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Selective precipitation of phenolic compounds from *Achillea millefolium* L. extracts by supercritical anti-solvent technique

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Abstract

In this work the fractionation of an ethanolic extract of *Achillea millefolium* L. (yarrow) using supercritical carbon dioxide anti-solvent technique was studied, with the target of increasing the concentration of phenolic compounds in the precipitated fraction. The effect of pressure on the selective precipitation was analyzed, together with the morphology and particle size distribution of the precipitates.

In the range of pressures studied (10-20 MPa), up to a 3 fold increase of the total phenolic compound concentration was observed in the precipitates in comparison with the ethanolic yarrow extract. In addition, the selective fractionation of the main phenolic compounds identified in the extract (3,5-dicaffeoylquinic acid, a glycoside form of apigenin and luteolin, as well as the aglycones) was analyzed. Particle sizes around 250-330 μm were produced with a nozzle of 101.6 μm inner diameter, due to the formation of aggregates. In this respect, increasing pressure from 10 to 15 MPa resulted in smaller particles, while further pressure increasing had no significant effect on particle size decrease.

Keywords: Supercritical carbon dioxide; anti-solvent; yarrow; phenolic compounds.

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1. Introduction

Achillea millefolium L (yarrow plant) is a flowering plant in the Asteraceae family, native to the Northern Hemisphere and widely spread in Europe and Asia [1]. *Achillea millefolium* has traditionally been used in folk medicine for treatment of asthma, bronchitis, skin inflammation, hemorrhages, headaches, inflammation, dyspepsia and hepatobiliary diseases [2, 3]. Certain compounds present in genus *Achillea millefolium* such as phenolic compounds and those belonging to the essential oil fraction have been associated with health benefits. Particularly, flavonoids (luteolin, apigenin, luteolin-7-O-glucoside, apigenin-7-O-glucoside, among others) and phenolcarboxylic acids (e.g., chlorogenic acid), constitute one of the most important groups of pharmacologically active principles in yarrow [4].

The extraction of phenolic compounds from yarrow has been carried out using organic solvents, such as water, methanol, ethanol or their aqueous mixtures. The presence of these phenolic compounds has been associated with beneficial health properties, such as choleric [5], antioxidant [6], anti-inflammatory [7], antimicrobial [8] and antimutagenic activity [9]. Therefore, it would be interesting to obtain, in an efficient and green manner, extracts highly concentrated in these compounds. In this regard, the use of supercritical CO₂ (SCCO₂) acting as an antisolvent is presented as an alternative for the production of plant extracts with high concentration of phenolic compounds.

In recent years, the interest in the use of supercritical fluids in particle formation and encapsulation processes for bioactive compounds has increased. One of these processes involves the use of SCCO₂ as an anti-solvent (SAS process, Supercritical Anti-Solvent). Regarding the application of SAS process in food technology, this technique has been mainly studied to form and/or encapsulate micro- and nanoparticles from single food ingredients. In this regard, SAS technique has been studied for the precipitation of β -carotene, quercetin [10], trans-resveratrol [11], lycopene [12], among others. Nevertheless, SAS has not been

extensively used for the fractionation of mixtures with complex chemical composition. In this sense, the number of studies where SAS process has been used to obtain dry precipitates from plant extracts has recently increased. In general, the objective was increasing the concentration of certain compounds, such as phenolic compounds, producing precipitates with enhanced biological activities in comparison with the starting extracts. Some studies reported in the literature are summarized in Table 1. Osorio-Tobón et al. [13] examined the precipitation of curcuminoids from an ethanolic extract obtained by pressurized liquid extraction (PLE) from deflavored turmeric. Depending on the conditions, between 54-97 % of the total curcuminoids present in the liquid extract was precipitated, and the curcuminoid content was 2 times higher than that of the liquid extract injected. Villanueva Bermejo et al. [14] carried out the selective fractionation of an extract obtained by PLE with ethyl lactate from green tea leaves. SAS precipitation produced a caffeine reduction higher than 90 % with respect to the PLE extract, obtaining decaffeinated precipitates with 23 % mass of catechins. Visentin et al. [15] carried out SAS fractionation to concentrate carnosic acid from oleoresins obtained by extraction of rosemary leaves with ethanol. In this case, the fraction enriched in carnosic acid with high antioxidant activity was not the precipitate but the one soluble in SCCO_2 . At 30 MP, authors obtained amounts of carnosic acid ten times higher than that present in the crude extract. Marqués et al. [16] concentrated antioxidants from a defatted grape seed waste extract; at 15 MPa and 40 °C, 70 % recovery of polyphenols and concentrations up to 2.7 times higher than in the injected extract, were obtained. Chinnarasu et al. [17] obtained precipitates from eucalyptus leaves in which the plant was previously extracted by supercritical fluid extraction using ethanol as a cosolvent. The precipitates showed a higher antioxidant activity than the starting SFE extract. Wu et al. [18] studied the fractionation of a propolis extract obtained by Soxhlet extraction with ethyl acetate to achieve precipitates enriched in the anti-proliferative compound 3,5-diprenyl-4-hydroxycinnamic acid

(DHCA). Checking several CO₂ flow rates and feed concentrations, precipitates with DHCA content close to 36 % mass were attained.

In this work, the selective fractionation of a yarrow liquid extract obtained by Ultrasound-Assisted Extraction (UAE) was accomplished using SAS technology to produce a powdered precipitate, with high content of phenolic compounds. The antioxidant activity of yarrow extracts has been related with the content of phenolic compounds [19, 20] and thus the precipitates obtained in this work can be considered for use as a natural antioxidant food ingredient.

2. Materials and methods

2.1 Reagents and Chemicals

CO₂ (N38) was supplied from Carbueros Metálicos (Madrid, Spain). Ethanol (99.5 % purity) and sodium carbonate salt (≥ 99.5 %) were acquired from Panreac (Barcelona, Spain). Acetonitrile (HPLC grade) was obtained from Lab-Scan analytical sciences (Gliwice, Poland). Formic acid (≥ 98 % purity) was obtained from Merck (Darmstadt, Germany). Folin-Ciocalteu's reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). The standards luteolin-7-O-glucoside (≥ 98 %), apigenin-7-O-glucoside (≥ 99 %), 3,5-dicaffeoylquinic acid (≥ 95 %), luteolin (≥ 99 %) and apigenin (≥ 99 %) were obtained from Extrasynthèse (Lyon, France).

2.2 Vegetal raw material

Achillea millefolium sample from Bulgaria was obtained from an herbalist's local supplier (Murcia, Spain) and the water content was lower than 5 % wt. *Achillea millefolium* included inflorescences and upper leaves of the plant. The sample was ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain). After grounding, a small sample (50 g) of the vegetal material was sieved and the particle size distribution obtained resulted that 87 % of

the particles had a size between 250 and 500 μm . All samples were stored in polyethylene bags and kept at 4 °C until extraction.

2.3 Preparation of yarrow dissolution

Raw vegetal material was extracted by UAE using an ultrasonic device (Branson Digital Sonifier 250 model, Danbury, USA) with an electric power of 200 W and frequencies of 60 kHz. The extraction was carried out with ethanol and 30 min time, at 1:10 plant / solvent ratio and keeping constant the extraction temperature at 40 °C.

The obtained extract was evaporated by rotary evaporation until obtaining 2.5 L of extract final volume containing 17.9 mg/mL of total solid concentration (2.2 % wt.). The dissolution was stored at -20 °C for its use in the SAS process.

2.4 SAS process

Precipitation process was performed by means of a supercritical technology equipment Thar SF2000 (Thar Technology, PA, USA). The equipment comprised two pumps for feeding the supercritical CO₂ (SCCO₂) and the liquid solution, respectively, the precipitation vessel and two separators (S1 and S2), each of 500 mL capacity, with independent control of temperature and pressure, on-line with a demister unit (Figure 1). The demister unit is specially designed to separate liquid or solid particles from the outgoing stream, before driving CO₂ to the storage tank. The precipitation vessel (Figure 2) consists on a stainless steel precipitation cell of 273 mL volume where the SCCO₂ and liquid solution lines are connected at the top of the cell and the injection into the vessel for both fluids is produced in a co-current manner (coaxial nozzle). The precipitation cell is equipped with a 101.6 μm inner diameter nozzle for the injection of the liquid solution and a porous metallic frit (5 μm in diameter) that is located at the bottom of the precipitator to collect the precipitate.

A typical experiment starts by pumping the SCCO₂ into the precipitation vessel until the pressure and temperature conditions are attained. Then, a desired amount of yarrow solution

from UAE is pumped into the precipitator and once the extract fed ends, additional CO₂ is pumped during 15 min to wash out the residual solvent from the precipitator. During the process, both separators are kept at ambient pressure to recover the components soluble in the SCCO₂/organic solvent phase which do not precipitate in the precipitation vessel. Finally, the system is depressurized and the obtained fractions are collected.

Precipitations from the yarrow solution (17.9 mg/mL concentration) were carried out at pressures in the range of 10–20 MPa, 40 °C and 120 min processing time. The CO₂ and extract flows were 50 g/min and 1.6 g/min, respectively. The flow ratio employed (31.3 g/g) allow SCCO₂ to dissolve the organic solvent, obtaining completely dry precipitates at the studied conditions. The particles retained in the frit (precipitate) were collected. The extracts (ethanol together with non-soluble compounds in SCCO₂/organic solvent phase) obtained in the two separators were combined and the organic solvent was removed by rotary evaporation under vacuum to dryness. Samples were kept at -20 °C under darkness until analysis.

2.5 Total phenolic content

The total phenolic content in the fractions collected after SAS process was determined using Folin-Ciocalteu method [21]. Briefly 50 µl of extract were mixed with 250 µl of Folin Ciocalteu reagent and 3 mL of milliQ water and allowed to stand at room temperature for 3 min; 750 µl of sodium carbonate (20%) solution and 950 µl of milliQ water were added to the mixture. After 2 hours at room temperature and remained in darkness, absorbance was measured at 760 nm using spectrophotometer (Genesis 10 UV Scanning, Thermo Scientific). Results were expressed as mg gallic acid equivalents in 1 g of extract (mg GAE/g extract).

2.6 Identification and quantification of the main phenolic compounds by HPLC

The concentration of the main phenolic compounds in the precipitates and yarrow ethanolic extract was measured in an Agilent 1260 Infinity HPLC system (Agilent Technologies, CA,

USA) equipped with a DAD detector. The column employed was ACE C18 Excel (150 mm x 4.6 mm and 5 μ m). The composition of the mobile phase was (A) 0.1 % (w/v) formic acid in water (B) acetonitrile. The column was maintained at 35 °C, with a flow rate of 0.5 mL/min. The mobile phase gradient employed was as follows: initial 100 % A, 1 min 100 % A, 6 min 85 % A, 21 min 75 % A, 26 min 65 % A, 36 min 50 % A, 41 min 50 % A, 43 min 0 % A, 48 min 0 % A, 50 min 100 % A. The injection volume was 20 μ L and the detection was carried out at 320 nm. Phenolic compounds were identified by using standards.

2.7 Morphology and determination of particle size

Morphologies of particles collected from the precipitation vessel were visually studied by scanning electron microscopy (SEM) in a Hitachi S-3000N (Hitachi High-Technologies, Tokyo, Japan) equipment. Samples were placed on carbon tapes and then were coated with a thin gold layer by a sputter coater. Particle size distributions were measured by light scattering with a laser diffraction system Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK), equipped with a wet dispersion unit.

3. Results and discussion

3.1 Precipitation yield and phenolic compound recovery

When the ethanolic extract is feed into the precipitation chamber through the nozzle, small drops are produced. These drops get in contact with the anti-solvent (SCCO₂) and the ethanol is dissolved in the supercritical stream, leading to supersaturation of the solutes in the drop, and producing its nucleation and particle growth. Considering the low concentration of solids in the ethanolic extract (2.2 % mass) it is theorized that the ethanol + solutes + CO₂ system can be treated as the pseudo-binary CO₂ + ethanol system. Under this hypothesis, and according to the flow rates used in this work, if all the ethanol feed would dissolved in the SCCO₂ stream, its concentration in the supercritical phase would be 3.10 % by weight, what

means a homogeneous supercritical phase according to the phase equilibria of the binary CO₂ + ethanol [22] in the range of temperatures and pressures used in this work. Thus, it is reasonable to expect that will be collected a solid precipitate (dry particles).

Table 2 shows the effect of pressure on SAS precipitation of the yarrow extract solution at 40 °C. In general, 55-60 % of the total mass of solids pumped was recovered. It can be observed that total yields ($Y_T = \text{mass precipitated} / \text{mass of solids pumped}$) ranged from 6.6 % to 17.3 % in the precipitation chamber and were considerably lower than the yields obtained in the separator (between 43 % and 52 %).

Additionally, in the range of pressures studied, it was observed that an increase in pressure produced an increase of Y_T in the precipitation chamber, being 2.0 times higher when pressure was varied from 10 to 15 MPa, while minor increase was observed from 15 to 20 MPa. This effect was also observed by the authors [14] in the SAS precipitation of an ethanolic green tea extract; at 50 °C the precipitation yield increased from 40.3 to 58.6 % when pressure varied from 10 to 15 MPa and using a CO₂/dissolution flow ratio of 20.

Osorio-Tobón et al. [13] studied the effect of nozzle type (T-mixer and coaxial), temperature (40 °C and 60 °C), pressure (10 and 12 MPa) and CO₂/dissolution flow ratio (17 and 27), on the SAS precipitation of curcuminoids from an ethanolic turmeric extract. In general, and despite the minor variance in the pressures examined, the authors stated that “an increase in the process pressure caused a slight decrease in the global yield of solids at both temperatures but was still more pronounced for the 333 K temperature case”. Yet, despite this is really the general conclusion, in the case of the coaxial nozzle and using a CO₂/dissolution ratio of 27 at 40 °C, the experimental results reported by Osorio-Tobón et al. [13] indicate a slight increase of precipitation yield (from 40-45% to 47-49%) when pressure increased from 10 to 12 MPa. These process conditions are similar to those used in this work (coaxial nozzle, 40 °C and CO₂/dissolution ratio of 30), in which an increase in precipitation yield with pressure

was also observed. In another work, Marqués et al. [16] concluded that an increase of pressure from 10 to 15 MPa (40 °C and CO₂/dissolution \approx 47) resulted in minor yields of precipitate in the antisolvent precipitation of antioxidants from grape seeds. These results should not be considered contradictory, since the different CO₂/dissolution ratios signify different ethanol mole fraction in the supercritical phase, which indeed influence the solubility of the solutes present in the dissolution, which in turn are different according to the vegetal raw material used in the study. Therefore, the complex multicomponent structure of plant matrix, comprising substances in a wide polarity range and with different solubility in CO₂, seems to exert a strong effect regarding the behavior of precipitation yield due to pressure.

With respect to the concentration of total phenolic compounds (TPC), in all the experiments (Table 2) the precipitates presented higher concentrations in comparison with that obtained in the UAE yarrow extract (53 mg GAE/g). Concentrations of TPC between 125 and 152 mg GAE/g were obtained, which means an enrichment factor (E) of 2.3-2.9. That is, the selective precipitation of phenolic compounds in the precipitation chamber was produced ($E > 1$) obtaining a concentration of phenolic compounds around 5 times higher than those obtained in the separator ($E < 1$).

Regarding the precipitation yield of TPC ($Y_{\text{TPC}} = \text{mass of phenolic compounds precipitated} / \text{mass of phenolic compounds pumped}$) a behavior similar to Y_{T} was observed, since an increase in the precipitation chamber by increasing the pressure was produced. The highest Y_{TPC} was obtained at 20 MPa (40.9 %), being 2.2 times higher than that obtained at 10 MPa (18.9 %). However, this increased precipitation of phenolic compounds is accompanied by a lower concentration of TPC (125 mg GAE/g at 20 MPa and 152 mg GAE/g at 10 MPa).

3.2 Analysis of the main phenolic compounds by HPLC

Figure 3 shows the concentration of the main phenolic compounds identified in the yarrow UAE extract and in the powders obtained in the precipitation vessel. In general, the concentration increased in the precipitates compared to yarrow extract, so that by using SAS technique, the selective fractionation of these compounds was attained.

While results were practically identical between 15 MPa and 20 MPa (see Figure 3), differences from 10 to 15 MPa can be observed. At 15 MPa a considerable increase in the concentration of the five compounds identified in the precipitates was produced. In this case, the concentration ranged from 28.8 mg/g of luteolin-7-O-glucoside (3.3-fold increase with respect to the concentration in the yarrow extract) to 4.9 mg/g of apigenin (2.6-fold increase) being glycosylated forms the compounds that underwent the highest increase (3.3 and 4.1 for luteolin and apigenin glucosides, respectively). Nevertheless, only the selective concentration of glycosylated forms (5.1 and 4.2-fold increase for luteolin and apigenin glucosides, respectively) and 3,5-dicaffeoylquinic acid (5.2 times higher) was observed at 10 MPa. At this pressure, a rise in the concentration of the aglycones luteolin and apigenin was not produced; even a slight decrease in the concentration of apigenin was observed in the precipitate.

Flavonoid glucosides and phenolic acids are soluble in polar solvents, such as water or methanol, but are scarcely soluble in low-polar solvents such as ethyl ether or chloroform [23]. On the other hand, the flavonoid aglycone is generally not soluble in water, but is soluble in ethanol, acetone, ethyl acetate, ethyl ether, and other organic solvents [24]. Taking into account the low polarity of SCCO_2 , it is expected in the supercritical phase, a higher solubility of the flavones (apigenin and luteolin) than the solubility of the corresponding glucosides and/or phenolic acid. This trend was observed in this work, being the concentration of 3,5-dicaffeoylquinic acid and the apigenin and luteolin glucosides in the precipitates higher than those of the corresponding aglycones.

3.3 Analysis of the morphology and particle size of precipitates

The morphology of the particles obtained in the precipitates is shown in Figure 4 at the different precipitation pressures explored. In general, the structure of precipitates was characterized by an irregular morphology with rounded particles aggregates, i.e. of low angularity. Furthermore, it can be observed that the pressure did not produce a significant change in the particle morphology of the different precipitates. However, it was observed a significant variation in particle size, with smaller particles and more similar size distributions as pressure was increased in the precipitation chamber (see Figure 5). A similar effect was observed by Chinnarasu et al. [17] at the same pressure range and 35 °C for the fractionation of ethanolic extracts from eucalyptus leaves.

Figure 5 shows the particle size distribution and Table 3 the most significant statistical variables obtained for the different yarrow precipitates. As indicated above, the increase of 10 to 15 MPa caused the formation of smaller particles. However, there were hardly any differences with increasing pressure from 15 to 20 MPa, presenting most of particles obtained at these two pressures, sizes between 250-270 μm and obtaining very similar mean particle sizes (101-95 μm). This means a reduction of 18-24 % compared to the size obtained at 10 MPa (330 μm modal value) and a reduction of 49-52 % compared to the mean size at that pressure (269 μm). Furthermore, it can be observed in Figure 5 and Table 3 that a wider size distribution was obtained at 10 MPa with particle size ranging from 8 to 859 μm , while the range obtained at 20 MPa was 0.5 to 586 μm , which was identical to that obtained at 15 MPa. A similar morphology and particle sizes were reported by Osorio-Tobón et al. [13] and Visentin et al. [25] for the fractionation of a turmeric extract and rosemary extract, respectively. In this case, authors obtained large irregular aggregates from agglomerated particles probably due to the complex chemical composition of the vegetal extracts. In this way, the different molecules could interact with neighboring molecules thus forming these

structures. Nevertheless, further experiments would be necessary to determine the influence of other process parameters (such as temperature, flow ratio or solid concentration in the feeding solution) on the morphology and size of particles formed from yarrow extract.

Conclusions

SAS precipitation of an ethanolic yarrow extract resulted in a dry powder with rounded particles aggregates of irregular shape at the different pressures studied. Mean particle size was smaller and particle size distribution was more homogeneous when pressure increased from 10 MPa to 15 MPa, but practically no differences were observed when increasing pressure up to 20 MPa.

A selective precipitation of yarrow phenolic compounds was observed in the range of conditions studied, obtaining almost a 3 fold increase of the concentration of total phenolic compounds in the precipitates with respect to this concentration in the ethanolic yarrow extract. A selective fractionation of all identified phenolic compounds was produced at 15 and 20 MPa, whereas only luteolin and apigenin glycosylated forms and 3,5-dicaffeoylquinic acid were concentrated in the precipitates obtained at 10 MPa.

Considering the selective effect of yarrow SAS precipitation to concentrate its phenolic compounds, it can be highlighted the usefulness of this technique to produce high valued bioactive ingredients with potential application in food products or nutraceuticals. Further studies are under development to determine the antioxidant and anti-inflammatory activity of precipitates in comparison with the initial yarrow extract.

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Table 1. SAS precipitation of different dissolutions of vegetal extracts.

Vegetal extracts / solvent	Fractionated compounds	Process conditions	Reference
Turmeric / ethanol	Curcuminoids	10 and 12 MPa 40 and 60 °C	[13]
Green tea leaves / ethyl lactate	Caffeine and catechins	15 - 30 MPa 50 and 70 °C	[14]
Rosemary leaves / ethanol	Carnosic acid	15 - 40 MPa 50 °C	[15]
Grape seed wastes / ethanol	Phenolic compounds	8 - 15 MPa 35 - 50 °C	[16]
Eucalyptus globulus leaves / ethanol	Essential oil	10 – 20 MPa 35 and 50 °C	[17]
Propolis / ethyl acetate	3,5-diprenyl-4-hydroxycinnamic acid	20 MPa 55 °C	[18]

Table 2. SAS precipitation of yarrow ethanolic extract at 40 °C and CO₂/dissolution ratio of 31.3 g/g. Y_T: total precipitation yield; Y_{TPC}: precipitation yield of total phenolic compounds; TPC: total phenolic compounds concentration (mg GAE/g); E: enrichment factor.

Pressure (MPa)	Precipitation vessel				First separator			
	Y _T (%)	TPC (mg/g)	Y _{TPC} (%)	E	Y _T (%)	TPC (mg/g)	Y _{TPC} (%)	E
10	6.6	152.1	18.9	2.9	52.1	34.2	33.3	0.6
15	13.3	123.3	30.9	2.3	42.6	25.9	21.0	0.5
20	17.3	125.0	40.9	2.4	45.5	25.1	21.1	0.5

Table 3. Statistical variables related with the particle size distribution obtained in yarrow precipitates obtained by SAS technology at 40 °C.

Variable (μm)	P = 10 MPa	P = 15 MPa	P = 20 MPa
Mode	330	270	251
Mean	269	136	129
Median	258	101	95
Range	8 - 859	0.5 - 586	0.5 - 586

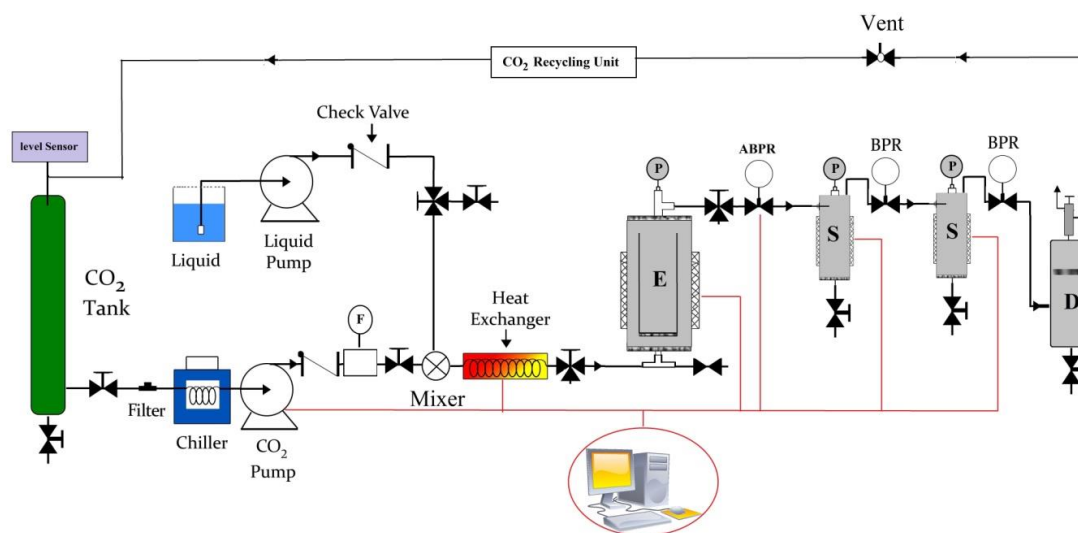


Figure 1. Schematic diagram of the supercritical anti-solvent equipment used for SAS precipitation. (F) mass flow meter, (E) precipitation cell, (ABPR) automatic back pressure regulator, (BPR) manual back pressure regulator, (P) manometer; (S) separator and (D) demister.

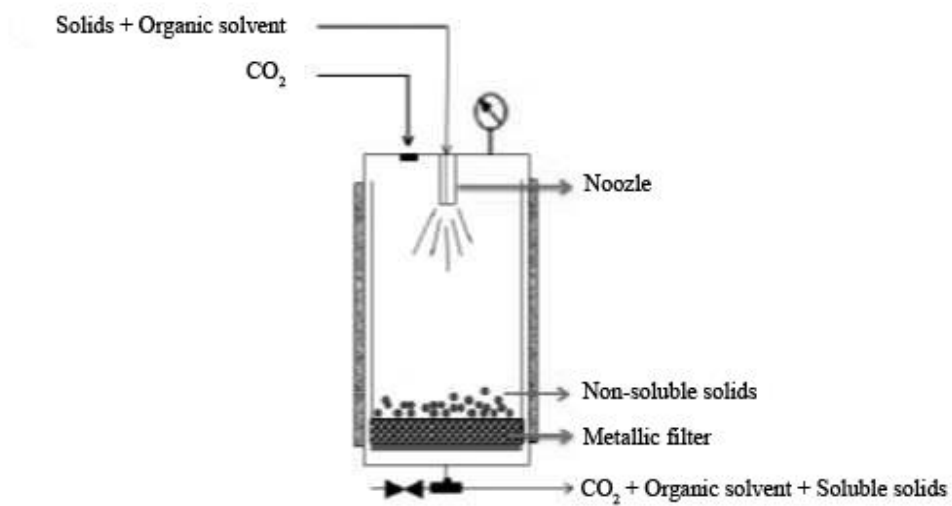


Figure 2. Scheme of the SAS precipitation cell of the supercritical anti-solvent equipment.

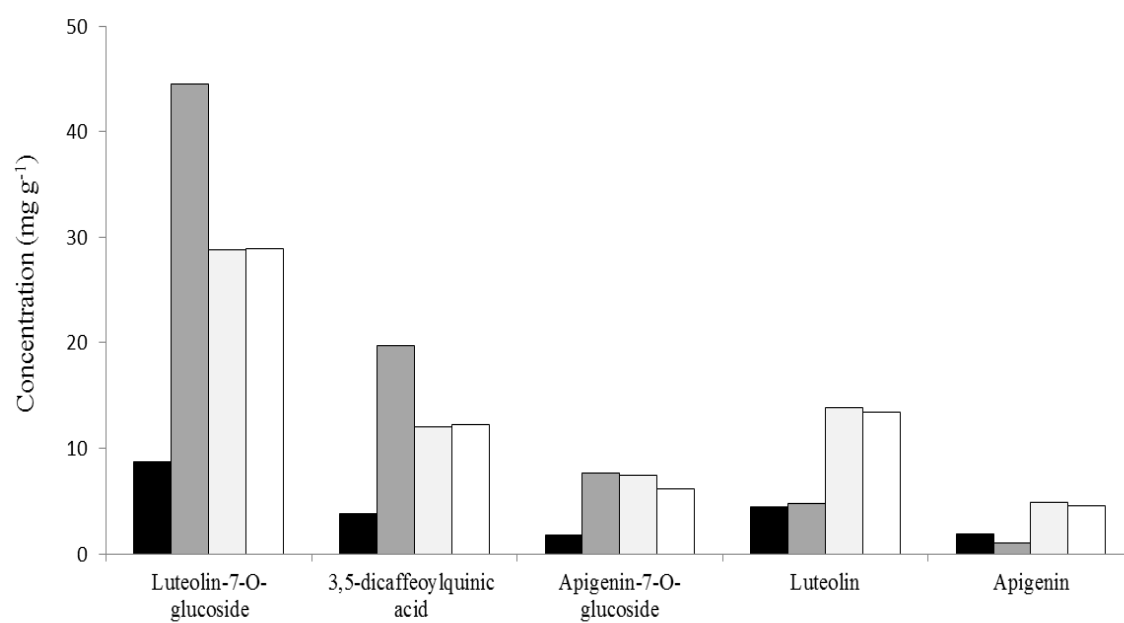


Figure 3. Concentration of main phenolic compounds identified in yarrow extract and SAS precipitates. (■) UAE extract; precipitate at (■) 10 MPa; (■) 15 MPa and (□) 20 MPa.

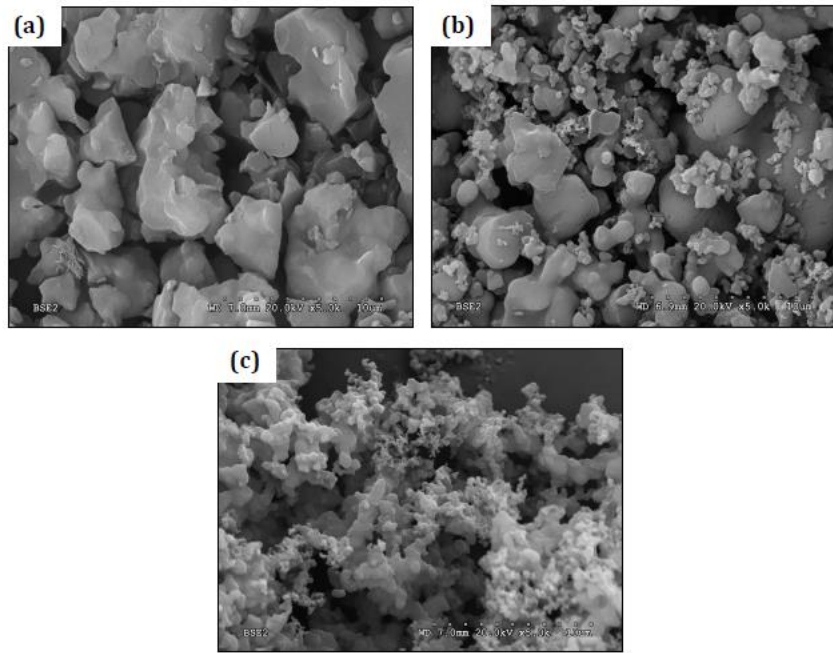


Figure 4. SEM images (x5000) of yarrow precipitates obtained by SAS technology at 40 °C and (a) 10 MPa, (b) 15 MPa and (c) 20 MPa.

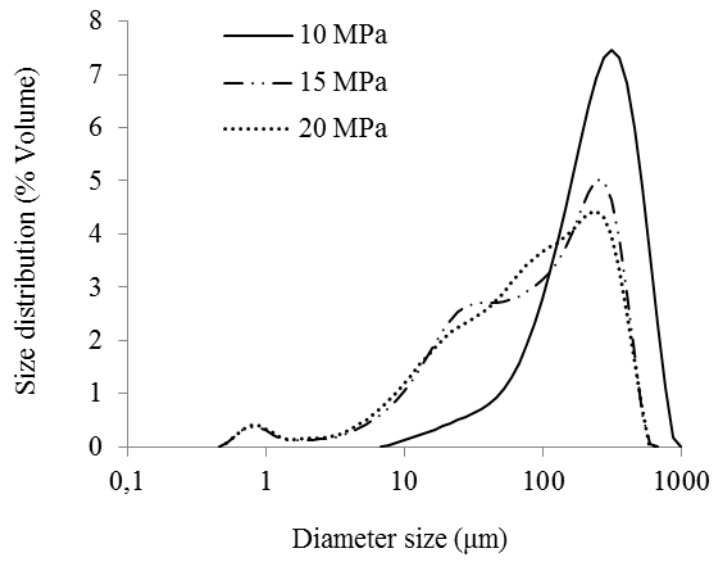


Figure 5. Particle size distribution of yarrow precipitates obtained by SAS technology at 40 °C and (—) 10 MPa, (— · —) 15 MPa and (•••••) 20 MPa.