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Supercritical carbon dioxide extraction of tagitinin C from *Tithonia diversifolia*

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Abstract

Different parameters as temperature, pressure, solvent mass and sample granulometry governing the extraction yield of tagitinin C from the aerial parts of *Tithonia diversifolia* were optimised.

An experimental design was carried out to map the effects of pressure (at 20.3, 30.4 and 40.5 MPa) and temperature (at 40, 60 and 80 °C) on the extraction yield of the active component and to determine the optimal conditions for the extraction of tagitinin C from *T. diversifolia*. The best conditions are met for a pressure of 35.0 MPa and a temperature of 68 °C.

The effect of the particle size was studied under low pressure (13.7 MPa) and temperature (40 °C) conditions, which failed to extract quantitatively the tagitinin C from leaves sieved to 250 μm size. The reduction of the particle size increased the extraction yield which became comparable to that of the optimised SFE for the particle in the range of 0–63 μm.

From the analysis of extraction kinetic curves of 200 mg of plant with supercritical carbon dioxide (range of 5–30 g), it appears that 15 g of this supercritical fluid is never limiting.

The optimised supercritical fluid extraction (SFE) was compared favourably to Soxhlet extraction with dichloromethane (S) and to maceration followed by lixiviation with diethyl ether (ML), which gave similar extraction yields but higher extract content of tagitinin C were found using SFE (15.6 and 30.7% w/w tagitinin C in S and ML extracts, respectively, versus 52.8% in SFE extract).

Keywords: Supercritical fluid extraction; Tagitinin C; *Tithonia diversifolia*; Sesquiterpene lactone

1. Introduction

Tithonia diversifolia (Asteraceae) is a shrub, which is native to Middle America and the West Indies. This plant has become naturalised around the tropics. Its aerial parts are traditionally used for the treatment of malaria in São Tomé e Principe.

Goffin et al. [1] isolated the tagitinin C (Fig. 1), a known sesquiterpene lactone [2,3], from the aerial parts of the plant and discovered its antimalarial activity against *Plasmodium falciparum*. Gu et al. recently showed significant antiproliferative activity of tagitinin C [4].

Supercritical fluid extraction (SFE) is known to be a fast and efficient method for the extraction of non-polar compounds from plant matrices. Carbon dioxide is the most widely used solvent for extraction of natural products for foods and medicines, under mild conditions. It is an inert, inexpensive, odourless, tasteless and environment-friendly solvent. Further, there is no solvent residue in the extract, since it is a gas in the ambient condition [5,6].

Several sesquiterpene lactones, such as santonin [7], parthenolide [8], costunolide [9] and artemisinin [10,11] were already extracted by SFE with good results.

In this work, the effect of various parameters such as pressure, temperature, mass of supercritical carbon dioxide ($SCCO_2$) and particle size was investigated on the extraction of tagitinin C from *T. diversifolia* in order to obtain less contaminated product in shorter time. In addition, SFE

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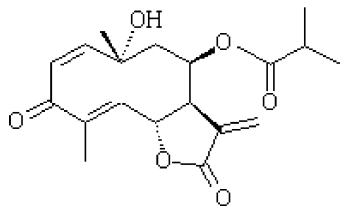

Fig. 1. Structure of tagitinin C.

was compared with conventional liquid solvent extraction processes (Soxhlet extraction with dichloromethane and maceration and lixiviation with diethyl ether).

2. Experimental

2.1. Reagents

Dichloromethane (p.a.), Uvasol® tetrachloroethylene and diethyl ether (p.a.) were obtained from Merck (Darmstadt, Germany).

The carbon dioxide, 99.98% (w/w) pressurised at 11.2 MPa with helium, was purchased from Air liquide (Liège, Belgium).

The tagitinin C, isolated and purified by Goffin et al. [1], was employed for the preparation of standards. Its purity (97.2%) was determined by HPLC normalisation procedure [12].

2.2. Plant material

The aerial parts of *T. diversifolia* (Asteraceae) were collected at the Democratic Republic of São Tomé e Principe in November 1997. A voucher specimen (MM 626) has been deposited at the Botanic Institute of the University of Coimbra. The moisture content was 8.19 ± 0.02 wt.%.

Unless otherwise specified, all plant samples were thoroughly ground and sieved under 250 μm size. This milling process was carried out without cooling system.

2.3. Apparatus

An Ultra Centrifugal Mill ZM 100 (Retsch, Germany) was used to grind and sieve plant samples.

Particle size analysis was performed by using a laser diffractometer Mastersizer 2000 with the Scirocco 2000 module (Malvern Instruments, United Kingdom).

Supercritical fluid extractions were performed using a stainless steel vessel of 1 mL in a Varian Star SFE Autoprep 44 (Suprex Corporation, Blacksburg, VA, USA). This device consists of mainly four modules: a modifier pump, a carbon dioxide pump, an oven and a collector module.

Fourier Transform Infrared quantification of tagitinin C was done using a Perkin-Elmer Spectrum GX spectrophotometer equipped with a MIRTGS detector, a shuttle (Perkin-Elmer Limited, Beaconsfield, UK) and a liquid transmission cell provided with sodium chloride windows (internal volume of 200 μL and a pathlength of 500 μm).

The modelling of the experimental design was realised by Origin 41® and Sigmaplot 2000® for Windows® program.

2.4. Experimental procedure

2.4.1. Particle size analysis

Three fractions of leaves (0.02–63, 63–125 and 125–250 μm) were obtained by sieving with a set of sieves of different size. Particle size analysis of these fractions was performed by using the laser diffractometer Mastersizer 2000. The sample was extemporaneously dispersed in compressed air at 0.4 MPa until an obscuration rate of 0.5–10% was obtained under a vibration rate of 50% of the track. Background and sample were measured for 12 s. Optical properties of the sample were defined as follow: refractive index 1.347 and absorption 0.1 (similarly to the particles named lactose in the Malvern software). Each sample was measured in triplicate.

2.4.2. Supercritical fluid extraction

For all experiments, the extraction vessel (1 mL) was packed with 200 mg of plant material. The extract was trapped by bubbling the carbon dioxide through 5 mL of tetrachloroethylene placed in a 10 mL volumetric flask.

After the collection, the flask was filled with tetrachloroethylene and left in the dark until quantification of tagitinin C by FT-IR.

The extraction kinetic curves were determined with a range of 5–30 g of $SCCO_2$ and with a flow rate equal to 1 mL min^{-1}. Each curves point is obtained by using fresh plant material.

The effects of two factors (pressure and temperature of $SCCO_2$) were investigated in the ranges of 20.3–40.5 MPa and 40–80 °C by a designed experiment. For all experiments, the amount and the flow rate of $SCCO_2$ were kept constant at 15 g and 1 mL min^{-1}, respectively. The center point and each corner point were carried out in triplicate. The extraction yield of tagitinin C was used as dependent variable.

2.4.3. Soxhlet extraction with dichloromethane

A sample of 200 mg of plant material was extracted in the dark with 150 mL of dichloromethane for 2 h in a Soxhlet apparatus.

A small Soxhlet (μSoxhlet) apparatus was also used to extract in the dark 200 mg of plant material with 5 mL of dichloromethane for 40 min.

The solutions were evaporated to dryness under reduced pressure, and the residues were solubilised in 10 mL of tetrachloroethylene using ultrasonic shaking during 5 min in an ultrasonic bath.

For the quantification of tagitinin C, the solution was placed in the liquid transmission cell and analyzed by the FTIR spectroscopy method [13].

2.4.4. Maceration and lixiviation with ether

The extraction and quantification procedures were previously described by Goffin et al. [12].

2.4.5. Quantification of tagitinin C by FTIR

The solutions obtained after extractions following the procedure 2.4.2, 2.4.3 and 2.4.4 were introduced in the liquid transmission cell. The absorbances at 1664.8 cm^{-1} were measured and compared with those of standard solutions containing 50, 75, 100, 125 and 150 $\mu g\,mL^{-1}$ of tagitinin C in tetrachloroethylene.

3. Results and discussion

3.1. Validation of the FT-IR spectroscopy method

A FT-IR spectroscopy method was previously validated in our laboratory for the determination of tagitinin C in ether extracts of *T. diversifolia* [13]. The same criteria of validation recommended by the International Chemical Harmonisation (ICH) [14] were applied for the extracts obtained using Soxhlet and SFE methods.

The calibration curve linearity for tetrachloroethylene solutions of tagitinin C was investigated with five calibration standards (50, 75, 100, 125, 150 $\mu g\,mL^{-1}$) carried out in triplicate, run on 3 different days. The absorbance at 1664.8 cm^{-1} was used. These results are shown in the first column of Table 1.

The coefficient of determination (r^2) was higher than 0.99 and each statistical test was passed. Thus, it results that the absorbance is proportional to the concentration of tagitinin C.

On the other hand, the calibration curve linearity of the standard addition technique was performed using extracts obtained with Soxhlet and SFE methods. These extracts were spiked with known amounts of tagitinin C (19.91, 55.17, 89.95 $\mu g\,mL^{-1}$ of tetrachloroethylene) carried out in triplicate. The absorbance at 1664.8 cm^{-1} was also used. The results, except for the intercept test, are displayed in the second and third column of Table 1. Indeed, the intercept test of the equations obtained with the standard addition technique was not determined. The coefficient of determination (r^2) was also higher than 0.99 and each statistical test was passed. These results also indicated that the absorbance is proportional to the added amounts of tagitinin C.

Concerning the comparison of slopes, the regression line for the standard solutions was: $A = 8.04 \times 10^{-5} + 9.20 \times 10^{-5}$ (sb $= 6.21 \times 10^{-7}$)C. This equation compares well with those obtained with Soxhlet extraction which was equal to $A = 0.005044 + 9.16 \times 10^{-5}$ (sb $= 1.52 \times 10^{-7}$)C and with supercritical fluid extraction equal to $A = 0.004642 + 9.20 \times 10^{-5}$ (sb $= 1.86 \times 10^{-7}$)C. According to Student's *t*-test, both slopes are similar with that of the standard solutions and thus these results suggest also the absence of matrix effect on the FT-IR method.

3.2. Tithonia diversifolia extracts by SFE

The extraction of natural products using supercritical fluid as carbon dioxide is a dynamic process and it can be useful in determining the extraction kinetic curves of tagitinin C from *T. diversifolia* under several conditions of pressure and temperature. These curves are shown in Fig. 2.

In both cases, a plateau is obtained after dynamic extraction using 15 g of $SCCO_2$. However the extraction yield of tagitinin C using supercritical carbon dioxide at 13.7 MPa and 40 °C was lower than that obtained at 30.4 MPa and 80 °C. This result can be explained by the incomplete mass transfer of tagitinin C from the matrix to $SCCO_2$ under low conditions of pressure and temperature.

Table 1
Linearity between the tagitinin C concentration and the absorbance at 1664.8 cm^{-1} for standard solutions [13] and standard addition technique on extracts obtained with Soxhlet extraction and supercritical fluid extraction

Parameters	Standard solutions	Standard addition technique	
		Soxhlet extraction	Supercritical fluid extraction
Coefficient of determination (r^2)	0.9994	0.9998	0.9999
Cochran test—homoscedasticity (g)	0.4482	0.6896	0.7421
Reference value (0.95)	0.6838	0.7679	0.7679
Significative slope test	21992	361163	244407
Reference value (0.95)	4.67	4.96	4.96
Intercept test	1.22	n.d.	n.d.
Reference value	2.16		
Validity test for the calibration linearity	1.85	1.24	0.92
Reference value	3.71	4.46	4.46

n.d.: not determined.

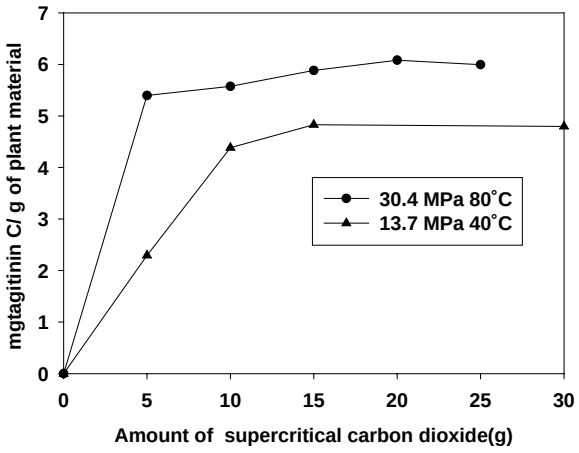

Fig. 2. Extraction kinetic curves of tagitinin C from *T. diversifolia* by SFE (flow rate 1 mL min^{-1}). Each measure was carried out in triplicate.

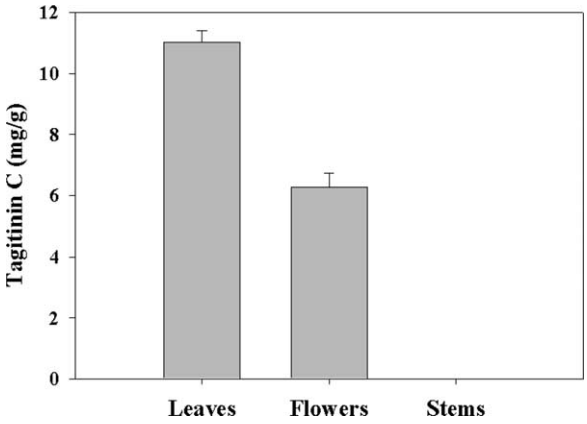

Fig. 4. SFE$_{optimised}$ (35.0 MPa, 68 °C, 15 g of CO_2, flow rate 1 mL min^{-1}) on leaves, flowers and stems of *T. diversifolia*. Each measure was carried out in triplicate.

The effects of the pressure and temperature in the extraction vessel were investigated at 20.3, 30.4 and 40.5 MPa, and at 40, 60 and 80 °C by a designed experiment. The amount of $SCCO_2$ was kept constant at 15 g for all experiments. Each corner and the center were carried out in triplicate. The extraction yield of tagitinin C was used as dependent variable.

The flow of the compressed fluid was maintained at 1 mL min^{-1}. Larger rate of gas could cause violent bubbling of the liquid collection solvent and leading to analyte losses via aerosol formation [6]. Fig. 3 shows the response surface estimated for the extraction yield of tagitinin C from the aerial part of *T. diversifolia* obtained with the designed experiment.

As one can see, the optimal conditions can be expected between 30.4 and 40.5 MPa and between 60 and 80 °C. These conditions are met at a pressure of 35.0 MPa and at a temperature of 68 °C.

The addition of a polar modifier to the $SCCO_2$ was examined using optimised conditions with the purpose of increasing the extraction yield of tagitinin C. Methanol was chosen because it is the most common entrainer for $SCCO_2$ and an excellent solvent of tagitinin C. Its concentration in the supercritical fluid was fixed at 1% (v/v) and 3% (v/v). Any increase of the extraction yield of tagitinin C was noticed in both cases. The effects of concentration and type of entrainer were not investigated further.

3.3. Distribution of tagitinin C in the plant

It is very important to know the distribution of the tagitinin C in the plant in order to choose the right part and to obtain herbal medicine with a high concentration of tagitinin C. This determination was realised on the leaves, the flowers and the stems of *T. diversifolia* using SFE$_{optimised}$ (35.0 MPa, 68 °C, 15 g of $SCCO_2$, flow rate 1 mL min^{-1}) (Fig. 4).

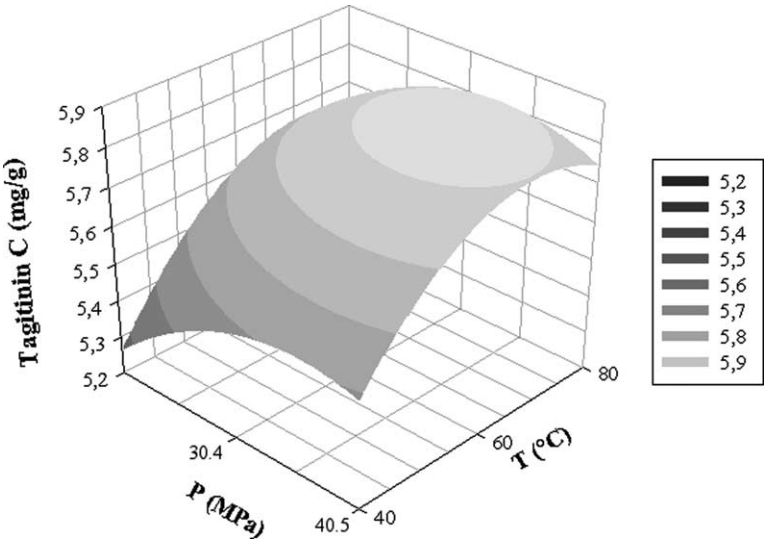

Fig. 3. Response surface estimated for the extraction yield of tagitinin C from the aerial part of *Tithonia diversifolia* (15 g of $SCCO_2$, flow rate 1 mL min^{-1}). Each corner point and the center point of this surface, representing the range of experimental conditions studied, were carried out in triplicate.

Table 2
The mean of the particle size of leaves of *T. diversifolia*

Range of the particle size (μm)	$d(0.5) \pm$ S.D. (μm)
0.02–63	24.02 ± 0.85
63–125	84.58 ± 3.23
125–250	162.63 ± 5.37

Each measure was carried out in triplicate.

From Fig. 4, it is seen that the leaves extract contains more tagitinin C than the flowers and the stems extracts. The absence of this compound in the stems extract seems to indicate that the stem does not contain it. Thus, the leaves should be chosen to obtain high-concentrated herbal remedies in tagitinin C.

3.4. Effect of particle size on the extract obtained by SFE

Previous experiments dealing with the extraction kinetic curves showed that the extraction yield of tagitinin C obtained under mild conditions of pressure (13.7 MPa) and temperature (40 °C) was lower. That can be explained by the fact that the $SCCO_2$ has not a sufficient solvent capacity for the solute to be extracted from the matrix plant. This is also true for the extraction of undesirable products. These mild conditions allow to improve the selectivity of SFE ($SFE_{selective}$).

In order to reduce the hindrance to the release of tagitinin C, the particle size of the solid feed must be reduced to provide a large surface area and a decrease of the intraparticle diffusional resistance. Thus, the particle size (size ranges: 0.02–63, 63–125, 125–250 μm) was investigated to improve the extraction yield of tagitinin C from *T. diversifolia*.

Initially, we used the aerial parts of the plant to obtain the three fractions (size ranges: 0.02–63, 63–125, 125–250 μm) of particle size. Unfortunately, we noticed that these ranges were not homogeneous because the large particles came essentially from the stem and the small particles came from the leaves. Following these results, we used the leaves of *T. diversifolia* to obtain homogeneous samples. The mean of the particle size of the leaves of *T. diversifolia* determined by laser diffractometry are shown in Table 2.

The influence of the particle size on the extraction kinetic curves was determined, as shown in Fig. 5.

As one can see, the particle size has a dramatic influence on the extraction yield and limits the extraction yield of tagitinin C in cases of small particles of 63–125 and 125–250 μm. From Figs. 5 and 6, it appears clearly that the smallest particles give the best extraction yield of tagitinin C. On the other hand, the decrease of the particle size of the sample can favour the channelling commonly related to a decrease of the extraction yield. Comparable extraction yields were obtained by $SFE_{optimised}$ of large particles of leaves (Fig. 4) and $SFE_{selective}$ of small particles of leaves (Fig. 5) indicating that channelling does not occur.

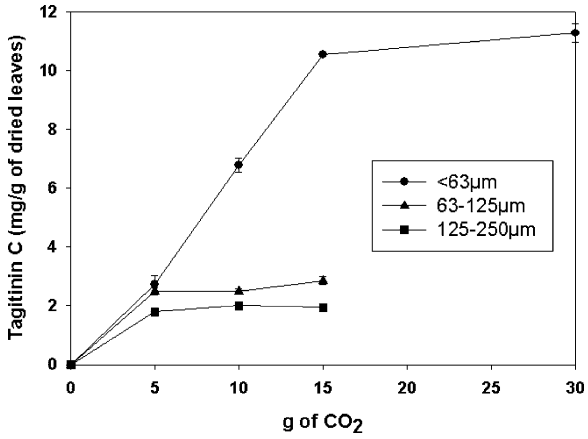

Fig. 5. Extraction kinetic curves of tagitinin C from dried leaves of *T. diversifolia* by $SFE_{selective}$ (13.7 MPa, 40 °C, flow rate 1 mL min^{-1}). Each measure was carried out in duplicate.

It seems interesting to notice that the extraction yield of leaves is more dependent on the granulometry than this of the aerial parts of the plant (Figs. 2 and 5). The particle size distribution of the powder of leaves and of the aerial parts of the plant was analyzed by laser diffractometry. Unfortunately, although the distribution peak was broad, any differentiation (bimodal distribution) between small and large particles was found. In order to find an explanation to the dependence of the extraction yield on the granulometry, the structure of the particles was investigated by a micrographic analysis of the different powders. It was observed that the powder of leaves seemed homogenous while the powder of the aerial parts contained a lot of very small particles and few large particles. The micrographic structure of small and large particles was different.

Taking into account that the stems were harder to ground than the leaves, it was reasonable to think that the small particles came from the leaves and that the large particles came from the stems. This high proportion of small particles

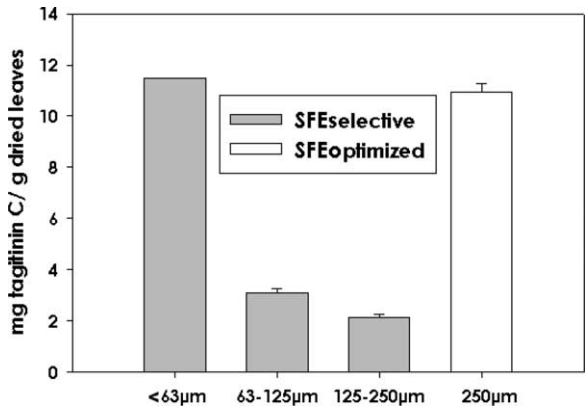

Fig. 6. Effect of particle size on extraction yield of tagitinin C from the dried leaves of *T. diversifolia*. $SFE_{selective}$ (13.7 MPa, 15 g of $SCCO_2$, flow rate 1 mL min^{-1}, 40 °C). $SFE_{optimised}$ (35.0 MPa, 15 g of $SCCO_2$, flow rate 1 mL min^{-1}, 68 °C). Each measure was carried out in duplicate.

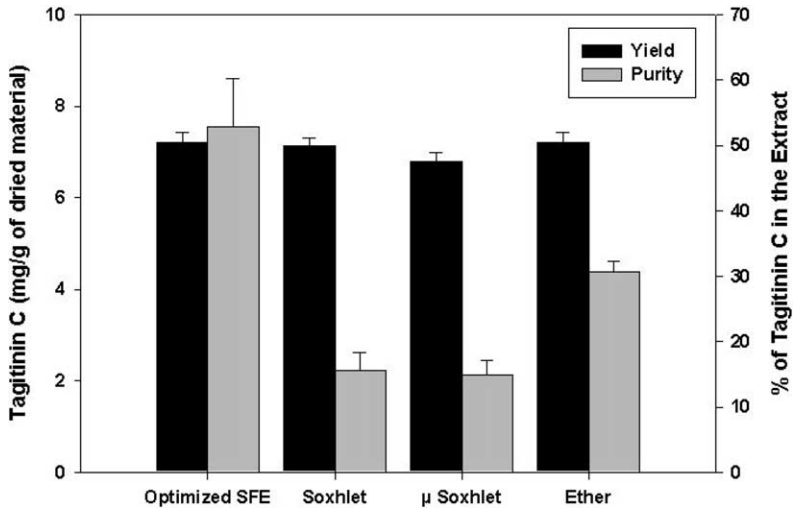

Fig. 7. Comparison of different extraction methods: extraction yields of tagitinin C from the dried aerial parts of *T. diversifolia* (left scale) and percent tagitinin C in the extract (w/w) (right scale). Each measure was carried out in triplicate.

of leaves in the powder can explain the results obtained in Section 3.2. Since fine particles of leaves have a high content of easily extractible tagitinin C while large particles of stems do not contain tagitinin C.

3.5. Comparison of the different extraction methods

The yield and the selectivity of extraction can vary with the extraction method. These parameters were investigated with the Soxhlet or the μSoxhlet extraction with dichloromethane, with the extraction by maceration and lixiviation with diethyl ether and with the $SFE_{optimised}$. The results are shown in Fig. 7.

As it can be seen from Fig. 7, the yield obtained by the $SFE_{optimised}$ was similar to those obtained by the classical extraction methods. However the supercritical extracts obtained were slightly yellow whereas dichloromethane and ether extracts were dark yellow. Moreover, the concentration of tagitinin C in the $SFE_{optimised}$ extract was higher than that of the classical extraction methods because dichloromethane and diethyl ether dissolves pigments from the plant together with the active component. Although it is difficult to compare solvent polarities, a feeling for the range of polarities can be based on the dielectric constant. The dichloromethane and diethyl ether dielectric constant are equal to 9 and 4.3, respectively.

Compared to these solvents, the $SCCO_2$ dielectric constant is ranging from 1 to 2 [15] depending on the temperature and the pressure of this supercritical fluid. The low dielectric constant of diethyl ether and $SCCO_2$ allows to have a better selectivity than that obtained with dichloromethane.

According to the poor selectivity of the dichloromethane extraction, we tried to optimise it with a small Soxhlet apparatus using only 5 mL of dichloromethane by cycle. After eight cycles corresponding to 40 min, the extraction yields of tagitinin C between both Soxhlet extraction methods were comparable. But, unfortunately, both concentrations of tagitinin C were also comparable (Fig. 7). These results lead us to think that dichloromethane dissolves rapidly pigments from the plant.

Unfortunately, the selectivity was not improved using low conditions of pressure and temperature on smallest particle size. This one was equal to $51.10 \pm 7.76\%$ (percent of tagitinin C in the extract) and similar to that obtained with the best conditions of extraction. The decrease of the particle size facilitates the extraction of tagitinin C but also the extraction of undesirable products.

4. Conclusion

This study has confirmed the feasibility of $SCCO_2$ extraction of tagitinin C from aerial parts of *T. diversifolia* and the use of the FT-IR method to determine the amount of this compound in the extracts.

The conditions of pressure and temperature of $SCCO_2$ were optimised using an experimental design to obtain the best yield of extraction of tagitinin C which was comparable to that of the classical methods of extraction but with an improvement of the selectivity. Furthermore the time of extraction used is much shorter than that of other methods.

In addition to temperature, pressure of the supercritical fluid, the particle size of the matrix was shown to have a dramatic influence on the extraction efficiency of tagitinin C. This parameter allowed reducing the pressure and the temperature of $SCCO_2$ while keeping an extraction yield comparable to that obtained with higher conditions.

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