 bar led to a decrease in the extraction yield. This phenomenon is attributed to the decrease in the density of the supercritical fluid and, therefore, its dissolving power. On the basis of these results, it is not advisable to work at 50 °C and 100 bar, since the yields are very low. Nevertheless, at higher pressure (400 and 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 compounds.

These results are consistent with those reported in the literature [13,14].

At 300 bar the extraction yield is independent of the temperature. There is compensation between the decrease in the supercritical carbon dioxide density and the increase in vapour pressure of the compounds as the temperature increases. For this reason, it is very important to study the behaviour of systems at this pressure.

Bearing in mind the results mentioned above, it was pertinent to carry out general biological assays on the extracts obtained at 300 bar, given that the temperature had very little effect on the extraction yield, and also those obtained at 500 bar, as these give the highest extraction yields.

4.2. Analysis of the experimental design

The results from the analysis of the experimental design are shown in Table 3. The estimated effects and interactions between the range of variables studied and the analysis of variance of the extraction process are also given. The sign associated with each of the effects indicates a positive or negative influence on the yield caused by the variable in question. The degree of significance of each factor is represented in Table 3 by its p-value; when a factor has a p-value of less than 0.05 it influences the process in a significant way for a confidence level of 0.95.

The results obtained show that pressure, temperature, pre-treatment and the combined interactions pressure/temperature and pressure/pre-treatment have a significant influence (95% confidence level) on the process and that the influence is positive.

Empirical correlations were obtained using the experimental data and the program Statgraphics. These correlations relate the variables that influence the extraction process of bioactive compounds with supercritical carbon dioxide. The expression obtained is given below (Eq. (1)).

$$R = 10.02 \times 10^2 - 1.91P - 27.96T - 37.73A - 2.04 \times 10^{-4}P^2 + 7.09 \times 10^{-2}PT + 0.46PA + 0.11T^2 + 0.56TA \quad (1)$$

where R is the extraction yield [mg extract/100 g dry sample], A the pre-treatment, T the temperature [°C] and P is the pressure [bar]. The resulting correlation coefficient is 0.97.

Eq. (1) is represented graphically in Fig. 2 for the dry sample under different operating conditions. A detailed analysis of the graph indicates that the highest yield is obtained at 50 °C and 500 bar; other aspects, such as bioactivity and economic considerations, must also be taken into account when choosing an appropriate pressure for a process.

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 [7]. 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 at 300 and 500 bar from congealed and dry samples are represented in Fig. 3. The experimental error was around 5.9%. It can also be seen from Fig. 3 that there is a clear difference between the activity levels of the extracts obtained at the different pressures selected.

The extracts obtained at 500 bar from dry samples exhibited −100% activity at 1000 ppm and values near to −50% for the 250 ppm dilution. The extract obtained with congealed samples at 500 bar and 50 °C also showed −100% activity at 1000 ppm and values near to –50% for the 125 ppm dilution. These extracts showed the best bioactivity profiles in that the activity level did not decrease markedly upon dilution.

The extracts obtained at 500 bar and 35 °C with congealed samples exhibited a different type of behaviour. In these cases, the activity level decreased drastically on dilution. The activity

profiles of the extracts obtained at 300 bar also show a marked decrease in the activity level on dilution.

The results obtained in the cluster analysis are represented in Fig. 4 along with the order in which the eight experiments for the general bioactivity assay are grouped. 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 [15]. Samples extracted at 300 bar and congealed samples extracted at 35 °C and 500 bar formed a conglomerate that shows a marked decrease in activity as the dilution increased.

5. Conclusions

On the basis of the experimental results, it can be concluded that, out of all of the processes reported here using supercritical carbon dioxide as an extraction solvent, the best extraction yields were obtained at a pressure of 500 bar and a temperature of 50 °C on dried samples. However, the best activity profiles were obtained from congealed samples extracted at 500 bar and 50 °C.

A cluster analysis was carried out in order to assess whether a subdivision of the results into groups was possible. It was found that the two experiments carried out at 500 bar on dried samples gave similar results to those from the extract obtained at 500 bar and 50 °C on congealed samples. It is advisable to work on dried samples on the basis that the extraction yields are higher and the activity profiles are acceptable. The leaves were dried in the field and, as a result, such a process is viable from an industrial point of view.

The laboratory-scale study on the congealed samples was nonetheless valuable as it highlighted the better biological activity profile shown by the extract obtained at 50 °C and 500 bar. This aspect could be of particular interest for the identification of the substances extracted.

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