



Alkaloids from *Chelidonium majus* L.: Fractionated supercritical CO₂ extraction with co-solvents



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ABSTRACT

Chelidonium majus L. is rich in many isoquinoline alkaloids which are responsible for the anti-inflammatory, antitumor and cytotoxic bioactivities known for this plant. The main alkaloids identified in *C. majus* L. include chelidonine, berberine, coptisine, sanguinarine and chelerythrine, which are present in different parts of the plant. In this work, alkaloids from *C. majus* aerial and terrestrial parts were extracted using a two step extraction procedure which consisted in a first step (SFE) using only supercritical carbon dioxide (scCO₂) as solvent followed by a second step (ESE) using scCO₂ and a co-solvent mixture composed by an alcohol (ethanol or isopropanol) and diethylamine (alkaline conditions). The effect of operation temperature, pressure, solvent density and solvent pH on extraction yields, kinetic profiles and alkaloids' selectivity was evaluated. Results showed that SFE presents high selectivity for alkaloids (particularly for chelidonine) at solvent densities in the range 813–850 kg/m³ and for both the aerial and terrestrial parts of the plant. The highest alkaloids extraction yield was observed at higher solvent density conditions using the scCO₂/isopropanol/diethylamine mixture (ESE extraction step). Chelidonine was found to be highly soluble in scCO₂. Therefore, the fractionated high pressure extraction procedure proposed in this work can be successfully applied to separate enriched chelidonine fractions, while other alkaloids can be obtained using basified isopropanol as co-solvent. This procedure demonstrates that alkaloids fractionation during extraction is an important tool to be used before further purification/isolation steps.

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1. Introduction

Chelidonium majus L. (commonly known as “greater celandine”) is a perennial plant native from Europe and Western Asia and widely extended to America. A specific feature of this botanical family is the production of outflowing yellow-orange coloured latex, present both in roots and aerial parts, that has been used for centuries in Western phytotherapy and Chinese traditional medicine, mainly due to its antimicrobial and anti-inflammatory properties and its healing activity against skin affections [1,2]. Colombo and Bosisio reviewed the studies reported in the literature concerning the bioactivity of *C. majus* extracts, including anti-inflammatory, antitumor and cytotoxic activity [3]. Authors compared the activity of the plant extracts with their composition

(major purified compounds) and concluded that alkaloids are the main responsible for those activities [3,4].

C. majus latex and herbal preparations are rich in many isoquinoline alkaloids (more than 20 have been identified), which belong to three main groups: (a) benzophenanthridines, like chelidonine, sanguinarine and chelerythrine, (b) protopine and derivatives, and (c) protoberberines, like berberine and coptisine [5]. The chemical structures of some of these compounds are shown in Fig. 1. The relative concentration and distribution of these alkaloids in *C. majus* depends on several factors. Typical alkaloid content in aerial parts are between 0.5 and 1.5% (w/w), according to several authors [6,7] while Suchomelová et al. [8] have reported up to 8% (w/w) total quaternary alkaloids in the methanolic extract of *C. majus* roots. It has been reported that chelidonine, berberine and coptisine are the main alkaloids in the aerial parts of the plant, while sanguinarine and chelerythrine are predominant in the roots [9].

Barbosa-Filho et al. have reported an exhaustive review concerning the anti-inflammatory activity of alkaloids, mainly of isoquinoline

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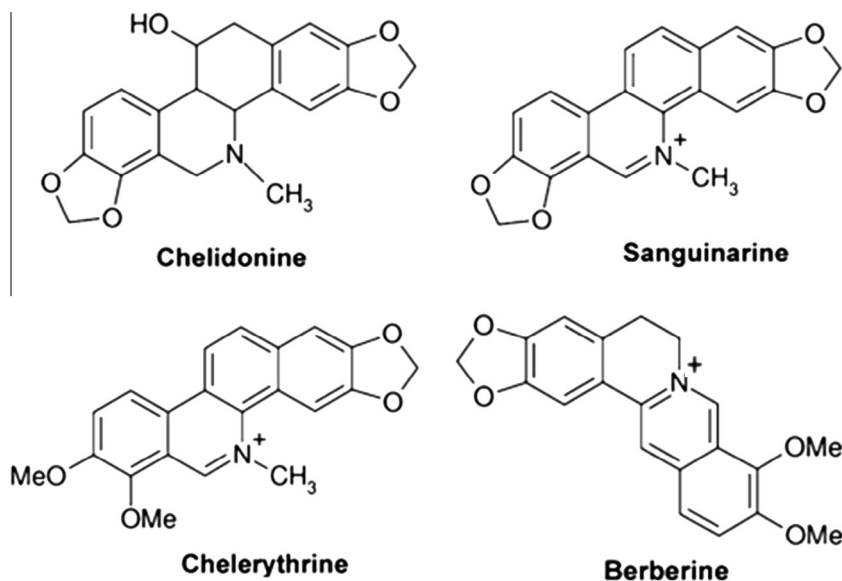


Fig. 1. Chemical structure of main *C. majus* alkaloids.

alkaloids [10]. However, the mechanism responsible for the anti-inflammatory activity of these alkaloids is not yet completely elucidated. Penciková et al. studied the effect of sanguinarine and chelerythrine on gene expression of several pro- and anti-inflammatory cytokines, concluding that sanguinarine presents high anti-inflammatory potential, even comparable to commercial prednisone [11]. Then et al. also reported significant antioxidant activity of *C. majus* ethanolic and aqueous extracts [12].

Due to the promising pharmacological applications of alkaloids, the study and development of techniques for their extraction and purification has become an important research field over the last years. Comparative extraction assays using traditional processes such as infusion, pressing and solvent extraction, as well as novel techniques, such as supercritical fluid extraction (SFE) and microwave assisted extraction have been reported. Then et al. concluded that extract yield, composition and alkaloid content are highly dependent on the extraction method and that SFE using supercritical CO₂ (scCO₂) and propylene glycol as co-solvent, and microwave extraction seem to be more selective towards some specific alkaloids, such as coptisine [13].

SFE of alkaloids with pure CO₂ generally presents lower yields than other solvent extraction methods. When polar co-solvents like water or alcohols are used, total extraction yield increases significantly, due to the increase of solvent phase polarity and diffusional properties. Moreover, the choice of a suitable solvent mixture can enhance the extraction selectivity for some specific compounds or group of compounds, with influence on the biological activity of the extracts [14]. General methods to extract/isolate alkaloids are already reported in the literature frequently using an alcohol (methanol or ethanol) as extraction solvent. The obtained solution could be further basified, generally with ammonium hydroxide, and the separation of alkaloids will depend on their differential basicity [15]. Choi et al. have demonstrated that SFE yield of alkaloids (hyoscyamine and scopolamine) from *Scopolia japonica* Maxim. notably increased when a mixture of methanol and diethylamine (10% v/v) was used as co-solvent (operating at 333 K and 34 MPa) [16]. According to these authors, this happens because these alkaloids naturally occur in plant tissues in the form of salts (hydrochlorides) presenting very low solubility in non-polar solvents like scCO₂ (which is also acidic). Therefore, the use of a basified co-solvent allows the extraction of alkaloids in the form of free bases, which explains the observed higher alkaloids extraction

yields. In fact, quaternary alkaloids exhibit pH-dependent equilibrium between the ionized or iminium form (at pH < pKa) and the neutral or amine form (at pH > pKa), with pKa values typically above 10 [17]. More recently, Xiao et al. tested several co-solvents for the SFE of isoquinoline alkaloids and concluded that the use of diethylamine (10%, v/v), as basifying agent, and water (1%, v/v) as polar co-solvent, enhanced alkaloid extraction yield and selectivity (~50% of the total extract) after 2 h of extraction at 343 K and 20 MPa [18].

Based on these facts, this work aims to study the Supercritical Fluid Extraction (SFE) and Enhanced Solvent Extraction (ESE) of *C. majus* aerial and terrestrial parts in order to obtain fractionated extracts. The effect of temperature, solvent density and basified co-solvents on total extraction yield, kinetic profiles and extract composition was investigated and compared with traditional extraction methods (Soxhlet with ethanol and low pressure solvent extraction with water). Extraction kinetic profiles are also presented and modelled to provide useful information for process design and optimization.

2. Materials and methods

2.1. Samples and chemicals

Samples of *C. majus* were harvested in the district of Guarda, Beira Interior (Portugal). Samples were separated as aerial parts (leaves and stems) and terrestrial parts (roots), dried in an oven at 323 K and comminuted separately originating two distinct raw materials. Particle size distribution was analyzed using a series of sieves and the 18/60 mesh fractions were selected for extraction purposes. The final moisture of the dried samples was determined by thermogravimetric analysis (TGA Q500, TA Instruments, USA).

Carbon dioxide (≥99.5%, Praxair, Spain), ethanol (≥99.5%, p.a., Panreac Quimica SA, Spain), isopropanol (≥99.9%, LC-MS, Fluka, Germany), methanol (≥99.9%, HPLC grade, Carlo Erba, Italy), ethyl acetate (≥99.5%, p.a., Panreac Quimica SA, Spain), dichloromethane (≥99.9%, p.a., Sigma-Aldrich, USA), diethylamine (DEA, ≥99%, Carlo Erba, Italy), acetonitrile (≥99.9%, HPLC grade, Carlo Erba, Italy), formic acid (~98%, p.a., Fluka, Switzerland), ammonium acetate (≥97%, Sigma-Aldrich, Germany), sodium 1-heptanesulfonate (Sigma-Aldrich, Germany), triethylamine

($\geq 99\%$, Sigma-Aldrich, Germany), and milli-Q water were used as solvents for the extraction experiments and extract analysis. Four alkaloid standards were analyzed, namely berberine (CAS number 633-65-8), chelidone (CAS number 476-32-4), chelerythrine chloride ($\geq 95\%$, CAS number 3895-92-9) and sanguinarine chloride hydrate ($\geq 98\%$, CAS number 5578-73-4), which were purchased from Sigma-Aldrich, Germany.

2.2. High-pressure extraction

High-pressure extraction experiments were performed using a modified lab-scale semicontinuous extraction system (Separex, France) shown schematically in Fig. S1. The comminuted raw material ($5\text{--}6 \times 10^{-3}$ kg) was placed into a thermostated stainless steel cell with an internal volume of 20×10^{-6} m³, provided with a filter at the inlet, acting as solvent diffusor, and another at the outlet, to avoid particles to be dragged out with the solvent stream. Pressure was kept by a back-pressure regulator and was measured by a manometer. High-pressure liquid pumps (Separex, France) were used to deliver scCO₂ and co-solvents. The outlet CO₂ flow rate was determined using a gas flow meter (Alexander Wright, London, UK). Extracts were collected into previously weighed refrigerated flasks at different time intervals. An adsorbent packed column (Porapak Q 80/100 mesh, Supelco, Bellefonte, USA) was placed after the collector flask in order to prevent extract losses in the solvent stream.

C. majus extraction was fractionated according to the following procedure: a first SFE step using pure scCO₂ and a second ESE step using scCO₂ and a basified co-solvent mixture composed of ethanol or isopropanol and DEA 9:1 (v/v). The scCO₂:co-solvent mixture ratio was 9:1 (w/w) for all the experiments. In the first SFE step, runs were performed at five different CO₂ density conditions, ranging from 325 to 929 kg/m³ (according to NIST), corresponding to a combination of three pressures (10, 20 and 30 MPa) and three temperature conditions (308, 318 and 328 K). Temperature and pressure were controlled to within 0.1 K and 0.5 MPa, respectively. A first static period of 30 min was applied, in order to improve contact between the raw material and the solvent, after which dynamic extraction was carried out during 300 min. The solvent (s) mass flow rate was set to an average value of 8×10^{-5} kg/s. Extracts were collected and weighed in separate flasks every 15 min and recovered by re-dissolution in ethyl acetate. After this first set of extraction runs, scCO₂ extracted raw material was blended and homogenized, independently of the SFE process conditions, and further extracted in a second ESE step in which two different pressure conditions (12 and 35 MPa) were tested at fixed temperature (328 K). The extraction procedure was performed with an initial static period of 30 min followed by a dynamic period of 210 min.

The residual extracts collected during depressurization and recovered after cleaning the tubing lines (with ethanol and pressurized CO₂) and the adsorbent column (washed with methanol) were stored and quantified separately. All experimental runs were carried out in duplicate. Extracts containing ethanol, methanol, ethyl acetate or co-solvent mixture were evaporated in a rotovap with vacuum control (Rotovapor R-210, Büchi, Switzerland). All the dry extracts were stored in a freezer at 255 K and protected from light until analysis.

2.3. Soxhlet and low pressure water extraction methodologies

For comparison purposes, Soxhlet extraction with ethanol (SoE) and low pressure solvent extraction with water (LPSE) were also performed. SoE was performed using a solid-to-solvent ratio of 1:8 (w/v) under reflux during 180 min. The aqueous extract was obtained using a Schilcher apparatus [19] and according to the

AOAC 962.17 method [20], using a solid-to-solvent ratio of 1:20 (w/v) and an extraction time of 180 min. Both extractions were performed in duplicate.

2.4. Kinetic extraction curves correlation

SFE and ESE kinetic curves (accumulated extract mass in dry basis vs time) were fitted by three straight lines, by minimizing the least regression error using the *fminsearch* function from Matlab (2007) [14,21]. The lines correspond to a first constant extraction rate (CER) period, a falling rate period (FRP) and a diffusion-controlled rate period (DP), respectively. The corresponding kinetic parameters for the CER period: mass transfer rate (M_{CER}), mass ratio of solutes in the solvent phase at the outlet (Y_{CER}), duration of CER period (t_{CER}) and the corresponding extract yield (R_{CER}), were determined according to Rodrigues et al. [22].

2.5. Thin layer chromatography (TLC)

Semi-quantitative thin layer chromatographic analysis of SFE, ESE and SoE extracts, as well as alkaloid standards, was performed using silica gel plates (20 × 20 cm, 0.2 mm thickness, Fluka, Germany). Two solvent mixtures were tested as mobile phase, based on literature reports: (A) isopropanol/formic acid/water 90:1:9 (v/v/v), proposed by Wagner et al. [23] for alkaloid analysis, and (B) acetonitrile/methanol/ammonium formate aqueous solution (0.03 M, pH = 2.8) 34:18:48 (v/v/v), originally optimized for HPLC analysis of *C. majus* alkaloids by Sarközy et al. [6]. Compounds were detected at 365 nm without any chemical treatment.

2.6. Liquid chromatography

Alkaloid quantification was performed by ultra-fast liquid chromatography (UFLC). The UFLC system was equipped with a LiChrosorb RP18 column (250 mm × 4.6 mm i.d., 5 μm particle size), a diode array detector (SPD-M20A) and a HPLC pump (LC-20AD) from Shimadzu (Japan). The mobile phase was prepared with (A) heptanesulfonic acid (0.01 M) and triethylamine (0.1 M) in water acidified with formic acid (pH 2.5) and (B) acetonitrile. A gradient elution was performed as follows: 0 min: 75% A; 1 min: 68% A; 2 min: 57.5% A; 4.5 min: 40% A; 12–40 min: 20% A (based on Suhomelová et al. [8]). Column temperature was 308 K, flow rate was 1 ml/min, and detection wavelengths were 242 nm (chelidone), 268 nm (chelerythrine), 276 nm (sanguinarine) and 344 nm (berberine), based on results reported by Pavão and Pinto [24]. The extracts were diluted in methanol at a concentration of 2 mg/ml and the injection volume was 20 μl. Standard calibration curves were used to quantify these four alkaloids. This analysis was performed in duplicate.

3. Results and discussion

3.1. Extraction yield and kinetics

The moisture of the dried raw materials was equal to $7.3 \pm 0.1\%$ (w/w) for the aerial parts and $7.4 \pm 0.5\%$ (w/w) for the terrestrial parts. The experimental extraction conditions (temperature, pressure, solvent density and flow rate) for all extraction procedures are indicated in Table 1.

Extraction yields calculated from the mass of the extract recovered, for each process and set conditions, are shown in Fig. 2, as a function of each corresponding solvent density. SFE results are zoomed for better comparison. SFE and ESE global yields, which also consider the volatile fraction recovered from the adsorbent

Table 1
C. majus extraction yields for different extraction processes and/or experimental conditions and kinetic parameters obtained from fitting of the extraction kinetic curves. Average solvent flow rate was approximately 8×10^{-5} kg/s.

Extraction process and solvent	Solvent density (kg/m ³)	Total yield (%)	$M_{\text{CER}} \times 10^8$ (kg/s)	$Y_{\text{CER}} \times 10^4$ (kg/kg)	R_{CER} (%wt., d.b.)	t_{CER} (s \times 60)	Fitting error (%)
<i>Aerial parts</i>							
scCO ₂ – 328 K/10 MPa	325	0.60 ± 0.10	0.39	0.47	0.46	190.7	1.7
scCO ₂ – 308 K/10 MPa	713	0.60 ± 0.01	0.41	0.50	0.35	96.9	3.4
scCO ₂ – 318 K/20 MPa	813	0.64 ± 0.02	1.97	2.42	0.35	16.6	0.6
scCO ₂ – 328 K/30 MPa	850	0.86 ± 0.03	2.39	3.02	0.39	15.2	0.8
scCO ₂ – 308 K/30 MPa	929	0.69 ± 0.02	1.91	2.41	0.11	6.8	5.2
scCO ₂ /EtOH/DEA – 328 K/12 MPa	506 ^a	3.8 ± 0.5	11.04	13.07	2.07	12.0	1.4
scCO ₂ /EtOH/DEA – 328 K/35 MPa	881 ^a	4.0 ± 1.0	7.51	9.55	2.27	25.8	1.3
scCO ₂ /Isop/DEA – 328 K/12 MPa	506 ^a	2.4 ± 0.5	11.85	14.51	1.12	15.0	1.1
scCO ₂ /Isop/DEA – 328 K/35 MPa	881 ^a	2.8 ± 0.3	5.91	7.39	1.65	23.8	0.7
H ₂ O – LPSE	740	29.9 ± 0.5	–	–	–	–	–
EtOH – Soxhlet (SoE)	970	7 ± 1	–	–	–	–	–
<i>Terrestrial parts</i>							
scCO ₂ – 328 K/10 MPa	325	0.12 ± 0.04	0.04	0.05	0.10	208.5	5.5
scCO ₂ – 308 K/10 MPa	713	0.44 ± 0.04	0.51	0.62	0.18	41.9	2.3
scCO ₂ – 318 K/20 MPa	813	0.50 ± 0.10	0.99	1.24	0.23	26.8	1.5
scCO ₂ – 328 K/30 MPa	850	0.46 ± 0.05	1.89	2.29	0.22	13.9	1.3
scCO ₂ – 308 K/30 MPa	929	0.5 ± 0.1	0.29	0.36	0.26	101.2	3.8
scCO ₂ /EtOH/DEA – 328 K/12 MPa	506 ^a	5.4 ± 0.1	8.16	9.93	3.02	36.4	2.3
scCO ₂ /EtOH/DEA – 328 K/35 MPa	881 ^a	3.8 ± 0.2	6.35	7.78	2.29	33.5	2.1
scCO ₂ /Isop/DEA – 328 K/12 MPa	506 ^a	2.8 ± 0.4	6.12	7.57	1.45	22.6	0.5
scCO ₂ /Isop/DEA – 328 K/35 MPa	881 ^a	2.9 ± 0.2	4.76	5.97	1.87	41.4	1.1
H ₂ O – LPSE	740	65 ± 2	–	–	–	–	–
EtOH – Soxhlet (SoE)	970	11 ± 1	–	–	–	–	–

^a Density value estimated for the binary system alcohol + CO₂.

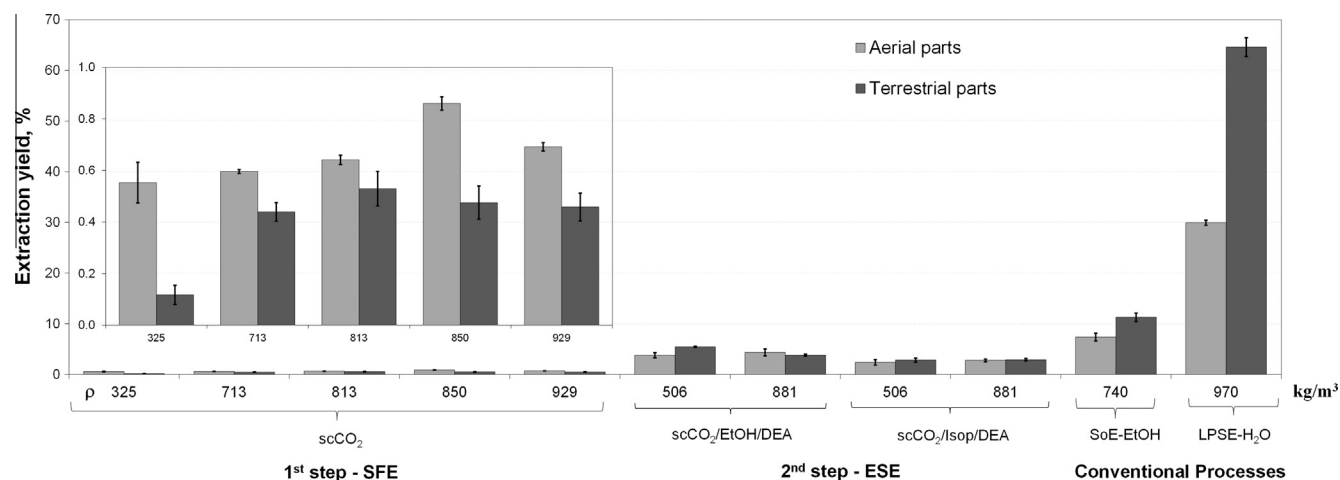


Fig. 2. Global extraction yields of *C. majus* aerial and terrestrial parts obtained using different extraction procedures (SFE, ESE and conventional) and experimental conditions. SFE results are zoomed in the inner graphic for better comparison.

column and the extract mass recovered after cleaning the extraction column and tubing lines, are shown in Fig. S2.

According to Fig. 2 it can be seen that global extraction yields are about one order of magnitude higher for ESE (~2–7% w/w) than for SFE (~0.2–1.0% w/w). This suggests that the raw material is rich in polar and hydrophilic compounds (such as phenolics and carbohydrates) and other high-molecular weight compounds commonly present in vegetable raw materials (like waxes, oleoresins and pigments), which are not easily extracted by pure scCO₂ during the 1st step, even at high solvent densities, while the addition of a polar co-solvent mixture significantly enhances their solubilization and co-extraction [25,26]. Comparison with SoE and LPSE (ethanolic and aqueous extracts) results, which present the highest extraction yields, confirms this premise.

SFE yields are higher for the aerial parts, suggesting higher composition in lipophilic compounds. In contrast, water extraction yields are remarkably higher for the terrestrial parts, while ESE and SoE processes present comparable extraction yields for both parts of the plant. Solvent power enhancement with density is a common feature in supercritical fluid extraction processes, usually reflected in higher extraction yields. This is explained in terms of an enhanced capacity of the solvent to swell the plant matrix and to solvate solute molecules and a higher solubility in the fluid phase, as discussed by many authors [26–28]. Besides the effect of density, temperature can have a significant influence both on solutes solubility (by increasing their vapor pressure) and on solvent transport properties. In fact, scCO₂ viscosity and surface tension, as well as density, are reduced with temperature while

diffusivity is increased, enhancing mass transfer from the plant matrix [29]. In this work, higher extraction yields were obtained at CO₂ densities above 700 kg/m³, compared to the low-density condition (325 kg/m³ at 328 K/10 MPa). However, the influence of temperature seems to predominate over density effects in the high-density range (700–930 kg/m³), as the highest yield was not obtained at the highest density condition (308 K/30 MPa), but at a somewhat lower value (850 kg/m³ at 328 K/30 MPa), mainly for the extraction of the aerial parts.

Regarding the 2nd (ESE) step, extraction yields obtained when using basified ethanol as co-solvent were higher than those obtained with basified isopropanol, both for aerial and terrestrial parts. This effect can be explained in terms of solvent's polarity, since ethanol is more polar than isopropanol and therefore enhances the extraction of polar and high molecular weight compounds, increasing the global extraction yield. Moreover solvent mixture density slightly increases extraction yields from the aerial parts, while no clear effect is observed for the terrestrial parts.

It is important to notice that, to the best of our knowledge, there is no information about the high pressure phase behavior of CO₂ + alcohol + DEA ternary systems in the literature. However, data reported in literature for the phase behavior of CO₂ + ethanol [30] and CO₂ + isopropanol [31] systems indicate that a 10% (w/w) mixture of alcohol (either ethanol or isopropanol) and scCO₂ is homogeneous at the studied extraction conditions. Assuming that the presence of 1% DEA does not significantly affect equilibrium, it can be considered that the solvent mixture is also homogeneous within the experimental range, and therefore no extra mass transfer resistances between gas and liquid phases (which could reduce extraction efficiency) are present. Consequently, the differences observed in extraction yields were here mainly ascribed to solvent density and polarity effects. However, in the case of the roots, other factors may also be playing a role, since the extraction process might be also influenced by a different morphology of the plant matrix: at 12 MPa the lower solvent viscosity may enhance matrix permeation and solute extraction.

Finally, it should be mentioned that according to Fig. S2 high molecular weight compounds extracted from both raw materials remained in the tubing line after dynamic extraction and mainly after the 2nd (ESE) step. Also, the amount of volatile compounds recovered in the adsorbent column indicates that extracts recovery procedure was not completely efficient and requires further improvement.

Fig. 3 shows the kinetic extraction curves measured for SFE and ESE of *C. majus* aerial and terrestrial parts in terms of extraction yield as a function of the specific mass of solvent, i.e., mass of solvent (or solvent mixture) per unit mass of raw material (in dry basis). As can be seen in Table 1, correlated kinetic parameters strongly depend on extraction conditions. In the case of the 1st step (SFE) it can be seen that at higher solvent densities (≥ 813 kg/m³) the constant extraction rate (CER) period is completely developed within the studied time interval. On the contrary, at lower solvent density, it was necessary to further extend the extraction period up to ~ 700 min in order to clearly identify the CER period duration (data not shown in the Figure). It is not clear that the diffusional period is attained in all cases, suggesting that some scCO₂-extractable material still remains in the plant matrix after the extraction experiments. The highest mass transfer rate (M_{CER}) and the highest mass ratio of solute in solvent phase (Y_{CER}) are attained at 328 K/30 MPa for both parts of the plant. As discussed previously, this condition also corresponds to the highest extraction yield (although not to the highest studied solvent density). On the other hand, the lowest M_{CER} and Y_{CER} values were observed at the lowest density conditions (328 K/10 MPa and 308 K/10 MPa): in these cases (densities below ~ 800 kg/m³), solvent density seems to predominate over temperature effects. The

duration of the CER period (t_{CER}) is reduced as solvent density increases. However, the influence of operation conditions on the extraction yield (R_{CER}) during the CER period is not clear: R_{CER} is within the range 0.11–0.46% (w/w) for aerial parts and 0.10–0.26% (w/w) for terrestrial parts, and no apparent correlation was observed with temperature and/or solvent density.

Regarding the 2nd (ESE) step, M_{CER} and Y_{CER} parameters decrease in all cases when pressure is increased from 12 to 35 MPa at constant temperature (323 K), for both co-solvent mixtures. The viscosity of the solvent mixture increases with pressure (as reported in the literature for alcohol + CO₂ systems at high pressure conditions [32], which may be the cause of the observed reduction of mass transfer rate and extraction yield. In accordance, t_{CER} increases in most cases. From the kinetic extraction profiles, it can be seen that only approximately half of the total yield is achieved during the CER period. In general, the kinetic parameter values are lower for the terrestrial parts: this behavior, also observed in the 1st SFE step, may be connected with mass transfer resistances specific of the plant matrix structure. The higher M_{CER} and Y_{CER} values observed when using ethanol + DEA as co-solvent (compared to using isopropanol + DEA) suggests that the polarity of the fluid phase is an important parameter in the global extraction efficiency.

Therefore, from the standpoint of the extraction global efficiency, SFE should be conducted at 328 K and 30 MPa, conditions at which extraction yield and mass transfer in the CER period are maximized. Moreover, if a cosolvent is used, in order to enhance extraction efficiency, basified ethanol should be preferred to isopropanol. However, besides global extraction yield, extract alkaloids content (or the selectivity of the extraction method towards alkaloids) is also fundamental in order to select the best extraction conditions.

3.2. Alkaloids composition

The presence of typical alkaloids in the extracts was suggested by preliminary TLC results and confirmed after the UFLC-UV analysis. Fig. 4 shows an example of typical TLC and schematic sketches plates of SFE extracts obtained at 308 K/30 MPa and SoE (ethanolic) extracts, using two eluting solvent mixtures (as described in Section 2). In all extracts, a bright yellow fluorescent zone appears when the plate is observed at 365 nm (without treatment), closely followed by a greenish zone. According to Wagner et al. [23], these zones typically correspond to chelerythrine and sanguinarine. SFE extracts showed also an intermediate orange zone that may correspond to berberine, partially overlapped by sanguinarine. For the profiles obtained with mobile phase A, these zones were distributed at retention factors (R_f) ranging between 0.12 and 0.23, and appeared both in SFE and SoE extracts of aerial and terrestrial parts. Two pale blue and white zones appeared in the R_f range 0.7–0.9, which cannot be attributed to alkaloids. Similar coloured zones were obtained using mobile phase B, but the elution order was inverse: alkaloids appeared in the R_f range 0.45–0.55, showing higher affinity for this solvent mixture, but separation efficiency was somewhat lost. Alkaloids identification and retention factors were confirmed by running standards under the same conditions. TLC profiles of ethanolic and SFE extracts are qualitatively similar, when comparing the presence and order of coloured zones, which confirms that an alkaloid fraction is extracted by both procedures. In SoE profiles, the yellow (chelerythrine) and green (sanguinarine) zones are broader and more intense, while in SFE profiles they are less bright and the intermediate orange zone is more clearly distinguishable. In addition, any difference was observed between aerial and terrestrial extract profiles, indicating that the same alkaloids are present in both raw materials. The same pattern was observed for ESE extracts, although the coloured bands were narrower and less intense.

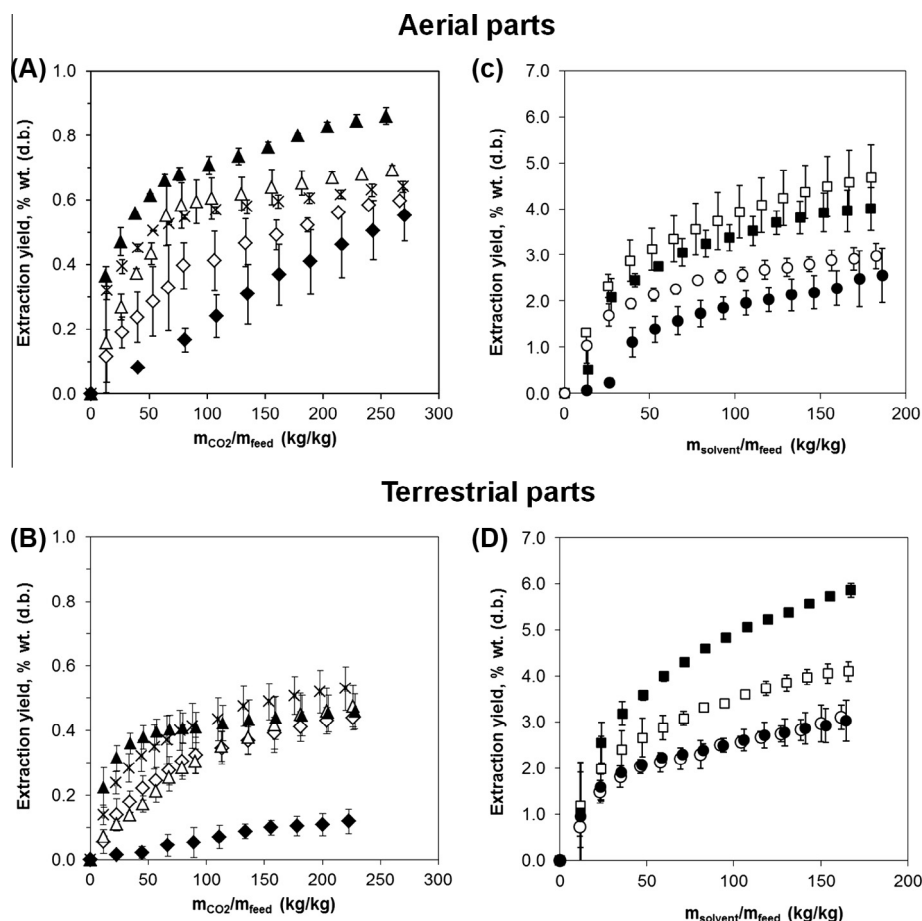


Fig. 3. Extraction kinetic profiles of *C. majus* aerial and terrestrial parts: (A and B) SFE (scCO₂): (◇) 325 kg/m³ (308 K/10 MPa), (△) 929 kg/m³ (308 K/30 MPa), (×) 813 kg/m³ (318 K/20 MPa), (◆) 325 kg/m³ (328 K/10 MPa), (▲) 850 kg/m³ (328 K/30 MPa); (C and D) ESE at 328 K: (●) ethanol/DEA/506 kg/m³ (12 MPa), (○) ethanol/DEA/881 kg/m³ (35 MPa), (■) isopropanol/DEA/506 kg/m³ (12 MPa), (□) isopropanol/DEA/881 kg/m³ (35 MPa).

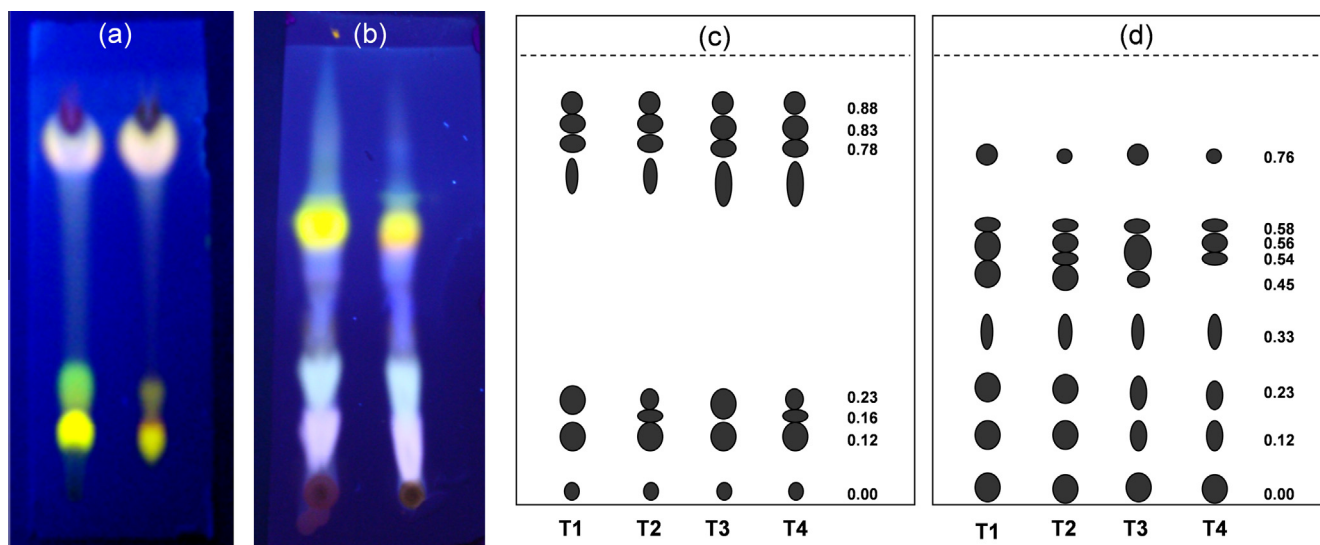


Fig. 4. TLC analysis of *C. majus* extracts. (a and b) Typical chromatograms obtained using mobile phase A and B, respectively. (c and d) TLC sketches for the following extracts: T1 - SoE extract from aerial parts; T2 - SFE extract from aerial parts; T3 - SoE extract from terrestrial parts; T4 - SFE extract from terrestrial parts; using mobile phase A and B, respectively.

This analysis also provides some information regarding suitable solvents for alkaloid extraction. Mobile phase B is more polar, as it contains about 50% water, and has higher ionic strength than mixture A, which contains 90% isopropanol. This indicates that *C. majus*

alkaloids are preferably extracted when using higher water content and ionic strength conditions. As mentioned before, low pH values increase alkaloids ionization making them more soluble in aqueous mixtures. In the case of mobile phase (A), isopropanol is not an

Table 2Extraction selectivity for individual alkaloids present in extracts from *C. majus* aerial and terrestrial parts (expressed in mg of alkaloid/g of extract).

Samples and process conditions (solvent, temperature and pressure)	Solvent density (kg/m ³)	Chelidonine (mg/g)	Chelirithrine (mg/g)	Sanguinarine + berberine (mg/g)	Total quantified alkaloids (mg/g)
<i>Aerial parts</i>					
scCO ₂ – 328 K/10 MPa	325	10.42 ± 0.03	12.31 ± 0.03	6.56 ± 0.02	29.28 ± 0.07
scCO ₂ – 308 K/10 MPa	713	78.58 ± 0.74	17.14 ± 0.28	9.88 ± 0.04	105.59 ± 1.06
scCO ₂ – 318 K/20 MPa	813	113.18 ± 3.07	3.27 ± 0.43	4.44 ± 0.65	120.89 ± 4.15
scCO ₂ – 328 K/30 MPa	850	132.11 ± 0.14	4.99 ± 1.25	4.88 ± 0.28	141.97 ± 1.67
scCO ₂ – 308 K/30 MPa	929	107.71 ± 1.45	3.31 ± 0.01	3.38 ± 0.35	114.39 ± 1.81
scCO ₂ /EtOH/DEA – 328 K/12 MPa	506 ^a	67.29 ± 0.79	4.06 ± 0.18	20.75 ± 0.54	92.10 ± 1.51
scCO ₂ /EtOH/DEA – 328 K/35 MPa	881 ^a	56.99 ± 0.10	2.36 ± 0.92	8.38 ± 0.66	67.73 ± 1.68
scCO ₂ /Isop/DEA – 328 K/12 MPa	506 ^a	75.79 ± 0.74	1.53 ± 0.18	1.67 ± 0.11	78.99 ± 1.02
scCO ₂ /Isop/DEA – 328 K/35 MPa	881 ^a	43.15 ± 0.18	1.40 ± 0.06	1.44 ± 0.10	45.98 ± 0.34
H ₂ O – LPSE	740	2.92 ± 0.04	0.13 ± 0.01	1.24 ± 0.03	4.29 ± 0.08
EtOH – Soxhlet (SoE)	970	37.52 ± 0.18	3.34 ± 0.10	18.77 ± 0.19	59.63 ± 0.47
<i>Terrestrial parts</i>					
scCO ₂ – 328 K/10 MPa	325	56.69 ± 0.38	54.84 ± 0.03	56.14 ± 0.17	167.67 ± 0.58
scCO ₂ – 308 K/10 MPa	713	370.45 ± 6.45	9.21 ± 0.71	14.16 ± 0.63	393.83 ± 7.79
scCO ₂ – 318 K/20 MPa	813	569.41 ± 0.62	7.60 ± 0.03	8.84 ± 0.22	585.85 ± 0.87
scCO ₂ – 328 K/30 MPa	850	528.40 ± 1.75	8.10 ± 0.12	12.47 ± 0.68	548.97 ± 2.56
scCO ₂ – 308 K/30 MPa	929	399.61 ± 0.59	11.37 ± 0.39	17.01 ± 0.42	428.00 ± 1.40
scCO ₂ /EtOH/DEA – 328 K/12 MPa	506 ^a	110.57 ± 1.83	2.65 ± 0.03	2.85 ± 0.24	116.07 ± 2.10
scCO ₂ /EtOH/DEA – 328 K/35 MPa	881 ^a	0.90 ± 0.08	3.10 ± 0.11	2.39 ± 0.19	6.39 ± 0.38
scCO ₂ /Isop/DEA – 328 K/12 MPa	506 ^a	138.77 ± 0.73	2.59 ± 0.26	2.47 ± 0.06	143.83 ± 1.05
scCO ₂ /Isop/DEA – 328 K/35 MPa	881 ^a	485.85 ± 0.73	10.66 ± 0.05	11.57 ± 0.02	508.08 ± 8.09
H ₂ O – LPSE	740	6.82 ± 0.17	0.09 ± 0.01	0.86 ± 0.01	7.76 ± 0.19
EtOH – Soxhlet (SoE)	970	87.32 ± 0.64	10.78 ± 0.12	12.19 ± 0.04	110.29 ± 0.80

^a Density value estimated for the binary system alcohol + CO₂.**Table 3**Extraction efficiency for individual alkaloids from *C. majus* aerial and terrestrial parts (expressed in mg of alkaloid/g of raw material in dry basis).

Samples and process conditions (solvent, temperature and pressure)	Solvent density (kg/m ³)	Chelidonine (mg/g)	Chelirithrine (mg/g)	Sanguinarine + berberine (mg/g)	Total quantified alkaloids (mg/g)
<i>Aerial parts</i>					
scCO ₂ – 328 K/10 MPa	325	0.06 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.18 ± 0.03
scCO ₂ – 308 K/10 MPa	713	0.51 ± 0.00	0.11 ± 0.00	0.06 ± 0.00	0.70 ± 0.01
scCO ₂ – 318 K/20 MPa	813	0.78 ± 0.04	0.02 ± 0.00	0.03 ± 0.01	0.84 ± 0.05
scCO ₂ – 328 K/30 MPa	850	1.23 ± 0.04	0.05 ± 0.01	0.05 ± 0.00	1.32 ± 0.03
scCO ₂ – 308 K/30 MPa	929	0.80 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.85 ± 0.01
scCO ₂ /EtOH/DEA – 328 K/12 MPa	506 ^a	2.75 ± 0.39	0.17 ± 0.03	0.85 ± 0.13	3.77 ± 0.56
scCO ₂ /EtOH/DEA – 328 K/35 MPa	881 ^a	2.66 ± 0.41	0.11 ± 0.03	0.39 ± 0.03	3.16 ± 0.41
scCO ₂ /Isop/DEA – 328 K/12 MPa	506 ^a	1.90 ± 0.43	0.04 ± 0.01	0.04 ± 0.01	2.02 ± 0.45
scCO ₂ /Isop/DEA – 328 K/35 MPa	881 ^a	1.28 ± 0.13	0.04 ± 0.01	0.04 ± 0.01	1.37 ± 0.14
H ₂ O – LPSE	740	0.94 ± 0.00	0.04 ± 0.00	0.40 ± 0.02	1.39 ± 0.01
EtOH – Soxhlet (SoE)	970	2.98 ± 0.30	0.27 ± 0.04	1.49 ± 0.14	4.73 ± 0.47
<i>Terrestrial parts</i>					
scCO ₂ – 328 K/10 MPa	325	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.21 ± 0.07
scCO ₂ – 308 K/10 MPa	713	1.76 ± 0.17	0.04 ± 0.00	0.07 ± 0.00	1.87 ± 0.18
scCO ₂ – 318 K/20 MPa	813	3.26 ± 0.42	0.04 ± 0.01	0.05 ± 0.01	3.35 ± 0.43
scCO ₂ – 328 K/30 MPa	850	2.61 ± 0.32	0.04 ± 0.01	0.06 ± 0.01	2.20 ± 0.30
scCO ₂ – 308 K/30 MPa	929	2.05 ± 0.27	0.06 ± 0.01	0.09 ± 0.01	2.72 ± 0.34
scCO ₂ /EtOH/DEA – 328 K/12 MPa	506 ^a	6.48 ± 0.06	0.16 ± 0.01	0.17 ± 0.02	6.80 ± 0.08
scCO ₂ /EtOH/DEA – 328 K/35 MPa	881 ^a	0.04 ± 0.01	0.13 ± 0.01	0.10 ± 0.00	0.26 ± 0.01
scCO ₂ /Isop/DEA – 328 K/12 MPa	506 ^a	4.22 ± 0.63	0.08 ± 0.02	0.08 ± 0.01	4.37 ± 0.67
scCO ₂ /Isop/DEA – 328 K/35 MPa	881 ^a	15.10 ± 1.52	0.33 ± 0.03	0.36 ± 0.03	15.80 ± 1.58
H ₂ O – LPSE	740	4.75 ± 0.02	0.06 ± 0.00	0.60 ± 0.02	5.41 ± 0.04
EtOH – Soxhlet (SoE)	970	10.62 ± 0.71	1.31 ± 0.08	1.48 ± 0.12	13.42 ± 0.90

^a Density value estimated for the binary system alcohol + CO₂.

efficient solvent for ionized alkaloids. This further supports the hypothesis that alkaloids' extraction with lower-polarity solvents may be enhanced by operating at basic pH conditions.

Based on this preliminary TLC analysis, alkaloids were quantified by UFLC-UV. Berberine and sanguinarine were quantified together (as sanguinarine equivalent) due to the limited resolution between them on the chromatographic conditions employed. The amounts of chelidonine, chelirithryne and berberine + sanguinarine were quantified per mass of extract (as a measure of extraction selectivity) and per mass of raw material (as a measure of extraction efficiency) and the results are reported in Tables 2 and 3,

respectively. The results presented in both tables permit to identify the experimental conditions that originate maximum alkaloids extraction yields, which are achieved using co-solvents (Table 3) and those that originate higher alkaloids selectivity, which are achieved using only scCO₂ as solvent (Table 2). Both data is important to select the proper extraction methodologies/conditions depending on the envisaged application. The composition of each of these alkaloids in the extracts is shown in Fig. 5.

As can be seen in Table 2, 1st (SFE) step extracts are richer in alkaloids (mainly for terrestrial parts) than those obtained with polar solvents or solvent mixtures (up to 140 mg alkaloids/g

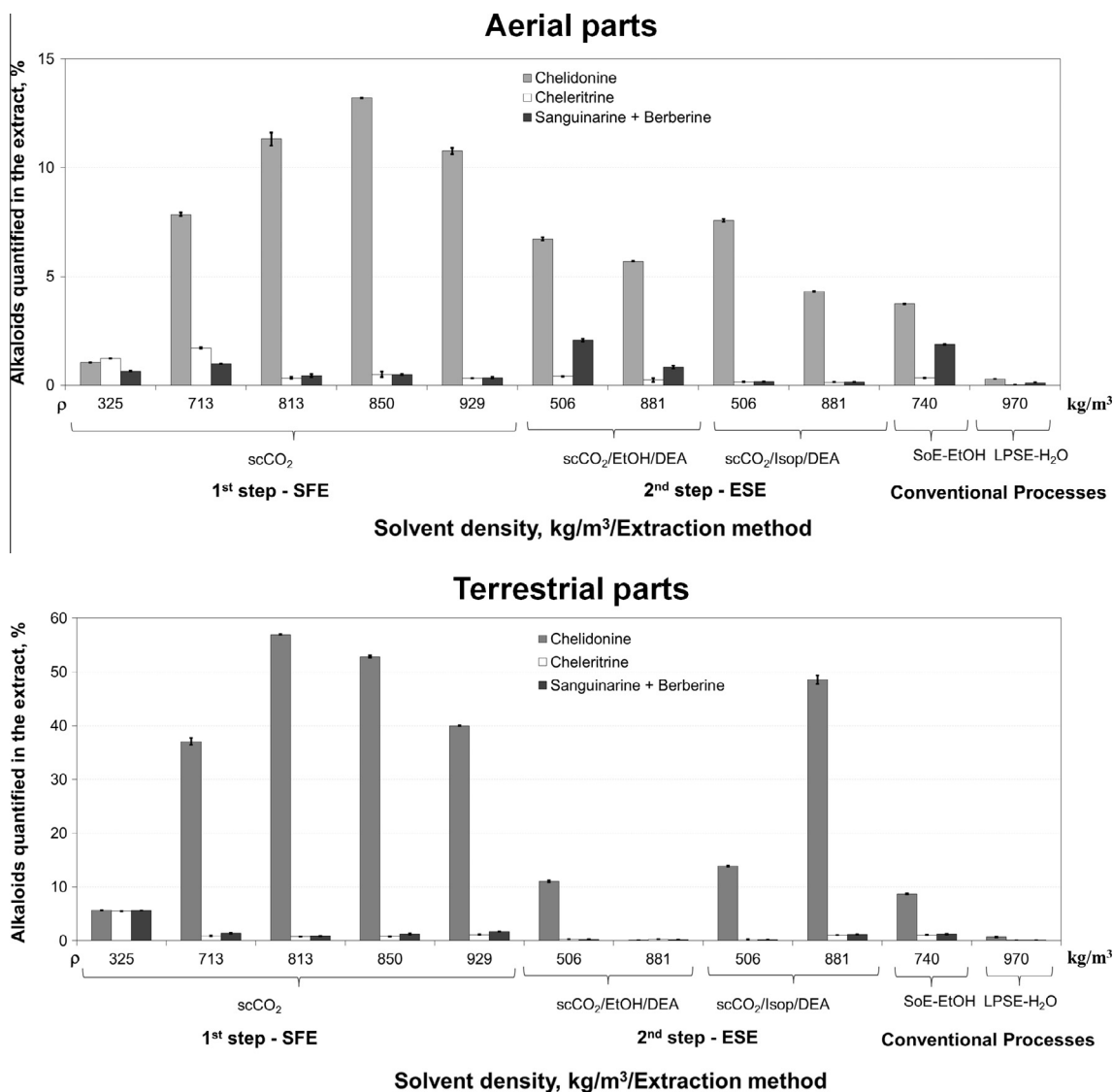


Fig. 5. Quantification of alkaloids extracted from *C. majus* aerial and terrestrial parts using SFE, ESE and conventional extraction methodologies (LPSE and SoE).

extract for aerial parts and 585 mg/g for terrestrial parts). An exception was observed for the extract obtained from the terrestrial parts of the plant using scCO₂/isopropanol/DEA as co-solvent mixture at 328 K/35 MPa, which also presented high selectivity for alkaloids. Alkaloids selectivity is affected by scCO₂ density in the same way as extraction yield, being highest at 328 K/30 MPa. Although SFE seems to be more selective, it originates considerably lower alkaloid extraction yields (Table 3), mainly for the aerial parts. Considering that aerial parts presented higher SFE global extraction yields, it can be concluded that other non-polar or low polarity compounds, such as waxes and lipids, are co-extracted with alkaloids, as suggested by the waxy appearance of these extracts. Moreover, and though alkaloids are considered the main active components, phenolic compounds such as caffeic, ferulic, *p*-coumaric, gentisic and *p*-hydroxybenzoic acids, as well as some derivative esters, have been also identified in *C. majus* [33].

Extracts obtained from the 2nd (ESE) step present in general lower selectivity for alkaloids (Table 2), but higher alkaloid extraction yields when comparing with the 1st (SFE) step (Table 3). Results obtained for the ethanolic extracts (SoE) are similar to those described for ESE extracts while those obtained for the aqueous extracts (LPSE) presented low selectivity for alkaloids, yielding

extracts with low alkaloid content (~4 mg/g for aerial parts and ~8 mg/g for terrestrial parts).

To sum up, the methodologies that presented higher selectivity for alkaloids were SFE at scCO₂ densities of 850 kg/m³ (328 K/30 MPa), which yielded ~142 mg/g extract, and 813 kg/m³ (318 K/20 MPa), which yielded ~585 mg/g extract, for the aerial and terrestrial parts of the plant, respectively. The highest alkaloids extraction yield (15.8 mg/g raw material) was obtained for the terrestrial parts when using ESE and scCO₂/isopropanol/DEA as co-solvent mixture at a solvent mixture density of 881 kg/m³ (328 K/35 MPa). This extract also presented high selectivity for alkaloids (508 mg alkaloids/g of extract), comparable to SFE results. These values are 8–10 times higher than other previously reported in the literature [6,8], and this variation can be related to the use of different extraction strategies and/or edaphoclimatic conditions of *C. majus* production.

Fig. 5 shows the extraction profiles of the target alkaloids as a function of the studied extraction methodologies/conditions. It can be seen that the concentration of chelidonium is several times higher than chelerytrine, sanguinarine and berberine in almost all extracts (up to 90% of total alkaloids), especially for those obtained from the 1st SFE step. This difference is more remarkable

for the extracts obtained from the terrestrial parts of *C. majus*, suggesting a natural higher biosynthesis of chelidonine in this part of the plant [9].

Overall, it can be concluded that SFE extracts are highly selective for alkaloids and especially for chelidonine. The apparent solubility of this alkaloid in scCO₂ increases with solvent density up to 813–850 kg/m³ and then decreases, in the same way as the global extraction yield. The use of basified alcohols as co-solvents enhances alkaloids' recovery from the raw materials, although the obtained extracts are less concentrated in alkaloids due to the co-extraction of other compounds.

4. Conclusions

In this work a fractionated extraction methodology was used to obtain alkaloids enriched fractions from *C. majus* L. A first SFE extraction step yielded extracts with high alkaloid content (up to ~140 and 550 mg/g for aerial and terrestrial parts, respectively) when operated at 328 K and 30 MPa, conditions that combine a high solvent density (850 kg/m³) and a positive effect of temperature on solutes vapor pressure and CO₂ diffusional properties. However, this first step was not sufficient for an exhaustive extraction of alkaloids; therefore a second ESE extraction step using basified alcohols was studied. Remaining alkaloids were extracted during this step, although other polar and high molecular weight compounds were co-extracted in higher extent, providing higher global extraction yields but less concentrated extracts (regarding alkaloids). Results obtained for quaternary alkaloids (chelidonine, chelerythrine and berberine + sanguinarine) quantification permitted to conclude that the 1st SFE step was especially selective for chelidonine. This procedure demonstrates that alkaloids fractionation during extraction is an important tool to be used before further purification/isolation steps.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.seppur.2016.04.006>.

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