Supercritical fluid extraction of bioactive compounds from sunflower leaves: comparison of analytical and pilot-scale extraction
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Supercritical fluid extraction of bioactive compounds from sunflower leaves: comparison of analytical and pilot-scale extraction

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

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

Abstract

Supercritical fluid extraction (SFE) from natural products has been widely studied as an alternative to the use of traditional techniques in the production of bioactive compounds. It has been demonstrated that this process is particularly useful when treating thermolabile compounds and solvent pollution has to be avoided.

SFE can be applied to systems of different scales, from analytical scale, to pilot plant scale and up to large industrial scale.

In most of these studies carbon dioxide was used as the solvent because of its relatively low critical temperature (31.1 ºC), 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 modifier, water is would be a good cosolvent in natural products.

The work described here involved the extraction of bioactive compounds from the sunflower (Helianthus annuus L) with CO_2 and CO_2+H_2O.

The extractions were carried out in an analytical Isco extractor with one extractor with a maximum capacity of 10 ml and in a pilot plant from Thar Technology provided with an extraction vessel with a capacity of 2 L. The experimental data obtained of both were compared and the implications of this analysis on the development of scale-up procedures were also discussed.

Keywords: supercritical fluid extraction, *Helianthus annuus L.*, analytical scale, pilot plant scale

1. Introduction

Interference of weeds with agricultural crops causes huge economic losses to farmers in two ways. Firstly, it reduces crop quality and quantity, and secondly it increases the cost of labour and herbicides to control them [1]. In the 60 years since its introduction, the area of chemical weed control has been a dynamic research field requiring constant innovation. The need for better tools, possessing broader weed control spectra and appropriate environmental behaviour, in conjunction with an increasing effort to cope with evolved weed resistance to herbicides has primarily been addressed by massive synthetic chemistry programs that have generated numerous compounds.

In this regard, greater attention has recently been paid to the use of allelopathic plants and their products to manage weeds in a sustainable manner [2]. A number of plants have been shown to control agricultural weeds when used as mulch under field conditions without affecting the growth and yield of the crop [3, 4]. It is therefore worthwhile to explore plants with strong allelopathic activity for the control of agricultural weeds. Chemical studies of *Helianthus annuus* L (sunflower) have shown that this specie is a rich source of compounds with a wide spectrum of biological activities, including potential allelopathy.

In allelopathy studies, bioassays are useful tools for the screening of plant species for allelopathic potential to follow 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) is very dependent on the bioassay results.

The traditional methods for the extraction of plant materials include steam distillation and organic solvent extraction using percolation, maceration or Soxhlet techniques. These procedures, however, have distinct drawbacks such as time-consuming and labour-intensive operations, handling of large volumes of hazardous solvents and extended concentration steps that can result in the loss or degradation of target analytes. Moreover, there is increasing interest in alternative extraction technologies that consume smaller quantities of organic solvent because of the rising solvent acquisition and disposal costs and regulatory restrictions [5].

Supercritical fluids have been shown to exhibit several advantages in the extraction of natural products from plant matrices. The combined liquid-like solvating capabilities and gas-like transport properties of supercritical fluids make them particularity suitable for the extraction of diffusion-controlled matrices such as plant tissues. Moreover, the solvent strength of a supercritical fluid can be easily tuned by simply changing the applied pressure and/or temperature.

Carbon dioxide, the most commonly used supercritical fluid, has the advantages of being non-flammable, fairly non-toxic, cost-effective and easily removed from the extract following decompression. Finally, due to its relatively low critical temperature (31.1 ºC), thermal sample decomposition is reduced. Pure CO_2, however, is not an

appropriate extraction fluid for polar analytes and retentive matrices. In order to enhance the solvating power of CO_2, the addition of a small amount of a modifier solvent is required [6].

Supercritical fluid extraction (SFE) can be applied to systems on a range of different scales, for instance from an analytical scale (less than a gram to a few grams of sample) to pilot plant scale (several hundred grams or kilograms of sample) and even up to large industrial scale (tons of raw material).

The work described here involved the extraction of bioactive compounds from the sunflower (*Helianthus annuus* L) with CO_2 and CO_2+H_2O at analytical and pilot plant scales.

2. Materials and methods

2.1 Samples and chemicals

Leaves of *Helianthus 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 de 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 ºC
- sample dried at room temperature (25 ºC ± 1 ºC) until a constant weight was reached

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

Reagent	***Purity***	***Company***	***Use***
Carbon dioxide	99.995%	Carburos Metallic	Extraction at high pressure
Water	Milli Q		Cosolvent
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

Table 1. Reagents.

2.2 Analytical-scale SFE

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 CO_2 was introduced by syringe pump Isco model 260D, and the cosolvent was introduced by syringe pumps Isco model 100 DX,

both allowed a constant flow of solvents. A schematic diagram of the SFE apparatus used in this research is shown in Fig. 1.

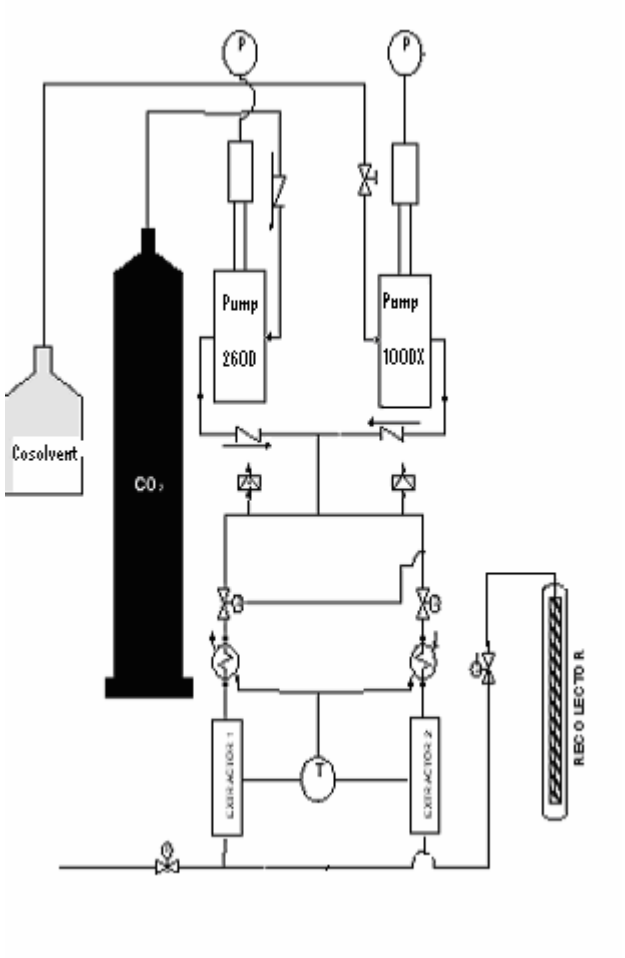

Figure 1. Schematic diagram of the analytical equipment.

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 minutes to reach the operating temperature. The pumps were loaded with carbon dioxide and water 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 and water.

When a balanced state had been attained, the micrometric valve was opened up from the thermostatically controlled restrictor (at 40 ºC) until a constant flow of 10 g/min was achieved. In order to determine the influence of cosolvent, water was tested at 5% of volume. The experiments on each sample were carried out in duplicate in order to evaluate the variability of the measurements.

In order to achieve complete extraction of the substances in question, a relatively long extraction time was used (5 hours). Extreme conditions of pressure were tried, 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 (400 bar). Experiments were carried out at the low temperatures of 35 and 50 ºC due to the possible degradation of substances. As the CO_2 evaporated at the restrictor outlet due to decompression, the extracted material was collected in a glass glass tubes containing methanol and were stored at 4 ºC 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 ºC.

2.3 Large-scale SFE

Preparative SFE experiments were performed on a pilot plant from Thar Technology (Pittsburgh, PA, USA, model SF2000) provided with a extraction vessel (capacity of 2 L) and two pumps with a maximum flow rate of 150 g/min of carbon dioxide and 50 g/min of cosolvent. A thermostated jacket allowed control of the extraction temperature. The cyclonic separator allowed periodical discharge of the extracted material during the SFE process. A schematic diagram of the SFE apparatus used in this research is shown in Fig. 2.

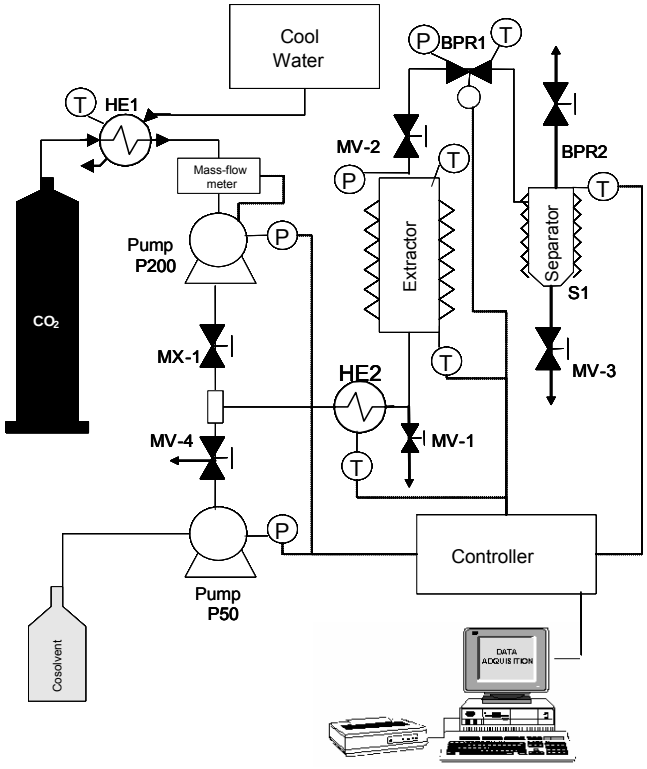

Fig. 2. Schematic diagram of the equipment used for the SFE.

The operating methodology involved loading the extraction cartridge with approximately 190 g of the sample, which had previously been homogenized in order to maintain a constant apparent density in all experiments. The valves MV-1 and MV-2 were closed and the valves MX-1 and MV-4 were opened.

When the heat exchanger (HE1) was at a temperature of approximately 1 ºC the CO_2 cylinder was opened. All of the operating conditions apart from the flow of CO_2 were established and when the system had stabilized the extractor was pressurized with CO_2. When the pressure in the extractor was near to the desired extraction pressure valve MV-2 was opened to start the flow of solvent. When a balanced state had been attained, BPR1 was opened automatically and the process began.

The extracts were collected in S1 and acetone was added to dissolve the extract. Valve MV-3 was subsequently used to collect the extracts in glass bottles and these were stored at 4 ºC with the exclusion of light. The acetone was later evaporated and the resulting extract weighed.

The experiments on each sample were carried out in duplicate in order to evaluate the variability of the measurements.

The extraction conditions were the same of analytical scale.

2.3 Coleoptile bioassay

Bioassays constitute an important tool to evaluate the inhibiting or stimulating activity in terms of growth of the substances extracted 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 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 per 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 ºC 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.

2.4 Scale-up criteria

Geometric dimensions

One scale-up criterion requires maintaining a constant geometric dimensiones. It is can be calculated from Eq. (1):

$$\left(\frac{H}{d}\right)_{extractor\ 1} = \left(\frac{H}{d}\right)_{extractor\ 2} \quad (1)$$

where H and d (cm) are height and diameter of each extractor.

Constant Re

The scale-up criterion of maintaining constant *Re* with a change in process scale is based on a combination of dimensional analysis and similarity, and one might reasonably expect to obtain similar flow patterns within the mixing volume provided the *Re* is the same at each scale of operation. Values of Reynolds number (*Re*) can be calculated from Eq. (2):

$$\mathrm{Re}_{extractor\ 1} = \mathrm{Re}_{extractor\ 2} = \frac{\rho_d\ D_p\ v}{\mu_d} \quad (2)$$

where ρ_d (g/mL) is the solvent density, μ_d (g/cms) the solvent viscosity, D_p (cm) diameter of particle and v (cm/min) is the average velocity of the solvent. Estimates of the average velocity can be calculated from Eq. (3):

$$v = \frac{Q_v}{A} \quad (3)$$

where Q_v (cm^3/min) is the volumetric flow rate and A (cm^2) is the cross-sectional area of both extractor.

Constant residence time

Another scale-up criterion requires maintaining a constant residence time tr. Values of residence time can be calculated from Eq. (4):

$$(t_r)_{extractor\ 1} = (t_r)_{extractor\ 2} = \frac{V\ \varepsilon}{Q_v} \quad (4)$$

where V (cm^3) is the volume of extractor, ε is the porosity and Q_v (cm^3/min) is the volumetric flow.

Other investigators [10] propose relations that allow predicting the behaviour of systems when increasing the scale. According to them is necessary maintaining constant in both extractors that relation 5:

$$\left(\frac{Q_m\ d}{M}\right)_{extractor\ 1} = \left(\frac{Q_m\ d}{M}\right)_{extractor\ 2} \quad (5)$$

where Qm (g/min) is the masic flow, d (cm) is the diameter of extractors and M (g) is the mass of sample.

3. Experimental results

The extraction yields expressed as *mg of extract/100 g of dry leaves* are shown in Figure 2 for an extraction time of 5 hours under different conditions of pressure, temperature and pre-treatment of the sample for each solvent system employed.

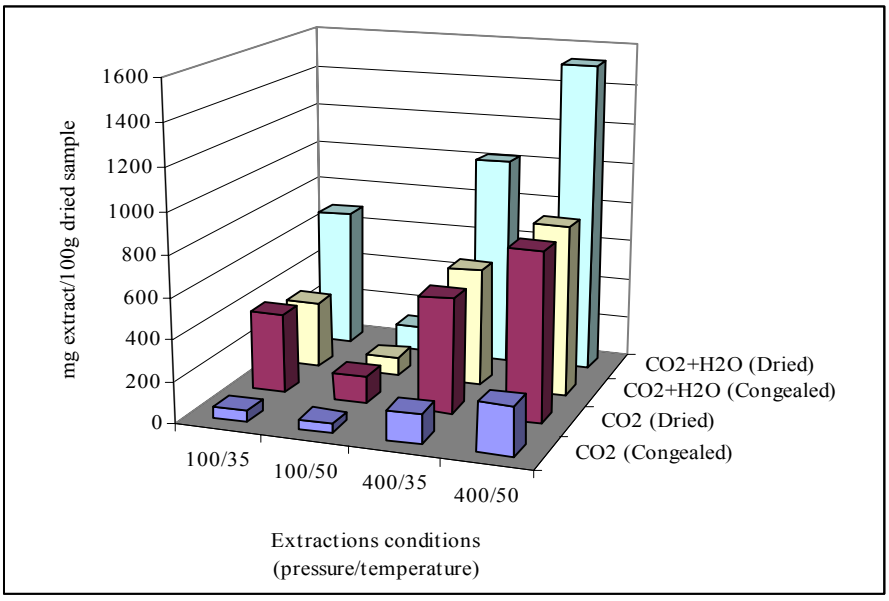

Fig 3. Extraction yields.

The results of the bioactivity assays for the extracts with the best extraction yields for each pre-treatment and the two systems solvents tested (400 bar of pressure at 50 ºC of temperatures) are shown in Fig 4.

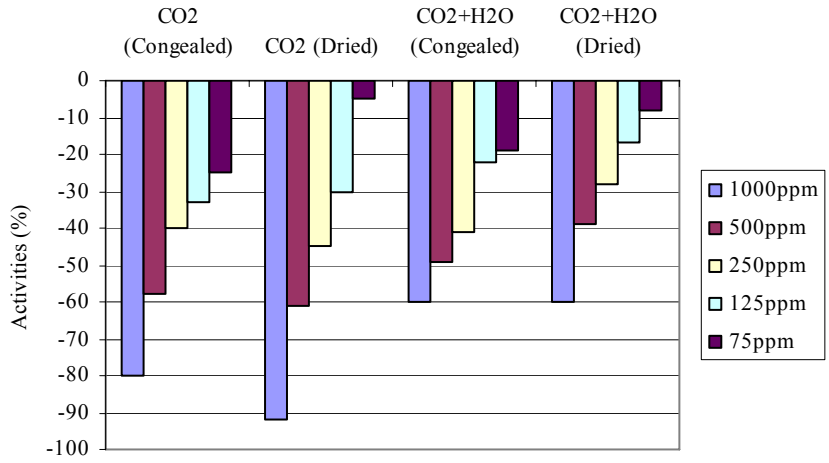

Fig 4. Bioactivities of extracts obtained at 400 bar.

Figures 5 and 6 shows a comparison on the yield of analytical and pilot plant scale at 50 ºC, 400 bar and 300 minutes.

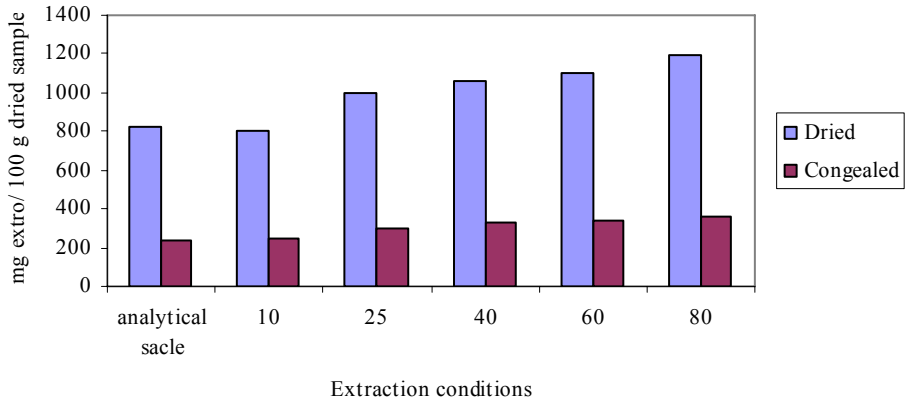

Fig 5. Comparison of the yields obtained in the extraction of bioactive substances with supercritical carbon dioxide on the two scales, to 50 ºC, 400 bar and 300 minutes.

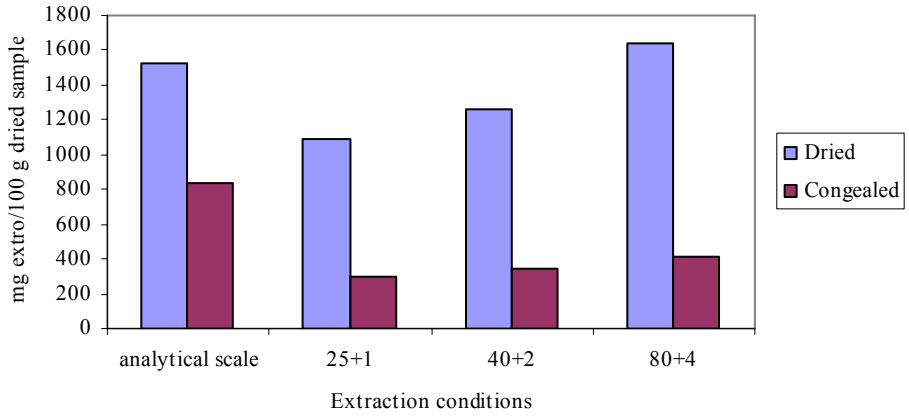

Fig 6. Comparison of the yields obtained in the extraction of bioactive substances with carbon dioxide and water on the two scales, to 50 ºC, 400 bar and 300 minutes.

Tables 2,3,4 and 5 shows parameters characteristic of the process of extraction from dried and congealed samples with supercritical carbon dioxide and mixtures of supercritical carbon dioxide and water.

	Analytical scale	Pilot plant scale				
H (cm)	5.7	31				
d (cm)	1.5	7				
H/d	**3.8**	**4.4**				
V (cm^3)	10	1200				
$\rho_{sólido}$ (g/ml)	0.25	0,25				
$Q_m CO_2$ (g/min)	0.4	10	25	40	60	80
M (g)	2.00	180				
$\rho_{aparente}$ (g/ml)	0.20	0.15				
ε	0.20	0.40				
v (cm/min)	0.24	0.28	0.70	1.12	1.60	2.26
Q_md/M (cm/min)	**0.30**	**0.38**	**0.97**	**1.55**	**2.33**	**3.11**
t_r (min)	**4.6**	**44.3**	**17.7**	**11.1**	**7.4**	**5.5**
Re	**1.0**	**1.2**	**3.0**	**4.8**	**6.8**	**9.7**

Table2. Parameters characteristic of the process of extraction from dried samples with supercritical carbon dioxide, ρ_{CO2}=0.924 g/ml; μ_{CO2}=1.0810^{-3}g/cms y D_p=0.3 cm.

	Analytical scale	Pilot plant scale				
H (cm)	5.7	31				
d (cm)	1.5	7				
H/d	**3.8**	**4.4**				
V (cm^3)	10	1200				
$\rho_{sólido}$ (g/ml)	0.20	0.20				
$Q_m CO_2$ (g/min)	0.4	10	0,4	10	0,4	10
M (g)	1.80	190				
$\rho_{aparente}$ (g/ml)	0.18	0.16				
ε	0.10	0.20				
v (cm/min)	0.24	0.28	0.24	0.28	0.24	0.28
Q_md/M (cm/min)	**0.33**	**0.37**	**0.33**	**0.37**	**0.33**	**0.37**
t_r (min)	**2.3**	**22.2**	**2.3**	**22.2**	**2.3**	**22.2**
Re	**1.0**	**1.2**	**1.0**	**1.2**	**1.0**	**1.2**

Table3. Parameters characteristic of the process of extraction from congealed samples with supercritical carbon dioxide, ρ_{CO2}=0.924 g/ml; μ_{CO2}=1.0810^{-3}g/cms y D_p=0.3 cm.

	Analytical scale	Pilot plant scale		
H (cm)	5.7	31		
d (cm)	1.5	7		
H/d	**3.8**	**4.4**		
V (cm^3)	10	1200		
$\rho_{sólido}$ (g/ml)	0.25	0,25		
Q_m CO_2+H_2O (g/min)	0.42	26	42	84
M (g)	2.10	190		
$\rho_{aparente}$ (g/ml)	0.21	0.16		
ε	0.16	0.36		
v (cm/min)	0.26	0.73	1.18	2.36
Q_md/M (cm/min)	**0.30**	**0.98**	**1.55**	**3.09**
t_r (min)	**3.3**	**14.5**	**9.0**	**4.5**
Re	**1.1**	**3.0**	**4.9**	**9.8**

Table4. Parameters characteristic of the process of extraction from dried samples with supercritical carbon dioxide and 5% of water as cosolvent, $\rho_{mixture}$=0.876 g/ml; $\mu_{mixture}$=1.0510^{-3}g/cms y D_p=0.3 cm.

	Analytical scale	Pilot plant scale		
H (cm)	5.7	31		
d (cm)	1.5	7		
H/d	**3.8**	**4.4**		
V (cm^3)	10	1200		
$\rho_{sólido}$ (g/ml)	0.20	0.20		
Q_m CO_2+H_2O (g/min)	0.42	26	42	84
M (g)	1.70	185		
$\rho_{aparente}$ (g/ml)	0.17	0.15		
ε	0.15	0.25		
v (cm/min)	0.26	0.73	1.18	2.36
Q_md/M (cm/min)	**0.37**	**0.98**	**1.59**	**3.18**
t_r (min)	**0.13**	**10.1**	**6.2**	**3.1**
Re	**1.1**	**3.0**	**4.9**	**9.8**

Table5. Parameters characteristic of the process of extraction from congealed samples with supercritical carbon dioxide and 5% of water as cosolvent, $\rho_{mixture}$=0.876 g/ml; $\mu_{mixture}$=1.0510^{-3}g/cms y D_p=0.3 cm.

4. Discussion of the results

4.1 Analytical scale experiment

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. 3), the best extraction yields were obtained for the dried samples. A freshly extracted sample has high moisture content and this can cause mechanical difficulties such as restrictor clogging due to ice formation.

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. This fact supports the observation that highly water-soluble solutes prefer to partition into the aqueous phase and remain in the extractor. The best extraction yields were achieved using 5% water as a modifier and drying the sample under different conditions of pressure and temperature.

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 3 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's dissolving power. On the basis of these results it is not advisable to work at 50 ºC and 100 bar since the yields are very low. Nevertheless, at higher pressure (400 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 ºC in order to avoid thermal degradation of the compounds.

4.1.2 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 its allelopathic potential.

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.

Figure 4 shows the activity profiles, with respect to the control, determined for the extracts obtained in the highest yields (to 400 bar) from samples treated in the two different ways. The samples extracted with CO_2 exhibited activity between -80% and -90% for 1000 ppm and give rise to values that are superior or near to –30% for the 125 ppm dilution. This shows that the activity level does not decrease drastically with dilution. The extracts obtained with CO_2 and water presents values –60% for the 1000 ppm and also shows a lower activity than the other samples at the 125 ppm dilution.

Therefore, the extracts obtained from CO_2, show better results in terms of bioactivity than the extracted obtained from CO_2 and 5% water, as can be seen in Fig 4.

4.2 Pilot scale experiment.

The results obtained in the analytical scale have been compared with those obtained using the pilot apparatus. We set temperature, pressure and percentage of cosolvent in at the same values used in the laboratory plant. Thus, the comparison was made between results obtained at the same operating conditions. In addition at pilot plant scale several flows were tested.

In Fig. 5 a comparison between the yields using CO_2 as solvent of extraction and the two apparatuses is reported. The results are very similar when 10 g/min is used at pilot plant.

Results obtained in Tables 2 and 3 confirm that dimensionless analysis (Reynolds) is the most relevant parameter for scale-up when CO_2 is using as solvent. The rations of the dimensions (H/d) are similarly in order to maintain geometric similarity. The equation 5 is similar in analytical and plant pilot scale.

Another important parameter is the residence time of the solvent in the extractor. Nevertheless, in this case this parameter is very difference between the analytical and pilot scale at 10 g/min, reason why it is not adapted to have presents on the process of sacle-up. Therefore, the scaling criterion of maintaining a constant residence time is not sufficient to ensure equivalent process during scale-up.

As can been seen in Fig. 6 the yields obtained at analytical scale with dried sample are similar when the flow is maintained at 84 g/min at pilot plant. Nevertheless, as can been seen in Tables 5 and 6, although the dimensions (H/d) continues being he himself, in this case is not similarity between the reason considered (Q_md/M and Re).

It is important to note that the effect that causes of cosolvent is different in analytical and pilot plant scale. It has been reported [5] 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. At analytical scale the passage of the solvents is descendent and the water is forced to leave the extractor increased solubility of the substances and therefore the yield. On the other hand, al pilot plant scale the passage of solvents are ascending and great part of this one does not solubility in CO_2 and remain in the extractor.

5. Conclusions

1. The use of water as modifiers increased the efficiency of extraction of the process study. The best yields were obtained using 5% water as a modifier.
2. At 400 bar the best yields were obtained at a temperature of 50 ºC, but at 100 bar the best yields were obtained at 35 ºC.
3. All of the extracts tested are bioactive, but samples obtained from CO_2, show the best activity profile.
4. The SFE method was scaled-up for preparative applications using a pilot plant. Large-scale SFE was technically feasible with pure CO_2 as the

extracting fluid. However, the use of CO_2 modified with water was not effective at the pilot plant scale.

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