Journal Pre-proof

Supercritical antisolvent fractionation of antioxidant compounds from *Lavandula luisieri* (Rozeira) Riv.-Mart

Carlota Giménez-Rota, Elisa Langa, José S. Urieta, María J. Hernáiz, Ana M. Mainar

PII: S0896-8446(20)30072-3

DOI: https://doi.org/10.1016/j.supflu.2020.104821

Reference: SUPFLU 104821

To appear in: *The Journal of Supercritical Fluids*

Received Date: 29 November 2019

Revised Date: 8 March 2020

Accepted Date: 11 March 2020

Please cite this article as: Giménez-Rota C, Langa E, Urieta JS, Hernáiz MJ, Mainar AM, Supercritical antisolvent fractionation of antioxidant compounds from *Lavandula luisieri* (Rozeira) Riv.-Mart, *The Journal of Supercritical Fluids* (2020), doi: https://doi.org/10.1016/j.supflu.2020.104821

Supercritical antisolvent fractionation of antioxidant compounds from *Lavandula luisieri* (Rozeira) Riv.-Mart.

Carlota Giménez-Rota [a,b], Elisa Langa [a,c], José S. Urieta [a], María J. Hernáiz [b], Ana M. Mainar [a,*]

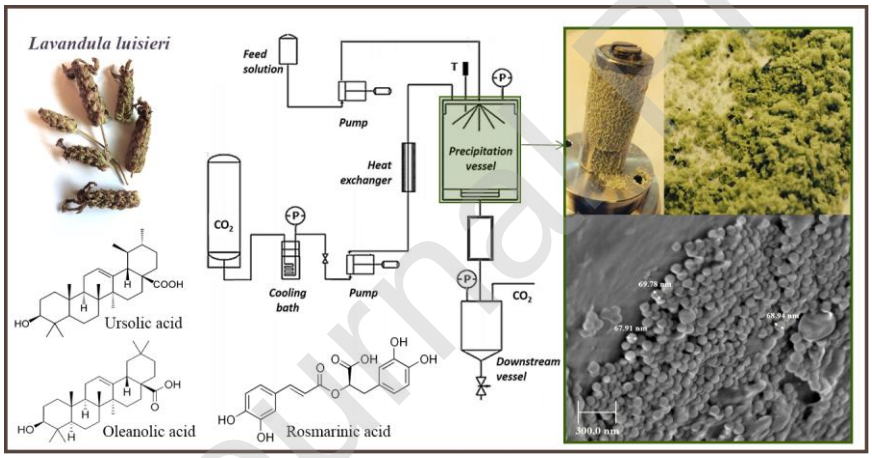
[a] GATHERS Group, Aragón Institute of Engineering Research (I3A), Universidad de Zaragoza, c/. Mariano Esquillor s/n, 50018 Zaragoza, Spain

[b] Chemistry in Pharmaceutical Science Department, Pharmacy Faculty, Complutense University of Madrid, Plaza Ramón y Cajal s/n, Madrid 28040, Spain

[c] Universidad San Jorge, Campus Universitario Villanueva de Gállego, Autovía A-23 Zaragoza-Huesca Km. 299, 50830 Villanueva de Gállego (Zaragoza), Spain

*Corresponding author. Tel.: +34 976761195; fax: +34 976761202. ammainar@unizar.es

Graphical abstract



Highlights

- Supercritical antisolvent fractionation was applied to *Lavandula luisieri* extract.
- Pressure and CO_2 flow rate influence in the fractionation process was evaluated.

- The fractionation of rosmarinic, oleanolic and ursolic acids was tracked.
- The best conditions for the supercritical fractionation were 130 bar and 30 g/min.
- A fine concentrated powder of *Lavandula luisieri* actives was produced.

ABSTRACT

There is a renewed research interest in food industry in natural additives to improve food shelf life and provide preventive or therapeutic effects on chronic ailments. The aim of this study was to optimise the concentration of three bioactives, rosmarinic, oleanolic and ursolic acids from *Lavandula luisieri* ethanolic extracts using the supercritical antisolvent fractionation technique. In order to evaluate the influence of pressure and CO_2 flow rate in the process, response surface methodology was employed. Actives quantification was accomplished using HPLC-Photodiode array. Rosmarinic acid, was completely retained and concentrated in the precipitation vessel, while oleanolic and ursolic acids distributed between both fractions. The supercritical antisolvent fractionation process is a useful and green technology to concentrate bioactive in a fine solid to be applied as natural preservative in food products. The optimum conditions for higher mass recovery and concentration of actives were 130 bar and CO_2 feeding flow rate 30 g/min, respectively.

Key words: Supercritical antisolvent fractionation, HPLC, Rosmarinic acid, Oleanolic acid, Ursolic acid.

Chemical compounds studied in this article

Rosmarinic acid (PubChem: 5281792); Oleanolic acid (PubChem: 10494); and Ursolic acid (PubChem: 64945)

Abbreviations

$scCO_2$, supercritical CO_2; SAF, Supercritical Antisolvent Fractionation; RA, Rosmarinic acid; OA, Oleanolic acid; UA, Ursolic acid; PDA; photodiode array, RSM, Response Surface Methodology; CCD, Central Composite Design; X_P, Pressure; $X_{Q_{CO_2}}$, CO_2 flow rate; FS, Feed Solution; PV, Precipitation Vessel; DV, Downstream Vessel; Y_{PV}%, Y_{DV}% and Y_{SAF}%, yields recovered in PV, DV and SAF fractions.

1. Introduction

Technological advances in food processing have increased the number and variety of additives used to produce the desired preservation or improvement of flavour, texture, appearance and nutritional value maintaining its safeness. Nevertheless, daily consumption of products with these substances have raised the consumers concern about their long-term consequences, demanding the substitution of artificial additives for natural ones [1]. In addition, society purpose to optimise wellbeing through healthy habits and diets with health-promoting properties, are a growing trend [2, 3]. The focus of many studies across the world, is targeted in searching natural alternatives to preserve food, and demonstrate their beneficial functionalities on food, not only improve or maintain their nutritional quality, but also add positive effect beyond its nutritional value [1, 4].

Plants are an inexhaustible natural source of compounds, such as polyphenols and terpenes with a wide range of beneficial bioactivities in human health: antioxidant, anticancer, anti-inflamatory, antimicrobial, antiviral, cardioprotective, neuro- and hepatoprotective [3, 5]. *Lamiaceae* is a highly distributed plant family, and one of the biggest in the plant kingdom, known for its content in polyphenolic compounds. In this work a member of this family, *Lavandula luisieri* (Rozeira) Riv.-Mart.,[6] an aromatic shrub endemic from to the south Iberian Peninsula, is studied. Although the essential oils of other *Lavandula* species present importance in the fragrance industry, *L. luisieri*, has not because of the presence of camphor. Nevertheless, it has a curious composition in a series of volatile substances with a 1,2,2,3,4-pentamethylcyclopentane (necrodane) structure [6], which gives to this plant the category of species since these compounds have only been found in the defensive secretions of the beetle *Necrodes surinamensis* [7, 8], and in the sex pheromone of the grape mealybug *Pseudococcus maritimus* [9]. Different extracts obtained from it have shown antifeedant, insecticide and antimicrobial effects [10, 11]. In addition rosmarinic acid (RA), tormentic acid, ursolic acid (UA) and oleanolic acid (OA) were isolated from the nonvolatile fraction [11, 12]. These polyphenol and triterpenoids have been reported to have several beneficial bioactivities such as antioxidant, anti-inflamatory, neuroprotective and hepatoprotective [13–15]. Rosmarinic acid is contained into Rosemary extract, another plant from the *Lamiaceae* family, which has been accepted by the EU food additive legislation as an effective and natural alternative to synthetic antioxidants [16]. As a consequence, there are several studies that analyse its extraction and concentration techniques [17], stability and pharmacokinetic profile that

ensure its antioxidant properties [18]. Regarding the identified triterpenes Ursolic and Oleanolic acids, it has been reported their potential application as antimicrobials because of their capacity to disrupt the peptidoglycan structure, and inhibit bacterial gene expression and biofilm formation [19]. Therefore, *L. luisieri* extracts containing a combination of these compounds could have several applications in the pharmaceutical, cosmetical or food fields.

Nevertheless, plant extracts have been obtained traditionally using techniques with two main limitations: high temperatures, as in hydrodistillation, which can cause actives principles degradation, and the use of organic solvents that are environmental pollutants. The use of supercritical CO_2 ($scCO_2$) is an alternative to obtain natural antioxidants from herbs and plants [19, 20]. The application of supercritical carbon dioxide as extraction solvent shows only one disadvantage: its low polarity. This means that the compounds that $scCO_2$ can extract are limited to nonpolar components with less antioxidant bioactivity or small volatile compounds [21, 22]. However, this lipophilic behaviour is useful when polar compounds have to be concentrated from an organic solution extract, as in Supercritical Antisolvent Fractionation (SAF) [23]. The obtained product is a dried powder avoiding solvent residues, whose shape and diameter can be modulated to improve its solubility or vehiculization along with polymers [24, 25].

In SAF technique an organic solution is continuously pumped and sprayed into a vessel with $scCO_2$. Molecules that are insoluble in this new solvent mixture of ethanol-$scCO_2$ precipitate as a solid, and the rest of them are dragged downstream. This technique has been applied by many researchers to fractionate and concentrate natural compounds, such as lignans from flaxseeds [26], flavonoids and polyphenols from *Vitis vinifera* seeds [27], or flavonoids from *Arrabidaea chica* leaves [28]. Sánchez-Camargo et al. and Visentin et al., and Quintana et al., [17, 29, 30] applied SAF process to rosemary extracts in order to concentrate their polyphenols, RA among them, and produced *raffinate* fraction with a higher antiproliferative and antioxidant activity than the original extract. Because of the distribution of RA, OA and UA in the plant kingdom and their many probed activities, also traditional techniques, such as ultrasound-assisted extraction and maceration, have been applied by other authors. Bernatoniene et al. [13] studied different techniques for the extraction of these three actives from Rosmarinus officinalis and achieved a highest yield of UA (15.8 ± 0.2 mg/g), RA (15.4 ± 0.1 mg/g), and OA (12.2 ± 0.1 mg/g).

According to these previous works on extraction and concentration of natural bioactives with supercritical techniques, the aim of this study was to optimise the pressure and CO_2 flow rate conditions in the Supercritical antisolvent fractionation of *L. luisieri* extract for a higher mass recovery and concentration of the three actives; rosmarinic, oleanolic and ursolic acids, into a solid powder.

2. Material and Methods

2.1 Plant material

Plant material was collected in 2009 in Zaragoza (Spain) from an adapted population of *L. luisieri*, original from Toledo (Spain). This adaptation was performed by Centro de Investigación y Tecnología de Aragón (CITA) (Spain).

Plant material was dried at room temperature and then pulverised. Its particle size distribution was carried out by a vibratory sieve shaker *CISA* model BA 300N, and the average diameter was calculated according to ASAEA S319.3 from the American National Standards Institute as shown in Eq. (1).

$$d_{mg} = \log^{-1}\left[\frac{\sum_i^n (w_i \log \bar{d}_i)}{\sum_i^n w_i}\right];\ \bar{d}_i = (d_i \cdot d_{i+1})^{0.5} \quad (1)$$

where d_i is the nominal mesh of the i^{th} sieve (mm), $d_{(i+1)}$ is the nominal mesh of the next larger sieve after the i^{th} sieve (mm) and w_i is the mass (g) of plant material retained by the i^{th} sieve.

The pulverised plant material was adjusted to a normal distribution and an approximately mean particle diameter of 0.33 mm to improve the extraction yield. Moisture content was tested five times using a *Sartorious* model MA 40 *Moisture Analyzer*, and the standard deviation was determined (10.6%, s = 0.3%). This pretreated plant material was kept in hermetically sealed food bags at −20 °C.

2.2 Chemicals and reagents

The solvents used in the extraction process were hexane (Panreac 99.0%) and ethanol (AnalaR NORMAPURE 99.96%). The SAF process was performed with CO_2 (ALPHA GAZ 99.8%) and ethanol (AnalaR NORMAPURE 99.96%). The chromatography mobile phase solvents were methanol (Scharlab 99.9%), water (MilliQ 18.2 MΩ·cm), phosphoric acid (Fluka 85.9%) and acetonitrile (Scharlab 99.9%). The

HPLC-PDA standards used were rosmarinic acid (RA, 99%), oleanolic acid (OA, 99.8%) and ursolic acid (UA, 99.7%), supplied by Sigma-Aldrich.

2.3 Maceration

100 g of plant material were stirred for 48 h at room temperature (25 °C) in 1 L absolute ethanol, after a previous clearance extraction using hexane under the same conditions. The extraction yields for the macerations, Y_i (wt.%), were calculated using Eq. (2). The extract obtained (ME) was used to prepare the feed solution (FS) for the SAF experiments.

$$Y_i(\text{wt.}\%) = \left(\frac{mass\ (g)_{\text{plant extract}}}{mass\ (g)_{\text{plant material}}}\right) \cdot 100 \qquad (2)$$

where *i* is the solvent of the extraction; hexane or EtOH, $mass_{\text{plant extract}}$ (g) is the mass of the dry extract after maceration, once the solvent had been removed, and $mass_{\text{plant material}}$ (g) was the initial mass of dried and pulverised plant.

2.4 Supercritical antisolvent fractionation process

L. luisieri ethanolic extract was fractionated using the SAF technique. The experiments of were performed in the "Green Chemistry Laboratory" (I3A Researching Institute at University of Zaragoza) using a scale apparatus previously described [31, 32]. A schematic structure is represented in Fig. 1. The main components of the device are: a CO_2 pump (mod. P200 max pressure 600 bar), an extract solution pump (Waters co-solvent pump series III maximum pressure 400 bar), a 0.5 L precipitation high pressure vessel (PV) with an injection nozzle (Ø =100 μm) in the top and a collection filter in the bottom, and a 0.5 L downstream low pressure separation vessel (DV). The pressure in PV was set with automated backpressure regulator (ABPR, TharSFC) and in DV with a manual backpressure regulator (BPR, CIRCOR Instrumentation Technologies). The experimental parameters of temperature, CO_2 flow rate, liquid solution flow rate, and PV pressure were controlled with the computer software Thar Instruments Process Suite. The equipment working limits are 400 bar and 120 °C.

Different SAF experiments were performed varying the PV pressure, from 80 to 150 bar, and the CO_2 flow rate from 10 to 30 g/min, the rest of variables were set at: extract solution concentration, 3% (wt.%); ethanolic solution flow rate, 0.45 mL/min and PV temperature, 40 °C. These settings were chosen according

to previous experience with the SAF equipment [32]. The fixed settings of ethanolic solution flow rate and temperature were chosen in order to maintain always a CO_2 molar fraction over 0.98 and ensure supercritical conditions of the CO_2-ethanol mixture in the precipitation vessel in all experiments performed [27]. The operational conditions of DV were also fixed at 35 bar and 25ºC, to achieve the recovery of the solvent and its separation from gaseous CO_2.

The experiments procedure, which was previously described by Langa et al. [32], consisted in three steps. Firstly, the experimental conditions were stabilized, pressure (bar), CO_2 flow rate (g/min), temperature (40 °C) and liquid flow rate (0.45 ml/min) with pure ethanol (≈60min). Secondly, the ethanolic extract was dissolved in 30 mL of ethanol at 3% (wt.%) and filtered through *NYLON* 0.45 μm pore size to constitute the feed solution (FS) to be pumped towards the precipitation vessel (PV) with a flow rate of 0.45 mL/min (≈60min). The insoluble compounds in the supercritical mixture precipitated in this high-pressure vessel. Those compounds that were still soluble in the ethanol-scCO_2 mixture were collected in the downstream vessel (DV). The manual backpressure regulator allowed the exit of the gas through the top of the vessel and the ethanolic solution of the dragged actives was recovered from the bottom. Finally, after the FS is entirely pumped, 30 mL of pure ethanol were pumped to ensure the complete injection of the FS, and later on, pure scCO_2 was injected (≈90 min), to eliminate the residual solvent and ensure its complete dragging to the DV. The ethanolic solution recovered in this vessel was dried using a rotavapor (model R-200) equipped with a heat bath (model B-490) a controller vacuum (model V-800) and a vacuum pump (model V-700) (Büchi, Marshall Scientific) at 70 mbar and 42 ºC and weight to determine the mass. The solid fraction from PV was directly weighted. The yields Y_{SAF}%, Y_{DV}%, Y_{PV}% were calculated using Eq. (3) and Eq. (4).

$$Y_i(\text{wt\%}) = (\text{mass fraction collected}_i / \text{mass of FS}) \cdot 100 \quad (3)$$

where *i* is the place of collecting: PV or DV

$$Y_{\text{SAF}}(\text{wt\%}) = Y_{\text{PV}}(\text{wt\%}) + Y_{\text{DV}}(\text{wt\%}) \quad (4)$$

The confidence interval of the obtained yields Y_{PV}%, Y_{DV}% and Y_{SAF}% from three experiment replicates was determined to measure the reproducibility of the SAF process applied.

Fractions from PV and DV and 1 mL sample from FS were collected and kept in ambar vials at -20 ºC until their analysis with HPLC-PDA.

2.5 HPLC analysis

The FS and its supercritical fractions PV and DV, were analysed by HPLC-PDA on a HPLC *Waters® Alliance 2695* with a *PDA Waters® 2998* detector. A *CORTECS®* C18 2.7 µm (4.6 × 150 mm) with a pre-column CORTECS® Pre-column VanGuard C18 2.7 µm (2.1 × 5 mm) was used. The compounds were eluted with an isocratic mobile phase methanol (MeOH): 0.5% H_3PO_4 in Milli-Q water (88:12) for 10 min at 0.8 mL/min flow rate. The detection wavelength was fixed at 330 nm for the first 6 min and at 210 nm for the last 4 min, in order to detect and quantify RA, OA and UA. Extract solutions (100 ppm approximately) were filtered through a *GH Polypropylene membrane ACRODISC* 13 mm pore size 0.2 µm filter. RA, OA and UA standards were run under the same chromatographic conditions in order to obtain their calibration regression which allows their quantification in the samples. The analyses were performed in triplicate.

2.6 Experimental design and statistical analysis

Response surface methodology (RSM) based on central composite design (CCD) [33] was employed to statistically evaluate and optimise the conditions of pressure (bar) in PV and CO_2 flow rate (g/min), for maximum yield recovery, in both vessels (Y_{SAF}%, Y_{DV}% and Y_{PV}%), as well as for a maximum concentration of *L. luiseri* bioactive compounds (RA, OA and UA). *Pressure* and *CO_2 flow rate* were coded as X_P and $X_{Q_{CO_2}}$, respectively. The range and levels of the variables used are gathered in Table 1.

A mathematical model for a two variable CCD is represented by Eq. (5)

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_{ii} X_i + \sum_{i=1}^{2} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{2} \beta_{ij} X_i X_j \quad (5)$$

where Y is an independent variable *(extraction yield)*, β_0 is the constant coefficient, β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients and β_{12} is an interaction coefficient, and X_i and X_j (X_P and $X_{Q_{CO_2}}$) are the independent variables whose influence is under study.

Response surface design following a central composite design was performed using the software *Minitab 17*, which propounded 11 random experiments with three central replicates (115 bar, 20g/min) according to the range levels of both variables previously set (Table 1). The statistical software was also used to determine the significance ($p<0.05$) of each coefficient in the model (Eq. 5) and the optimal conditions for the

maximum yield recovery or maximum bioactive compound concentration in *L. luisieri* extracts (RA, OA and UA).

2.7 Microscopy observations

A scanning electron microscope (SEM) was used to characterise the solid morphology obtained in the PV by SAF. It was performed by the Electron Microscopy service from Zaragoza University (Spain). To that extent, a LEO 420 version V2.04, ASSING, was used. Extracted solids were placed on a carbon tab previously stuck to an aluminium stub (Agar Scientific, Stansted, UK). Samples were overcoated with carbon using a sputter coater (mod. 108A, Agar Scientific), to have an approximated idea of the particles observed, some spheres of the obtained images were measured using the software *Smartiff image estimator*.

3. Results and Discussion

3.1 L. luisieri *maceration extraction yield and chemical characterization*

Plant material was submitted to two serial macerations. First of all, plant material was soaked into hexane in order to eliminate volatiles and degrease non-polar compounds such as cuticular waxes [20]. The extraction yield of this maceration with hexane, Y_{hex}, was 3.3%. This pretreatment reduce the dilution of the final bioactivity of the second maceration extract. Then, polar and bioactive compounds were obtained in a second maceration performed with ethanol. It is nontoxic solvent, easily biodegradable and it has a higher extractive capacity because it breaks the cell membrane of plant material [34]. This second extract was afterwards processed through Supercritical Antisolvent Fractionation, which only uses Carbon dioxide, also non toxic. The seriated treatment of natural resources allows its complete exploitation and it has been observed in a previous work performed [35, 36].

The extraction yield obtained, Y_{EtOH}%, was 12%, a total content in actives of 120 mg per g of dried *L. luisieri*. Julio et al. [11] obtained similar results when extracting from 2 different *L. luisieri* populations with a Soxhlet apparatus, 18% and 12% respectively, methodology that applies heat. In this work the extraction was performed at room temperature and in ambar bottles to avoid degradation from heat and light, obtaining extraction yield results comparable with soxhlet procedure. The different *L. luisieri* populations presented mainly quantitative differences among the founded actives, for example, in the relative content in rosmarinic (3.4% vs 7.3%) and oleanolic acid (3.5% vs 3.0%), but it was also noticeable the qualitative difference between them regarding ursolic acid, absent in one of the populations[11].

In this work, the ethanolic extract composition analysis, performed applying the method described in 2.7, allowed the identification and quantification of rosmarinic acid (RA), oleanolic acid (OA), and ursolic acid, (UA). Their retention times were 1.6 min, 7.5 min and 7.8 min, respectively, as can be observed in Fig. 2. RA was measured at 310 nm, and OA and UA were measured at 210 nm for a better peak definition and quantification. All identified compounds were quantified: RA 5.3% ±1.2, OA 2.4% ±0.8 and UA 5.1% ±1.2. Although in this work only three compounds were identified, Upson et al. [37] reported that an methanolic extract form Portuguese *L. luisieri* contained several types of flavonoids. Further studies should be perform to elucidate completely the composition of this extract. This maceration extract was submitted to SAF under different experimental conditions of pressure and CO_2 flow rate.

3.2 SAF mass recovery yields

Several experiments of supercritical antisolvent fractionation of *L. luisieri* ethanolic extract were performed varying the pressure and CO_2 flow rate conditions inside the ranges 80–150 bar and 10–30 g/min respectively. The other experimental parameters were fixed; temperature 40 °C to avoid degradation, liquid flow rate 0.5 mL/min to maintain CO_2 molar fraction over the critical point, and FS concentration 3% (wt.%). After every SAF experiment, the mass recovered in each fraction was quantified, and the yields Y_{PV}%, Y_{DV}% and the sum of them Y_{SAF}% were determined according to Eq. 4. These yield results are shown in Table 2, where they have been organised in ascending order of X_P and $X_{Q_{CO_2}}$ for an easier understanding of the data. The central experimental replicates yield measures with 95% confidence intervals, were Y_{PV}% 39.2% ± 3.6, Y_{DV}% 20.1% ± 0.98 and Y_{SAF}% 59.4% ± 4.2.

The conditions at which the highest mass recovery (Y_{SAF}%) was obtained were; 90 bar 27 g/min, 140 bar 27 g/min, and at 115 bar 30 g/min, Besides, as can be observed in table 2, for all measured yields, under the same X_P the yield increases with the $X_{Q_{CO_2}}$. According to these results, $X_{Q_{CO_2}}$ seems to have a marked effect in the mass recovery from *L. luisieri* ethanolic extract. A higher proportion of $scCO_2$ favoured compound precipitation in PV as well as dragging compounds to DV, resulting in an increased total mass recovery. Consequently, the overall mass losses in the SAF equipment are lower when $X_{Q_{CO_2}}$ is increased. Nevertheless, it was not achieved a complete recovery of the matter introduced in the equipment, since, it was always some material retained into the valves, pipes or filter of the SAF equipment. These results

correspond with a previous study reported by Martín et al. [38], although there have been reported other behaviours like with *Artemisia absinthium* [32]. These differences reported depend on the original plant material understudied, the composition of the extract to be fractionated and its solubility into the mixture ethanol-scCO_2.

Regarding the amount of material recovered in PV and DV, it is noticeable that Y_{PV}% is always higher than Y_{DV}% independent of the X_P and $X_{Q_{CO_2}}$ experimental conditions. The range of Y_{PV}% values was 27.7–55.6% (at 115 bar, 10 g/min and at 90 bar, 27 g/min, respectively), and the range of Y_{DV}% values was 8.8–30.9% (at 80 bar, 20 g/min and at 115 bar, 30 g/min, respectively). The range of Y_{SAF}% values was 46.9–82.6% (at 115 bar, 10 g/min and at 115 bar, 30 g/min, respectively). This can be interpreted as the ethanolic maceration extract of *L. luisieri* having a low solubility in ethanol-scCO_2 mixture. The initial composition of the FS affects to the final mass yields obtained in SAF fractions. While Marqués et al. [27] who studied the *Vitis vinifera* seeds extract, yield results correlate with the ones obtained in this work, in a previous study with *Artemisia absithium* ethanolic extract, the mass recover results were very different since the DV fraction yield was always higher than the PV fraction yield [32].

3.3 Actives supercritical fractionation

The ethanolic extract, containing RA 5.3% ±1.2, OA 2.4% ±0.8 and UA 5.1% ±1.2, was dissolved in ethanol at 3% (wt.%) to constitute the feed solution (FS) for each one of the 11 SAF experiments. FS provided two fraction after every experiment: PV, a solid that precipitates in the mixture ethanol-sc-CO_2, and DV, soluble compounds in the mixture collected as an ethanolic solution after depressurization and separation of CO_2. After each experiment, a fine yellow-green powder and a green solution were obtained in the PV and DV fractions respectively, and their content in RA, OA and UA quantified with HPLC-PDA and express in percentage. All chromatographic assays were performed in triplicate (total chromatographic assays n=99). In order to analyse the behaviour of each compound during the supercritical process, it was measured the concentration in PV and DV of each component regarding to the ethanolic extract used as feed solution (FS). To do so, the ratios PV/FS and DV/FS were defined according to equation (6) and when any of these ratios was >1 an enrichment of a compound was assumed. The percentage of RA, OA and UA in each fraction and

their ratio regarding the initial FS, are shown in Table 2, where results have been organised in ascending order of X_P and $X_{Q_{CO_2}}$ for an easier understanding of the data.

$$(PV\ or\ DV)/FS = \frac{mg\ \text{of active}/g\ \text{of}\ PV\ \text{or}\ DV}{mg\ \text{of active}/g\ \text{of}\ FS} \qquad (6)$$

The chromatographic analysis revealed that RA is completely retained in the PV, regardless of the CO_2 pressure and flow rate conditions. RA% in the solid fraction was always higher than in the initial FS therefore, RA ratio PV/FS was always ≥1 (1.1–2.3) (Fig.2). Due to RA complete precipitation in the first vessel, PV, it concentrates its quantity regarding to the FS, providing a solid extract with higher proportion in this antioxidant than in the ethanolic extract. At the working conditions of 140 bar 27g/min of CO_2 flow rate, it was achieved the highest enrichment of RA regarding the initial FS (PV/FS), 2.3 times higher, which correspond with 99.2 mg/g of PV. According to these results, it can be said that RA, the most polar compound studied in this work, is insoluble in the ethanol-scCO_2 mixture. Previous studies applying this technology to Rosmarinus extract obtained also an RA enrichment regarding the initial feed solution, in the PV, *raffinate or precipitate* [17], [29]. Quintana et al. [17] produced a precipitated with a 2–3 fold enrichment of rosmarinic acid at a work range of 80-200 bar and 40-60 °C.

On the other hand, OA and UA are partially soluble in the mixture of solvents because they distributed between PV and DV (Fig. 2). The solubility of this triterpenes in the mixture ethanol-scCO_2 has been previously observed in the supercritical extraction of apple pomace and *Hedoytis diffusa* or snake needle grass [39, 40]. The highest UA extraction yield extracted from apple pomace was at 60 °C, 550 bar and ethanol 25% (w/w), while for OA from snake needle grass was 282 bar, 56 °C and ethanol 12.5% (v/v).

Besides, this separation between both fractions seems to be influenced by X_P and $X_{Q_{CO_2}}$ because their concentration distribution varied in every SAF experiment performed. This is shown in Table 2, where OA and UA concentrations (%) and therefore their ratios (PV/FS and DV/FS) varied. OA concentration ratios in PV were 0.53–2.00 which correspond to 1.49–3.40% of PV, while DV/FS ranged from 0.00 to 3.56, which corresponds to 0.00–5.86% DV fraction. UA and OA have a very similar chemical structure and same molecular weight, only differs on the position of a methyl group. As we can see in table 2, at some experimental conditions such as 115 bar 30 g/min or 150 bar 20 g/min both terpenes behaves similarly, they

precipitate in the first vessel when they find sc-CO2, and only a few proportion of them is dragged to the final DV fraction.

UA PV/FS varied from 0.90 to 2.72, which corresponds to 5.86–10.44 % of PV fraction, while DV/FS was mainly <1 (0.05–1.28) which corresponds to 0.20–5.22%. Between all experiment performed, at 140 bar 27 g/min OA and UA concentrates in both fractions along with RA in PV and a high mass recovery is obtained. The different distribution into SAF fractions of natural extracts differ among plant species depending on the solubility into the mixture ethanol-scCO2 of their main compounds. An analysis of the possible traces of the liquid solvent employed should be performed in further studies since the simultaneous concentration of this three natural bioactives could have interest for its industrial production and application in the alimentary or pharmaceutical fields as natural preservatives.

3.4 SEM image analysis/characterization

The microscopic observation of the precipitated solid obtained in the PV showed spherical morphologies and particles of nanometric order. Although an analysis of particle distribution was not performed, some micrographs of the powder recovered in some experiments for its observation. As an example, Fig. 3 is provided, where particle measured diameter observed was of 68-70 nm at 90 bar 27 g/min. The morphology and the size are highly influenced by the droplet formed by the injector and the liquid surface tension [41] but also by the experimental conditions. During the precipitation process, the $scCO_2$ diffuses and eliminates the ethanol that surrounds the extract very quickly forcing the solid to conserve its original shape and volume, and when the mixture ethanol-$scCO_2$ is over the critical point an increase of pressure leads to smaller particles would [42]. The mean size and particle size distribution of the precipitate should be further characterize to determine the influence of X_P and $X_{Q_{CO_2}}$ in the precipitation process for this *L. luisieri* extract.

3.5 Statistical analysis and SAF conditions optimization

In order to determine the statistical influence of X_P and $X_{Q_{CO_2}}$ variables, a surface response analysis of all these results was performed with the software *Minitab 17*. Y_{SAF}% Y_{PV}% Y_{DV}% and RA+OA+UA PV/FS were adopted as response variables and used to determine the coefficients of the equations. The level of significance of each equation factor (linear, quadratic and interaction), the final coefficient of determination (R^2) and the standard deviation (s) were obtained. The equations (6), (7) and (8) define the response surface

of the experimental yields, Y_{PV}% Y_{DV}% and Y_{SAF}% respectively, as a function of pressure and CO_2 flow rate. These equations are graphically represented in Fig. 4.

Total recovery of plant material (Y_{SAF}%) and its distribution between precipitation and downstream vessels (Y_{PV}% and Y_{DV}%) depended on X_P and $X_{Q_{CO_2}}$ experimental conditions.

According to the statistical analysis, the mass recovered in the PV is influenced by both linear factors and only quadratic X_P (Eq. 7). The graphical representation of this equation in Fig. 4.B predicts a higher Y_{PV}% at the lowest values of X_P and the highest $X_{Q_{CO_2}}$ of the range studied. At this same figure, a maximum recovery in DV at the highest values of $X_{Q_{CO_2}}$ can be observed, however in this case higher values of X_P are also required. Y_{DV}% depends on both linear and quadratic X_P and $X_{Q_{CO_2}}$ (Eq. 8). The solubility of compounds extracted with ethanol from *L. luisieri* seems to increase their solubility along with pressure, causing their pass through to the PV filter towards the DV fraction. See Fig. 4.C.

Finally, as Y_{SAF}% is the sum of both PV and DV yields, the influence of X_P and $X_{Q_{CO_2}}$ is a combination their effect in both fractions separately. In this case, Y_{SAF}% depends on the linear factors and the quadratic $X_{Q_{CO_2}}$ (Eq. 9). In the graphical representation, Fig. 4.A, of this equation, for a fixed pressure the yield increases with the $X_{Q_{CO_2}}$.

$$Y_{PV}\% = 67.6 - 0.737X_P + 1.196X_{Q_{CO_2}} + 0.00256X_P{}^2 \ (R^2 = 88.93\%, s = 3.5) \quad (7)$$

$$Y_{DV}\% = -32.9 + 1.003X_P - 1.920X_{Q_{CO_2}} - 0.00364X_P{}^2 + 0.0606X_{Q_{CO_2}}{}^2 \ (R^2 = 88.59\%, s = 2.7) \quad (8)$$

$$Y_{SAF}\% = 59.4 + 0.0164X_P - 1.92X_{Q_{CO_2}} + 0.0905X_{Q_{CO_2}}{}^2 \ (R^2 = 90.17\%, s = 4.4) \quad (9)$$

It was also analysed how experimental variables influenced the fractionation chemical composition. According to the results, the behaviour of RA, OA and UA did not respond individually to the CCD model applied in this work, nor its % in PV or DV, neither their concentration regarding FS, PV/FS or DV/FS. Nevertheless, when the three compounds are analysed together RA+OA+UA, their concentration in PV regarding the initial FS depend on the studied experimental variables X_P and $X_{Q_{CO_2}}$. This dependence, defined in Eq. 10, is graphically represented in Fig. 4.D.

RA+OA+UA PV/FS = $5.257 - 0.03213X_P - 0.2064X_{Q_{CO_2}} + 0.000649X_{Q_{CO_2}}{}^2 + 0.001625X_P \cdot X_{Q_{CO_2}}$ (R^2 = 96.24%, s = 0.05) (10)

According to this statistical prediction equation, the highest concentration of RA, OA and UA in the PV can be obtained at low X_P and $X_{Q_{CO_2}}$ (X_P < 90 bar and $X_{Q_{CO_2}}$< 12 g/min) or at high X_P and $X_{Q_{CO_2}}$ (X_P > 140 bar and $X_{Q_{CO_2}}$ > 27 g/min) which correspond with the results observed in table 2.

Under these experimental conditions, the actives solubility in the ethanol-scCO$_2$ mixture decreases. RA, OA and UA have been reported to present different biological activities; RA as anti-inflammatory, anti-allergy [43] and cytoprotective [18]; and OA and UA as hepatoprotective [44], antiinflamatory [15], antimutagenic [45], and antimicrobial [18, 35, 44]. Besides, because of the concomitant presence of OA and UA in many medicinal plant species, some studies have reported their positive effects when applied together. They have shown chemo-protective effects against DNA damage through oxidation [34, 35], and in vitro and in vivo anti-proliferatives [47]. Because of this, their concomitant concentration may be interesting for several applications. Thus, the antioxidant and antimicrobial properties of *L. luisieri* supercritical fractions have been reported. Both bioactivities were concentrated into the PV fraction regarding the initial ethanolic extract. The antioxidant activity was related to the enrichment in rosmarinic acid, while the terpenes oleanolic and ursolic acids seemed to be responsible of the inhibitory and bactericidal properties [31]. Therefore, the final enriched multifunctional product can be used for several applications.

According to the final optimisation analysis performed in this work, the theoretical conditions for a maximum extract recovery after the SAF process (Y_{SAF}%, Y_{PV}% and Y_{DV}% maximum) and a higher concentration of the three studied compounds in PV (RA+OA+UA PV/FS) are 130 bar and 30 g/min (composite desirability 0.9385), as represented in Fig. 5.

4. Conclusions

In the present work, an integrated process based on the use of supercritical antisolvent fractionation has been optimised to obtain *L. luisieri* extracts with concentrated composition in bioactives. By employing an RSM CCD it has been possible to optimise the yield recovery by modifying important factors involved in the SAF process (pressure X_P and scCO$_2$ flow rate $X_{Q_{CO_2}}$). Besides, the behaviour of three tracked compounds

identified from the ethanolic extract of *L. luisieri* along the supercritical process was also followed. The optimised conditions for a higher mass recovery and RA, OA and UA enrichment in PV were 130 bar 30 g/min. The application of the green SAF technology lets us obtain in the PV a fine solid product highly enriched, with potential for its industrial production and application in the alimentary or pharmaceutical fields as natural preservatives.

Declaration of interests

The corresponding author, on behalf of all authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Authors give thanks to MINECO-FEDER funds [project number CTQ2015-64049-C3-2-R] and Gobierno de Aragón-FSE-FEDER "Construyendo Europa desde Aragón" (Group E39_17R) for the financial support, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) and to J. Burillo from CITA (Centro de Investigación Agroalimentario de Aragón), for providing the plant material.

REFERENCES

[1] R. Gyawali y S. A. Ibrahim, «Natural products as antimicrobial agents», *Food Control*, vol. 46, pp. 412-429, dic. 2014, doi: 10.1016/j.foodcont.2014.05.047.

[2] R. Campos-Vega, G. Loarca-Piña, y B. D. Oomah, «Minor components of pulses and their potential impact on human health», *Food Res. Int.*, vol. 43, n.º 2, pp. 461-482, mar. 2010, doi: 10.1016/j.foodres.2009.09.004.

[3] S. Krüger, M. Mirgos, y G. E. Morlock, «Effect-directed analysis of fresh and dried elderberry (Sambucus nigra L.) via hyphenated planar chromatography», *J. Chromatogr. A*, vol. 1426, pp. 209-219, dic. 2015, doi: 10.1016/j.chroma.2015.11.021.

[4] A. Oniszczuk, M. Olech, T. Oniszczuk, K. Wojtunik-Kulesza, y A. Wójtowicz, «Extraction methods, LC-ESI-MS/MS analysis of phenolic compounds and antiradical properties of functional food enriched with elderberry flowers or fruits.», *Arab. J. Chem.*, doi: 10.1016/j.arabjc.2016.09.003.

[5] M. D. Kalim, D. Bhattacharyya, A. Banerjee, y S. Chattopadhyay, «Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine», *BMC Complement. Altern. Med.*, vol. 10, n.º 1, p. 77, dic. 2010, doi: 10.1186/1472-6882-10-77.

[6] M. C. M.I. García-Vallejo, García-Vallejo, J. Sanz, M. Bernabe, y A. Velasco-Negueruela, «Necrodane (1,2,2,3,4-pentamethylcyclopentane) derivatives in Lavandula luisieri, new compounds to the plant kingdom», *Phytochemistry*, vol. 36, n.º 1, pp. 43-45, may 1994, doi: 10.1016/S0031-9422(00)97009-2.

[7] T. Eisner, M. Deyrup, R. Jacobs, y J. Meinwald, «Necrodols: Anti-insectan terpenes from defensive secretion of carrion beetle (Necrodes surinamensis)», *J. Chem. Ecol.*, vol. 12, n.º 6, pp. 1407-1415, jun. 1986, doi: 10.1007/BF01012360.

[8] B. Roach, T. Eisner, y J. Meinwald, «Defense mechanisms of arthropods. 83. .alpha.- and .beta.-Necrodol, novel terpenes from a carrion beetle (Necrodes surinamensis, Silphidae, Coleoptera)», *J. Org. Chem.*, vol. 55, n.º 13, pp. 4047-4051, jun. 1990, doi: 10.1021/jo00300a020.

[9] B. A. Figadère, J. S. McElfresh, D. Borchardt, K. M. Daane, W. Bentley, y J. G. Millar, «trans-α-Necrodyl isobutyrate, the sex pheromone of the grape mealybug, Pseudococcus maritimus», *Tetrahedron Lett.*, vol. 48, pp. 8434-8437.

[10] N. Baldovini, S. Lavoine-Hanneguelle, G. Ferrando, G. Dusart, y L. Lizzani-Cuvelier, «Necrodane monoterpenoids from Lavandula luisieri», *Phytochemistry*, vol. 66, n.º 14, pp. 1651-1655, jul. 2005, doi: 10.1016/j.phytochem.2005.04.040.

[11] L. F. Julio *et al.*, «Comparative chemistry and insect antifeedant effects of conventional (Clevenger and Soxhlet) and supercritical extracts (CO2) of two Lavandula luisieri populations», *Ind. Crops Prod.*, vol. 58, pp. 25-30, jul. 2014, doi: 10.1016/j.indcrop.2014.03.021.

[12] A. González-Coloma, F. Delgado, J. M. Rodilla, L. Silva, J. Sanz, y J. Burillo, «Chemical and biological profiles of Lavandula luisieri essential oils from western Iberia Peninsula populations», *Biochem. Syst. Ecol.*, vol. 39, n.º 1, pp. 1-8, feb. 2011, doi: 10.1016/j.bse.2010.08.010.

[13] J. Bernatoniene, U. Cizauskaite, L. Ivanauskas, V. Jakstas, Z. Kalveniene, y D. M. Kopustinskiene, «Novel approaches to optimize extraction processes of ursolic, oleanolic and rosmarinic acids from Rosmarinus officinalis leaves», *Ind. Crops Prod.*, vol. 84, pp. 72-79, jun. 2016, doi: 10.1016/j.indcrop.2016.01.031.

[14] M. Ghasemzadeh Rahbardar, B. Amin, S. Mehri, S. J. Mirnajafi-Zadeh, y H. Hosseinzadeh, «Anti-inflammatory effects of ethanolic extract of Rosmarinus officinalis L. and rosmarinic acid in a rat model of neuropathic pain», *Biomed. Pharmacother.*, vol. 86, pp. 441-449, feb. 2017, doi: 10.1016/j.biopha.2016.12.049.

[15] J. Liu, «Pharmacology of oleanolic acid and ursolic acid», *J. Ethnopharmacol.*, vol. 49, n.º 2, pp. 57-68, dic. 1995, doi: 10.1016/0378-8741(95)90032-2.

[16] M. Petersen y M. S. J. Simmonds, «Rosmarinic acid», *Phytochemistry*, vol. 62, n.º 2, pp. 121-125, ene. 2003, doi: 10.1016/S0031-9422(02)00513-7.

[17] S. E. Quintana, D. Villanueva-Bermejo, G. Reglero, M. R. García-Risco, y T. Fornari, «Supercritical antisolvent particle precipitation and fractionation of rosemary (Rosmarinus officinalis L.) extracts», *J. CO2 Util.*, vol. 34, pp. 479-489, dic. 2019, doi: 10.1016/j.jcou.2019.07.032.

[18] S. Nunes *et al.*, «Therapeutic and nutraceutical potential of rosmarinic acid—Cytoprotective properties and pharmacokinetic profile», *Crit. Rev. Food Sci. Nutr.*, vol. 57, n.º 9, pp. 1799-1806, jun. 2017, doi: 10.1080/10408398.2015.1006768.

[19] K. I. Wolska, A. M. Grudniak, B. Fiecek, A. Kraczkiewicz-Dowjat, y A. Kurek, «Antibacterial activity of oleanolic and ursolic acids and their derivatives», *Cent. Eur. J. Biol.*, vol. 5, n.º 5, pp. 543-553, oct. 2010, doi: 10.2478/s11535-010-0045-x.
[20] M. M. R. de Melo, A. J. D. Silvestre, y C. M. Silva, «Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology», *J. Supercrit. Fluids*, vol. 92, pp. 115-176, ago. 2014, doi: 10.1016/j.supflu.2014.04.007.
[21] E. Reverchon y I. De Marco, «Supercritical fluid extraction and fractionation of natural matter», *J. Supercrit. Fluids*, vol. 38, n.º 2, pp. 146-166, sep. 2006, doi: 10.1016/j.supflu.2006.03.020.
[22] V. Pérez-Tortosa, A. López-Orenes, A. Martínez-Pérez, M. A. Ferrer, y A. A. Calderón, «Antioxidant activity and rosmarinic acid changes in salicylic acid-treated Thymus membranaceus shoots», *Food Chem.*, vol. 130, n.º 2, pp. 362-369, ene. 2012, doi: 10.1016/j.foodchem.2011.07.051.
[23] M. A. Meneses, G. Caputo, M. Scognamiglio, E. Reverchon, y R. Adami, «Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction», *J. Food Eng.*, vol. 163, pp. 45-53, oct. 2015, doi: 10.1016/j.jfoodeng.2015.04.025.
[24] P. Franco, E. Reverchon, y I. De Marco, «PVP/ketoprofen coprecipitation using supercritical antisolvent process», *Powder Technol.*, vol. 340, pp. 1-7, dic. 2018, doi: 10.1016/j.powtec.2018.09.007.
[25] G. Ozkan, P. Franco, I. De Marco, J. Xiao, y E. Capanoglu, «A review of microencapsulation methods for food antioxidants: Principles, advantages, drawbacks and applications», *Food Chem.*, vol. 272, pp. 494-506, ene. 2019, doi: 10.1016/j.foodchem.2018.07.205.
[26] G. Perretti, C. Virgili, A. Troilo, O. Marconi, G. F. Regnicoli, y P. Fantozzi, «Supercritical antisolvent fractionation of lignans from the ethanol extract of flaxseed», *J. Supercrit. Fluids*, vol. 75, pp. 94-100, mar. 2013, doi: 10.1016/j.supflu.2012.12.028.
[27] J. L. Marqués, G. D. Porta, E. Reverchon, J. A. R. Renuncio, y A. M. Mainar, «Supercritical antisolvent extraction of antioxidants from grape seeds after vinification», *J. Supercrit. Fluids*, vol. 82, pp. 238-243, oct. 2013, doi: 10.1016/j.supflu.2013.07.005.
[28] J. T. Paula, I. M. O. Sousa, M. A. Foglio, y F. A. Cabral, «Selective fractionation of extracts of Arrabidaea chica Verlot using supercritical carbon dioxide as antisolvent», *J. Supercrit. Fluids*, vol. 133, pp. 9-16, mar. 2018, doi: 10.1016/j.supflu.2017.09.021.
[29] A. P. Sánchez-Camargo *et al.*, «Supercritical antisolvent fractionation of rosemary extracts obtained by pressurized liquid extraction to enhance their antiproliferative activity», *J. Supercrit. Fluids*, vol. 107, pp. 581-589, ene. 2016, doi: 10.1016/j.supflu.2015.07.019.
[30] A. Visentin, S. Rodríguez-Rojo, A. Navarrete, D. Maestri, y M. J. Cocero, «Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process», *J. Food Eng.*, vol. 109, n.º 1, pp. 9-15, mar. 2012, doi: 10.1016/j.jfoodeng.2011.10.015.
[31] C. Giménez-Rota, S. Lorán, A. M. Mainar, M. J. Hernáiz, y C. Rota, «Supercritical Carbon Dioxide Antisolvent Fractionation for the Sustainable Concentration of Lavandula luisieri (Rozeira) Riv.- Mart Antimicrobial and Antioxidant Compounds and Comparison with Its Conventional Extracts», *Plants*, vol. 8, n.º 11, p. 455, nov. 2019, doi: 10.3390/plants8110455.
[32] Elisa Langa, Juan Ignacio Pardo, Carlota Giménez-Rota, Azucena González-Coloma, María J. Hernáiz, y Ana M. Mainar, «Supercritical anti-solvent fractionation of Artemisia absinthium L. conventional extracts: tracking artemetin and casticin | Elisa Langa | Request PDF», *Journal of Supercritical Fluids*, vol. 151, may 2019, doi: 10.1016/j.supflu.2019.05.003.
[33] Douglas C. Montgomery, *Design and Analysis of Experiments*, 9th ed. Wiley.
[34] A. Wüst Zibetti, A. Aydi, M. Arauco Livia, A. Bolzan, y D. Barth, «Solvent extraction and purification of rosmarinic acid from supercritical fluid extraction fractionation waste: Economic evaluation and scale-up», *J. Supercrit. Fluids*, vol. 83, pp. 133-145, nov. 2013, doi: 10.1016/j.supflu.2013.09.005.
[35] Hanna Pieper, «The utilization of the residues of viticulture via supercritical extraction: Antioxidative capacity of the extracts», vol. Bachelor Essay.
[36] Juan I. Pardo *et al.*, «Té de roca: Extractos supercriticos y actividad antioxidante», vol. VII reunión de expertos en tecnologías de fluidos comprimidos, 10-13 Junio.
[37] T. M. Upson, R. J. Grayer, J. R. Greenham, C. A. Williams, F. Al-Ghamdi, y F.-H. Chen, «Leaf flavonoids as systematic characters in the genera Lavandula and Sabaudia», *Biochem. Syst. Ecol.*, vol. 28, n.º 10, pp. 991-1007, dic. 2000, doi: 10.1016/S0305-1978(00)00013-2.
[38] L. Martín, A. González-Coloma, C. E. Díaz, A. M. Mainar, y J. S. Urieta, «Supercritical CO2 extraction of Persea indica: Effect of extraction parameters, modelling and bioactivity of its extracts», *J. Supercrit. Fluids*, vol. 57, n.º 2, pp. 120-128, jun. 2011, doi: 10.1016/j.supflu.2011.03.004.

[39] E. Ordonez-Quintana *et al.*, «Supercritical and subcritical extraction of ursolic acid and polyphenols from apple pomace: Effect of variables on composition and antioxidant capacity», *J. Food Process. Preserv.*, p. e14296, doi: 10.1111/jfpp.14296.
[40] M.-C. Wei, Y.-C. Yang, y S.-J. Hong, «Determination of Oleanolic and Ursolic Acids in Hedyotis diffusa Using Hyphenated Ultrasound-Assisted Supercritical Carbon Dioxide Extraction and Chromatography», *Evid.-Based Complement. Altern. Med. ECAM*, vol. 2015, 2015, doi: 10.1155/2015/450547.
[41] E. Reverchon y I. De Marco, «Mechanisms controlling supercritical antisolvent precipitate morphology», *Chem. Eng. J.*, vol. 169, n.º 1, pp. 358-370, may 2011, doi: 10.1016/j.cej.2011.02.064.
[42] A. Montes, C. Pereyra, y E. J. M. de la Ossa, «Mean Aspects Controlling Supercritical CO2 Precipitation Processes», *Heat Mass Transf. - Adv. Sci. Technol. Appl.*, may 2019, doi: 10.5772/intechopen.85735.
[43] J. Weiss, P. Takhistov, y D. J. McClements, «Functional Materials in Food Nanotechnology», *J. Food Sci.*, vol. 71, n.º 9, pp. R107-R116, nov. 2006, doi: 10.1111/j.1750-3841.2006.00195.x.
[44] G. A. Gutiérrez-Rebolledo, A. G. Siordia-Reyes, M. Meckes-Fischer, y A. Jiménez-Arellanes, «Hepatoprotective properties of oleanolic and ursolic acids in antitubercular drug-induced liver damage», *Asian Pac. J. Trop. Med.*, vol. 9, n.º 7, pp. 644-651, jul. 2016, doi: 10.1016/j.apjtm.2016.05.015.
[45] F. Aparecida Resende, C. A. M. de Andrade Barcala, M. C. da Silva Faria, F. H. Kato, W. R. Cunha, y D. C. Tavares, «Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice», *Life Sci.*, vol. 79, n.º 13, pp. 1268-1273, ago. 2006, doi: 10.1016/j.lfs.2006.03.038.
[46] J. A. Jesus, J. H. G. Lago, M. D. Laurenti, E. S. Yamamoto, y L. F. D. Passero, «Antimicrobial Activity of Oleanolic and Ursolic Acids: An Update», *Evidence-Based Complementary and Alternative Medicine*, 2015. [En línea]. Disponible en: https://www.hindawi.com/journals/ecam/2015/620472/. [Accedido: 10-jul-2018].
[47] C. Soica *et al.*, «The Synergistic Biologic Activity of Oleanolic and Ursolic Acids in Complex with Hydroxypropyl-γ-Cyclodextrin», *Molecules*, vol. 19, n.º 4, pp. 4924-4940, abr. 2014, doi: 10.3390/molecules19044924.

FIGURE CAPTIONS

Fig. 1. Scheme of the SAF plant. Feed solution reservoir (FS); liquid pump (P-LIQ); CO_2 reservoir (R), cooling bath (CB); CO_2 pump (P-SCF); heat exchanger (HE); precipitation vessel (PV); Thermopar (T); automated back pressure regulator (ABPR); back pressure regulator (BPR); downstream vessel (DV).

Fig. 2. Overlayed chromatograms of ME (red) PV (blue) and DV (black) at 115 bar and 20 g/min. Peak 1 RA (T_R = 1.626 min, λ = 310 nm), peak 2 OA (T_R = 7.468 min, λ = 210 nm) and peak 3 UA (T_R = 7.802 min, λ = 210 nm).

Fig. 3. Scanning electron micrograph from the solid obtained in the precipitation vessel. Minimum particle measured diameter of 68 nm at 90 bar 27 g/min (Mag= 40.00 K X)

Fig. 4. Response surface plots of the SAF fraction yields: A) Y_{PV}%; B) Y_{DV}%; C) Y_{SAF}% as a function of X_P and $X_{Q_{CO_2}}$; and D) concentration of RA+OA+UA in PV regarding FS as a function of X_P and $X_{Q_{CO_2}}$.

FIGURES

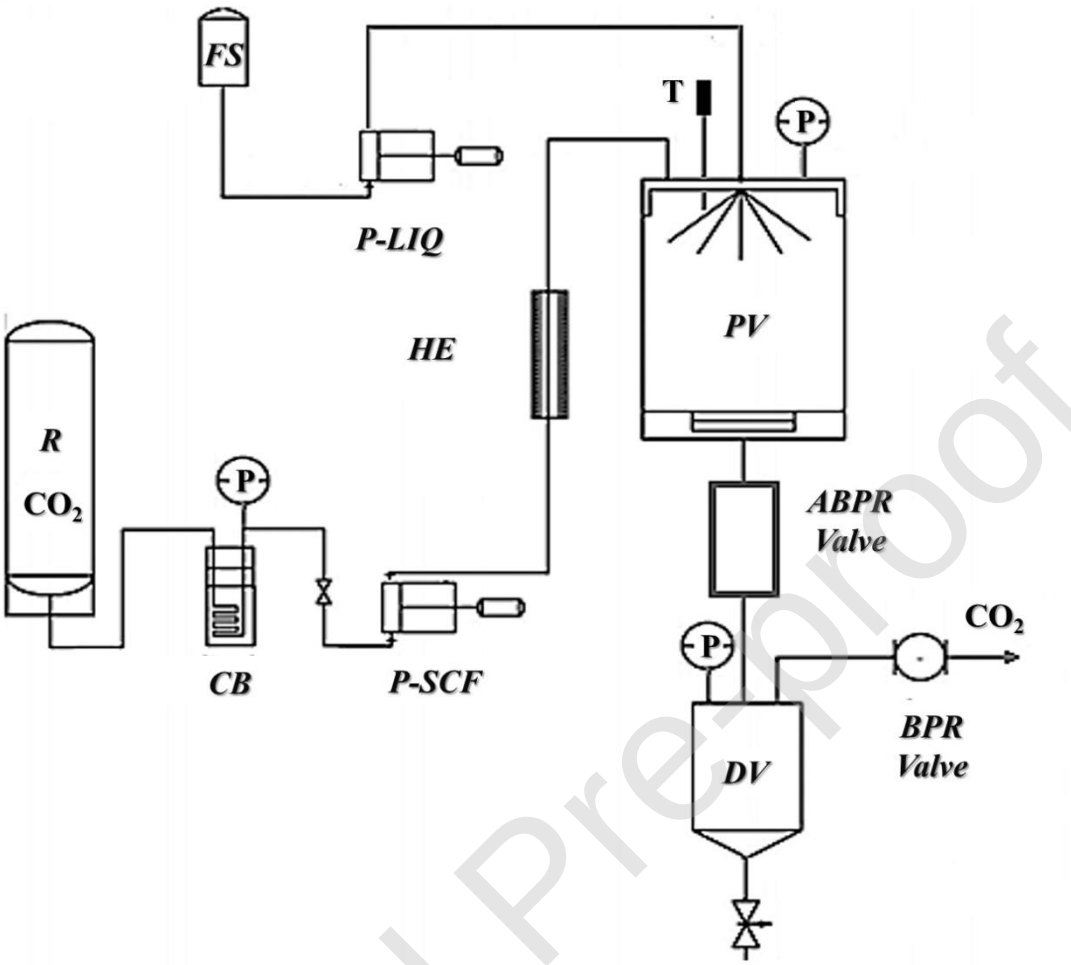


Figure 1

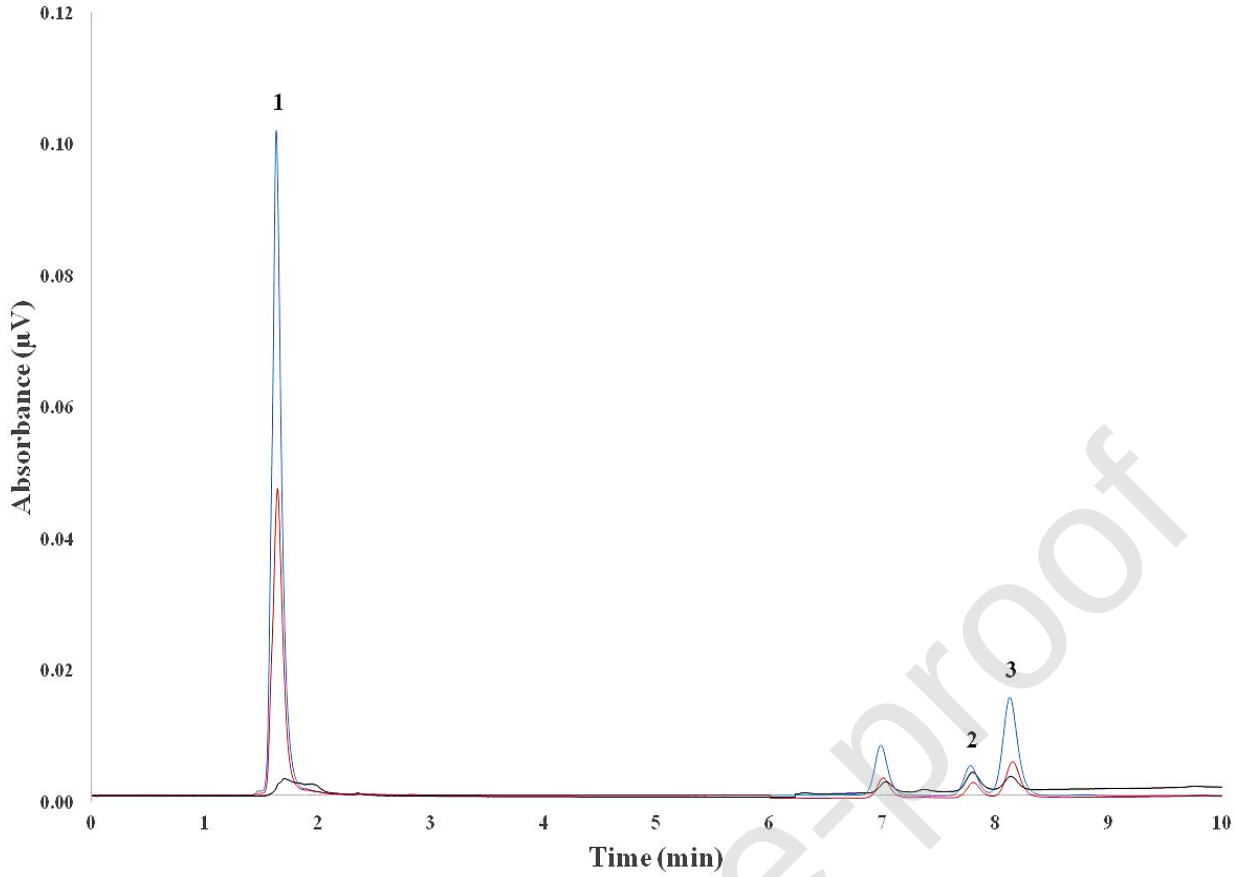


Figure 2

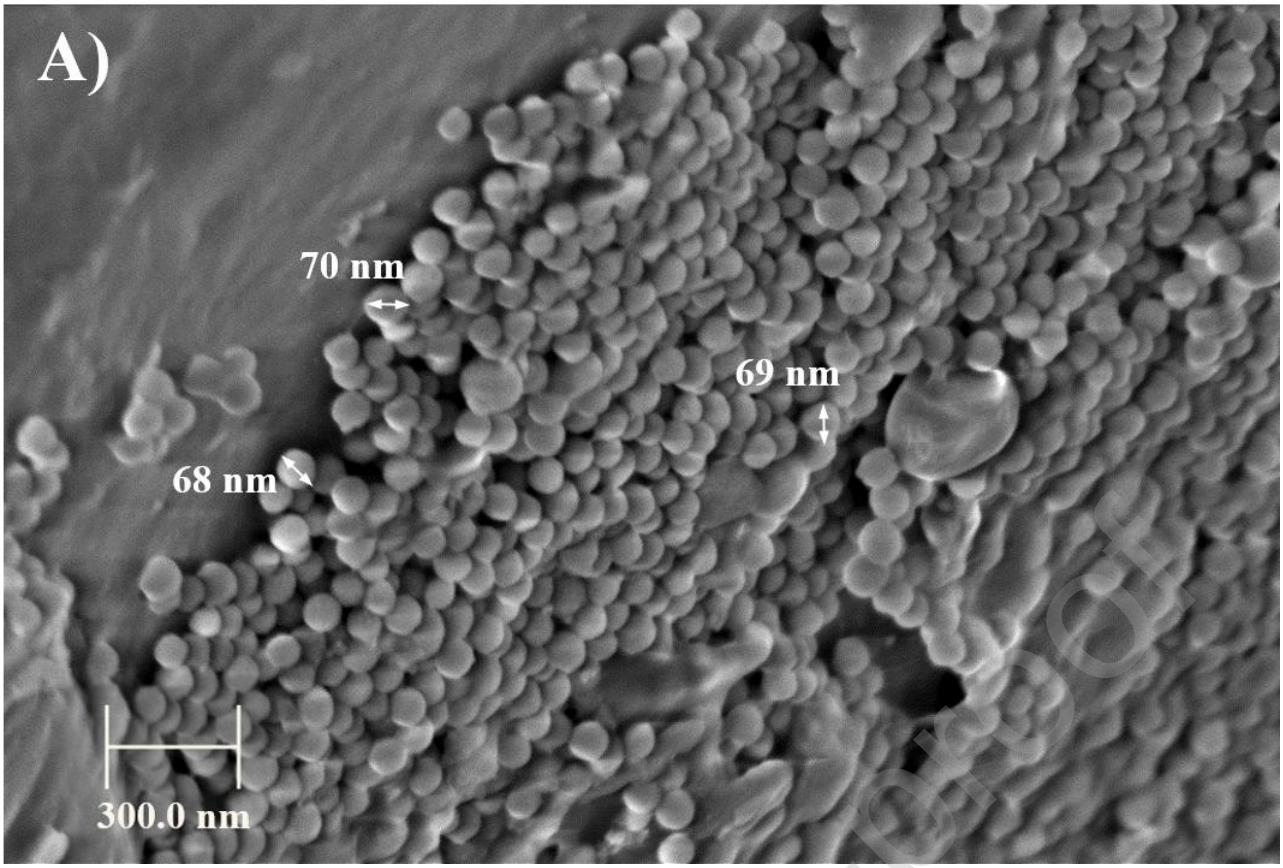


Figure 3

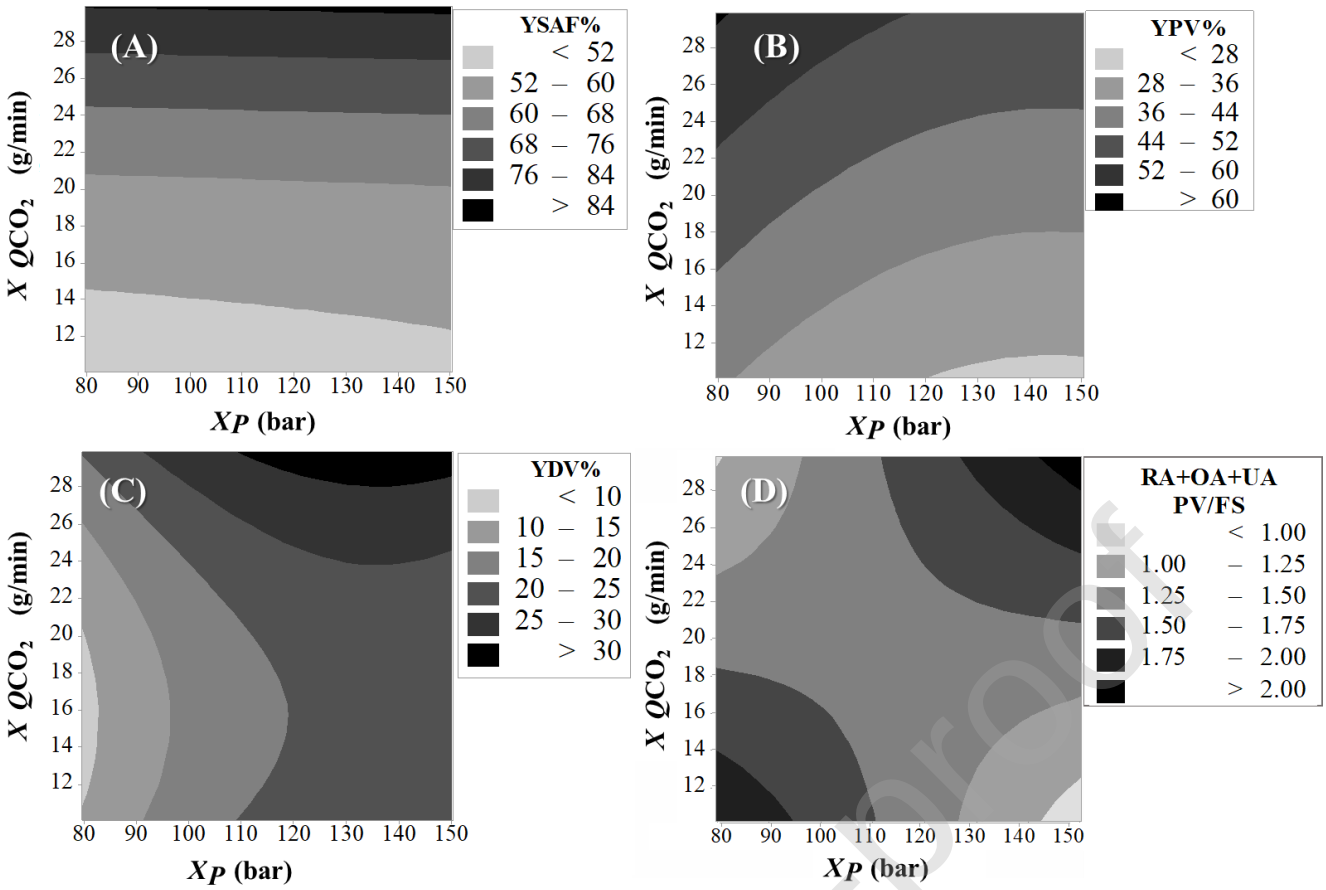


Figure 4

TABLES

Table 1. Codification and levels of the two independent variables for the SAF factorial design of experiments.

Variable	Symbol	Factor levels				
		{-1.44	−1	0	1	1.44}
Pressure (bar)	X_P	80	90	115	140	150
CO_2 flow rate (g/min)	$X_{Q_{CO_2}}$	10	13	20	27	30

Table 2. *L. luisieri* experimental SAF results. Experimental yields obtained in each experiment; Y_{SAF} %, Y_{PV} % and Y_{DV}% RA, OA and UA in % quantified in FS, PV and DV obtained in each experiment; and RA, OA and UA concentration in PV and DV regarding FS.

X_P	$X_{Q_{CO_2}}$	Y_{PV}%	Y_{DV}%	Y_{SAF}%	RA			OA					UA					RA+OA+UA
(bar)	(g/min)				FS (%)	PV(%)	PV/FS	FS(%)	PV(%)	DV(%)	PV/FS	DV/FS	FS(%)	PV(%)	DV(%)	PV/FS	DV/FS	PV/FS
80	**20**	48.0	8.8	56.8	5.1	8.9	**1.8**	1.9	1.8	5.5	**1.0**	**2.9**	4.6	7.0	4.6	**1.5**	**1.0**	**1.4**
90	**13**	39.0	15.4	54.4	4.0	7.9	**2.0**	1.5	3.3	2.4	**2.3**	**1.6**	3.8	10.4	1.8	**2.7**	**0.5**	**1.7**
	27	55.6	20.1	75.7	6.4	6.7	**1.1**	2.5	2.5	1.2	**1.0**	**0.5**	5.6	5.6	1.0	**1.0**	**0.2**	**1.2**
115	**10**	27.7	19.2	46.9	5.7	8.0	**1.4**	2.3	1.9	3.5	**0.8**	**1.5**	5.2	7.1	4.7	**1.4**	**0.9**	**1.4**
	20	38.7	19.2	57.9	4.0	8.9	**2.2**	1.5	2.1	5.0	**1.3**	**3.2**	3.9	6.5	3.9	**1.7**	**1.0**	**1.4**
	20	36.3	20.3	56.6	3.8	7.3	**2.0**	2.2	2.4	3.5	**1.1**	**1.6**	5.1	8.2	2.7	**1.6**	**0.5**	**1.4**
	20	42.6	20.9	63.6	6.3	8.1	**1.3**	3.7	2.4	3.2	**0.7**	**0.9**	7.7	7.0	2.3	**0.9**	**0.3**	**1.4**
	30	51.7	30.9	82.6	6.3	8.1	**1.3**	2.6	3.0	0.6	**1.2**	**0.2**	5.5	8.8	0.6	**1.6**	**0.1**	**1.6**
140	**13**	34.2	22.7	56.9	7.2	7.5	**1.1**	2.8	1.5	4.6	**0.5**	**1.7**	6.5	5.9	4.2	**0.9**	**0.7**	**1.2**
	27	50.6	29.6	80.2	4.4	10.0	**2.3**	1.7	2.0	5.9	**1.2**	**3.6**	4.1	10.3	5.1	**2.5**	**1.3**	**1.8**
150	**20**	33.9	20.3	54.2	4.6	6.9	**1.5**	1.7	3.4	0.0	**2.0**	**0.0**	4.3	7.8	0.2	**1.8**	**0.1**	**1.5**