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# Effect of the pre-treatment of the samples on the natural substances extraction from *Helianthus annuus* L. using supercritical carbon dioxide

L. Casas [a,*], C. Mantell [a], M. Rodríguez [a], M.D. Gordillo [a], A. Torres [b], F.A. Macías [b], E. Martínez de la Ossa [a]

[a] *Department of Chemical Engineering, Food Technology and Environmental Technologies, Faculty of Science, University of Cádiz, Box 40, 11510 Puerto Real, Cádiz, Spain*
[b] *Department of Organic Chemistry, Faculty of Science, University of Cádiz, Box 40, 11510 Puerto Real, Cádiz, Spain*

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**Abstract**

The extraction of bioactive compounds from sunflowers (*Helianthus annuus* L.) with supercritical carbon dioxide has been studied. The samples were treated in four different ways and the effects of two factors (pressure and temperature) were investigated at 100, 500 bar and 35, 50 °C. The best yields were obtained using a high temperature and a high pressure (50 °C and 500 bar). The dry samples produced better extraction yields than the moist samples. The bioactivities of the extracts were compared for the samples treated in different ways. The best activity profiles were obtained for the moist samples extracted at 35 °C and 500 bar.


*Keywords:* Sunflower; *Helianthus annuus* L.; Natural product; Supercritical carbon dioxide; Extraction; Bioactive

## 1. Introduction

Modern crop production technology depends on synthetic insecticides, herbicides and other chemicals. However, the economic and environmental costs of such procedures are rapidly increasing. The increasing dependence on chemicals for pest control poses a serious threat because, after pesticides have been applied to a field, some of their degradation products are adsorbed into the soil and persist for long periods of time. A major problem faced in chemical weed control is the development of resistant weed biotypes. The indiscriminate use of herbicides has resulted in an increasing incidence of resistance in weeds to some herbicide classes, shifts in weed populations to species that are more closely related to the crop they infest, and environmental pollution and its associated health hazards [1]. Studies on allelopathic interactions may help in overcoming such problems through the development of crop varieties that have a greater ability to smother weeds, the use of natural phytotoxins from plants or microbes as herbicides, or the use of synthetic derivates of natural products as herbicides [2].

The discovery of new allelochemicals from plants or microbes has attracted a great deal of attention in the last 20 years [3]. Allelochemicals have been implicated as biocommunicators and are potential sources of new structural types of pesticides with new modes of action that may be less harmful than those presently used in agriculture. These compounds also have potential as new pharmaceuticals. Allelopathy, an emerging branch of applied sciences that involves the study of biochemical plant–plant and plant–microorganism interactions, may help in providing a new generation of natural phytotoxins and mycotoxins as models for natural agrochemicals and pharmaceuticals [4,5].

* Corresponding author. Tel.: +34 9560 16579; fax: +34 9560 16411.
*E-mail address:* lourdes.casas@uca.es (L. Casas).

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Plants have their own defence mechanisms and allelochemicals are in fact natural herbicides. One way to use allelopathy in agriculture is through the isolation, identification and synthesis of active compounds from allelopathic plant species. Sunflower species are allelopathic in nature [4].

Since active compounds in herbal plants are usually present in low concentrations, a great deal of research has been done to develop more effective and selective extraction methods for the recovery of the desired compounds from the raw materials. In conventional extraction methods such as hydrodistillation (steam distillation) and solvent extraction, there are few adjustable parameters to control the selectivity of the extraction processes. Therefore, the development of alternative extraction techniques with better selectivity and efficiency is a highly desirable target. The high price of organic solvents and increasing concern over environmental factors also drive the development of new processing techniques. Since the late 1970s, supercritical fluid extraction (SFE) has been used to isolate natural products but for a long time this technique was only applied to a few products [6]. The development of processes and equipment over the last three decades is today beginning to pay off and industries are becoming more and more interested in supercritical techniques. Consequently, supercritical fluid extraction (SFE) was introduced as an environmentally responsible and efficient extraction technique for solid materials. This technique has been extensively studied for the separation of active compounds from herbs and other plants [7–10] as well as for the samples for the alimentary industry [11,12].

In most of these studies carbon dioxide is 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. The high quality of the products obtained by supercritical fluid extraction (SFE) was highlighted by List et al. [13], who observed that the extracted products do not need any particular refining operation as the vegetable matter does not experience any stress during treatment.

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 [14], 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 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 [15]. 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.

In allelopathy studies, bioassays are useful tools for 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. Now we are in the first level; it is the study of an efficient new extraction technique for solid extract without losing bioactivity. One follow level of the study will be to characterize the chemical structure and the biological activity profile of the final bioactive compounds found, and to compare these structures with the previously isolated ones using the conventional extraction methods.

The conventional extraction techniques have been extensively applied for the isolation of bioactive compounds from sunflower species, such as organic solvent extraction.

In continuation of the systematic study of the allelopathic activity of different cultivar of *Helianthus annuus* L., it reported the isolation, structural elucidation and allelopathic bioassay of many different compounds. According to their carbon skeleton, these compounds have been classified as terpenoids: monoterpenes, bisnorsesquiterpenes, sesquiterpenes, sesquiterpenes lactones, diterpenes, triterpenes and sterols; fatty acids, phenolic compounds: flavonoids, coumarins, and simple phenolic.

Sesquiterpenes are one of the most frequent terpenes implicated in allelopathic processes. In this way sesquiterpenes lactones and a number of compounds from novel sesquiterpenes family heliannuol have been isolated from sunflower cultivar (Figs. 1 and 2) [16,17].

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

## 2. Material 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 [4] (plants 1.2 m tall with flowers, 1 month before harvest) and were provided by Rancho de la Merced, Agricultural Research Station (CIFA), Junta of Andalucía, Jerez, Spain.

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

- Sample refrigerated at 4 °C for 30 days (denoted as R).
- Sample congealed at −25 °C for 30 days (C).
- Sample dried in an oven at 40 °C until a constant weight was reached (E).
- Sample dried at room temperature (25 ± 1 °C) until a constant weight was reached (P).

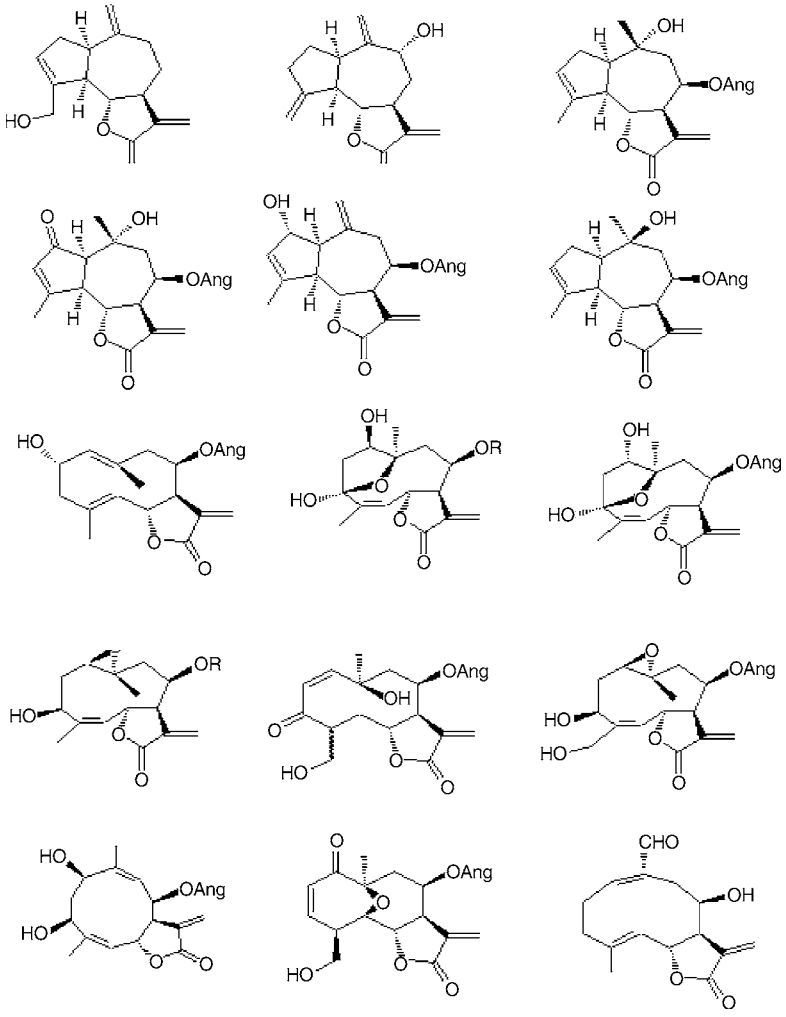

Fig. 1. Bioactive sesquiterpene lactones from sunflowers leaves.

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 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 pump (Isco model 260D), which allowed a constant flow of solvent.

The samples leave the vessel through a restrictor, which was thermostated coaxially to avoid obstructions due to the solidification of $CO_2$. Fig. 3 shows a schematic diagram of the SFE apparatus used in this research.

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 tried, with a lower limit of 100 bar chosen because it is near to the

Table 1
Reagents

| Reagent | Purity | Company | Use |
|---|---|---|---|
| Carbon dioxide | 99.995% | Carburos Metálicos | Extraction at high pressure |
| Methanol | PA | Panreac | Collection of extracts |
| 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 |

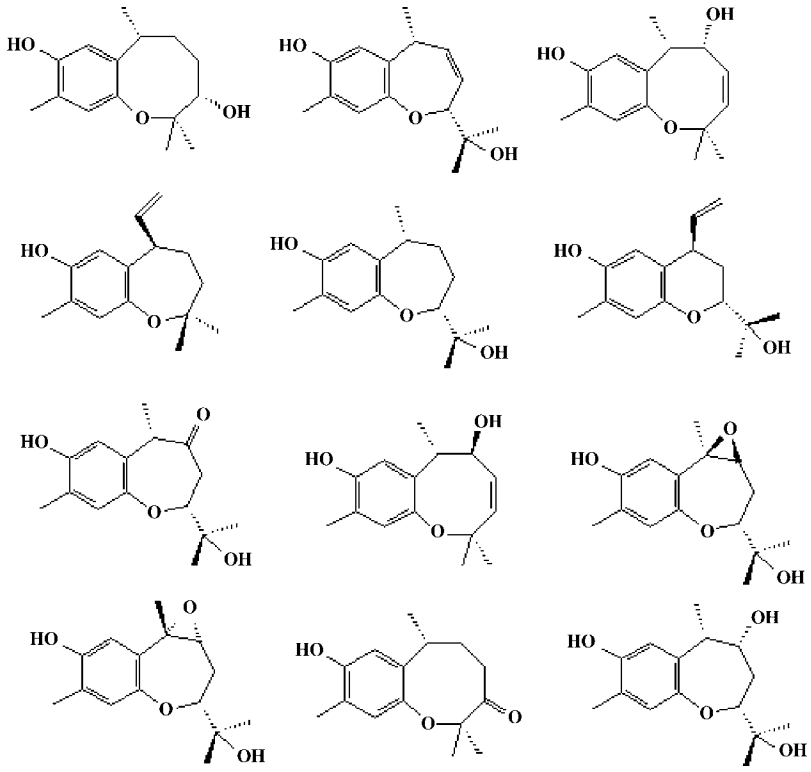

Fig. 2. Bioactive heliannouols from sunflowers cultivar.

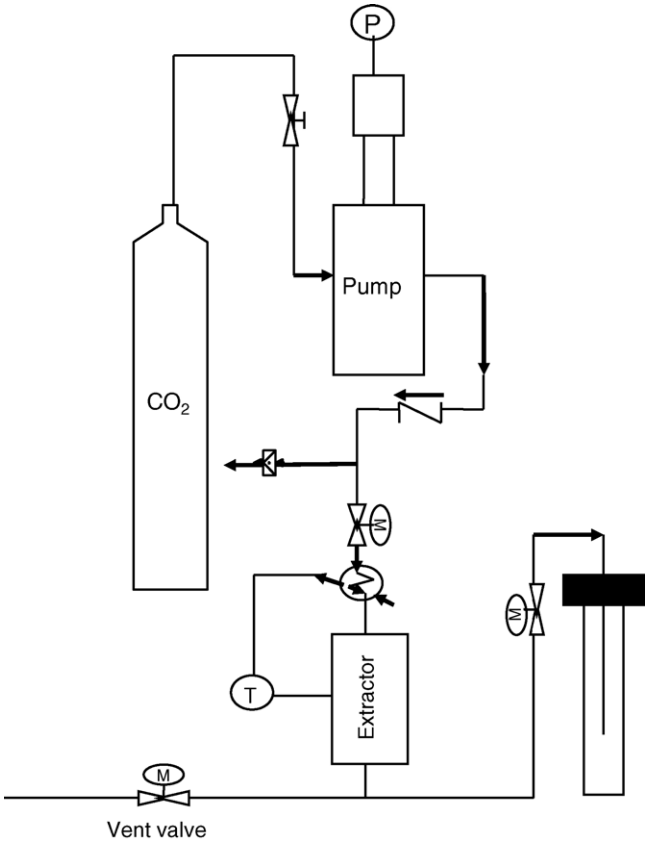


Fig. 3. Schematic diagram of the equipment used for the SFE.

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 low temperatures of 35 and 50 °C due to the possible 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 confirm the results. The extracts were collected in methanol and were stored at 4 °C with the exclusion of light. The methanol was later evaporated and the resulting extract weighed.

### 2.3. 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 section.

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 dimethyl sulfoxide

(DMSO) and diluted to the final bioassay concentration. Parallel controls were also run [18].

A sample (16 mg) of each crude extract obtained under the conditions described in the 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 6.00 rpm in a roller tube apparatus for 24 h at 22 °C in the dark. The coleoptiles longitudes 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.

### 2.4. Cluster analysis

Hierarchical cluster analyses 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

Fig. 4 shows extraction yields expressed as mg of extract/100 g of dry leaves for an extraction time of 5 h under different conditions of pressure, temperature and pre-treatment of the sample. The confidence interval for these results is 95%.

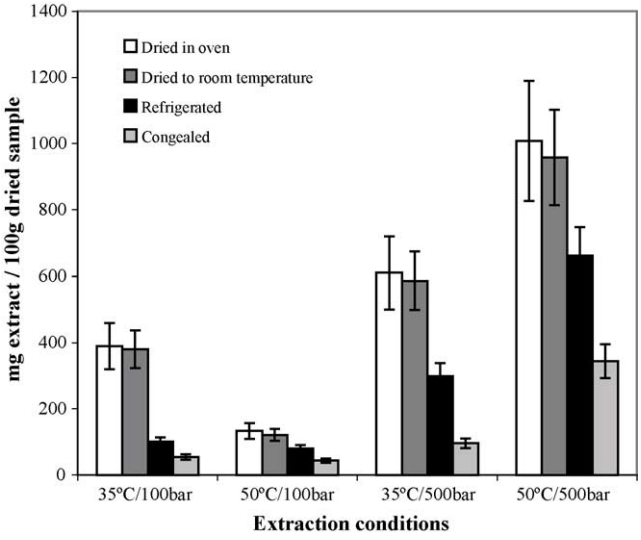


Fig. 4. Influence of pressure and temperature on extraction yield.

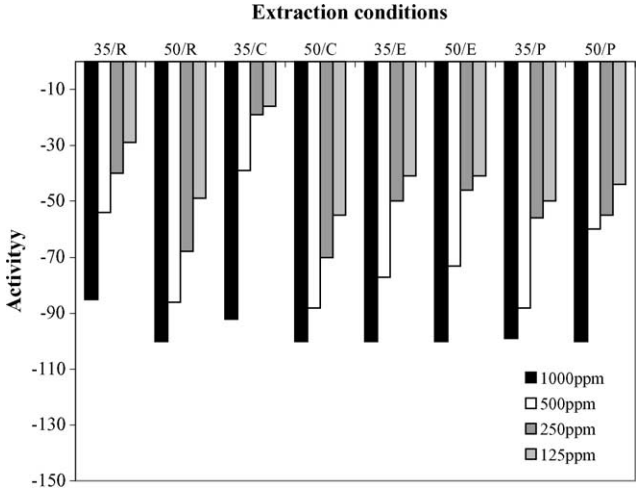


Fig. 5. Bioactivities of extracts obtained at 500 bar.

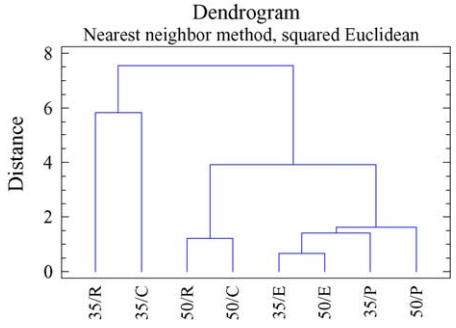


Fig. 6. Cluster analysis.

Fig. 5 shows the results of the bioactivity assays for the extracts with the best extraction yields for each of the four pre-treatment methods (500 bar of pressure at the two studied temperatures). 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.

Fig. 6 shows the statistical result of the cluster analysis applied to the activity data for each of the sets of conditions employed in the bioactivity study.

## 4. Discussion of the results

### 4.1. Extraction yield

The storage of the raw material once the leaves have 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.

According to our experimental data (Fig. 4), the best extraction yields were obtained for the oven-dried samples and those dried at room temperature. Significant differences at the 95% confidence level were not detected in the extraction yields obtained when the extraction processes were carried out with oven-dried samples and samples stored at room temperature. On the basis of these results, the most appropriate drying process will be defined by economic factors.

A freshly extracted sample has high moisture content (76%) and this can cause mechanical difficulties, such as restrictor clogging due to ice formation. It has been reported [7] that water is only 0.3% soluble in supercritical $CO_2$ but, despite this limited solubility, could play an important role in the extraction process. The moisture from the cooled and 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. The significant differences between extraction yields of the cooled and congealed samples can be attributed to the loss of moisture (15%) in the former case during storage.

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. 4 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, 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 (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.

### 4.2. Bioassay

It is 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 is its allelopathic potential [18].

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.

Fig. 5 shows the activity profiles, with respect to the control, determined for the extracts obtained in the highest yields (500 bar) from samples treated in four different ways. All extracts exhibited significant activity superior to 1000 ppm (−92%) and only the assays on samples obtained at 500 bar and 35 °C from refrigerated samples show a value down to this (−85%).

All of the extracts, with the exception of that obtained at 35 °C and 500 bar with the congealed samples, give rise to values that are superior or near to −40% for the 250 ppm dilution. This shows that the activity level does not decrease drastically with dilution. This extract is the only one that presents values down to −20% for the last dilution studied. The extract obtained at 35 °C and 500 bar from the refrigerated sample also shows a lower activity than the other samples (−29%) at the 125 ppm dilution (with the exception mentioned above).

The activity profiles of the extracts obtained from oven-dried sunflower leaves and those stored at room temperature are very similar. This shows that a temperature variation does not cause appreciable changes in the bioactivity of the samples. In contrast, in the case of the refrigerated and congealed samples, an increase in the temperature increases the activity profiles. The extracts obtained at 50 °C and 500 bar from the refrigerated and congealed samples show the best bioactivity profiles, but their extraction yields are surpassed by the extracts obtained from dry samples. Therefore, 500 bar, 50 °C and refrigerated samples are the recommended conditions to obtain the extracts because they show the best bioactivity profiles in spite of the fact that they do not show the maximum yield.

Fig. 6 shows the results obtained in the cluster analysis and the order in which the eight experiments for the general bioactivity assay are grouped. According to this analysis, eight clusters are initially formed. In the following step, samples dried in an oven at 35 and 50 °C, respectively, are united in a group, since they are very close to one another. A total of seven clusters are then left. Assays carried out at 50 °C on refrigerated and congealed samples are subsequently united to leave six groups. This process continues until a single large group is formed that contains all the cases. This final situation is shown in Fig. 6.

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 [19]. Samples refrigerated and congealed at 35 °C and 500 bar

are separated into two groups that show a marked decrease in activity as the dilution is increased. Samples congealed and refrigerated at 50 °C and 500 bar form a conglomerate that corresponds to a small diminution in the activity on increasing the dilution. This trend supports the analysis represented in Fig. 5.

## 5. Conclusions

The study described above allows the following conclusions to be drawn concerning the yield and activity of the extracts. The moisture present in the raw material (sunflower leaves) has a negative influence on the extraction process. This can be seen from Fig. 4, where extraction yields of congealed and refrigerated samples are low with respect to those obtained from previously dried samples under given conditions of pressure and temperature. The extracts obtained from moist samples, at 50 °C, show better results in terms of bioactivity than the compounds extracted from previously dried samples, as can be seen in Fig. 5. In these cases, it is appropriate to undertake an economic analysis in order to establish a balance between the operation costs and the quality of the resulting product.

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