GLUCOSINOLATES AND FATTY ACIDS EXTRACTION FROM *ERUCA SATIVA* THROUGH SUPERCRITICAL TECHNOLOGY

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ABSTRACT

Supercritical fluid extraction from freeze-dried and milled *Eruca sativa* leaves was assessed with the aim of obtaining two different extracts rich in valuable compounds. In a first step, a fraction containing 338.0 mg/g of lipids was obtained through pure supercritical CO_2 extraction. In a second step, a fraction rich in glucosinolates and phenolic compounds was extracted using supercritical CO_2 with a modifier. Three different co-solvents were compared: water, ethanol and methanol. Extraction curves were fitted by the model of broken and intact cells developed by Sovová and the main variables that affect the extraction process (pressure, temperature, co-solvent dosage and pre-treatment effect) were studied. The highest glucosinolate and phenol contents were obtained when water was used as co-solvent. Particularly, when the extraction was carried out at 250 bar and 65°C, a fraction containing a phenolic content of 122.8 mg/100 g and a glucosinolate content of 191.8 mg/100 g was extracted.

INTRODUCTION

Rocket salad (*Eruca sativa*) is a good source of glucosinolates, phenolic compounds [1] and free fatty acids [2]. The potential beneficial effects of glucosinolates and related compounds, in relation to several diseases (cancer, cardiovascular and neurological diseases) have been recently reported [3,4]. Indeed, some of the cancer chemoprotective activity of *Eruca sativa* and the other cruciferous vegetables is widely believed to be related to their content of minor dietary components such as glucosinolates [5]. By other hand, the erucic acid and other lipids obtainable from cruciferous oil are promising materials due to the physic-chemical characteristics of long chain fatty acids [6].

Hot aqueous methanol have been predominantly used to extract glucosinolates [1,7], however the use of methanol in food products is limited by strict legal statues [8]. Supercritical carbon dioxide could be an alternative and environmentally friendly technique, that offers several advantages over classical solvent extraction methods, since it is inert, non-toxic and allows extraction at lower temperature and relatively low pressure. In this study, a first step with pure CO_2 is performed to extract lipids from *Eruca sativa* leaves. However, the polar nature of glucosinolates and phenolic compounds makes it necessary the use of co-solvents, so to enhance the fluid affinity towards polar compounds. Water and ethanol are generally recognised as safe (GRAS), and environmental benign, and can therefore be used in food

extraction processes [9]. Supercritical fluid extraction of phenols from other vegetables has been already applied [9, 10]. However, to the best of our knowledge, supercritical technology has never been applied for extracting glucosinolates from vegetables so far. Also, as far as we know, this is the first work on supercritical fluid extraction from rocket salad.

MATERIALS AND METHODS

Materials

Eruca sativa leaves were supplied by the agri-company "La Marostegana", located in Piazzola di Brenta (Italy). CO_2 (4.0 type, purity greater than 99.99%) used as supercritical solvent was purchased by Rivoira. Ethanol (99.8%) and methanol (99.8%) used as co-solvents for supercritical extraction were purchased from Sigma Aldrich. Water was Milli-Q quality. Solvents used for the measures were provided by Carlo Erba, Prolabo, J.T. Baker and Lab-Scan.

Supercritical fluid extraction

Prior to the extraction, *Eruca sativa* leaves were mechanically ground with a kitchen grinder and stored at -25 °C. Then they were freeze-dried, milled with a mortar and sieved, obtaining a particle size less than 0.5 mm.

The supercritical extraction tests were performed by a laboratory scale equipment previously reported [11]. The method was applied as follows: the extractor was filled with 0.49±0.05 g of freeze-dried *Eruca sativa* powder. A thermo-resistance around the extractor maintained the desired temperature, which was measured in the internal flow before and after the vessel. CO_2 was compressed through a high pressure pump and pre-heated before flowing through the extraction vessel. A constant CO_2 flow rate of 0.3±0.05 kg/h was kept in every test. The co-solvent was pumped by an intelligent HPLC pump (Jasco PU-1580) and mixed with the CO_2 stream, before the extractor. After the extraction, the supercritical fluid was expanded. The lipids and the co-solvent were collected in 12 mL of a solvent (the one used as co-solvent). CO_2 gas at atmospheric temperature passed through a flow meter before being vented. The extract was collected and filtered through a 0.20 μm filter (Ministart). The co-solvent was evaporated by a rotatory evaporator.

According to Serra et al. [12], when a co-solvent was used, a two-step fractioned extraction methodology was employed, comprising a first CO_2 SFE step in order to extract the low polarity CO_2-soluble compounds and a second ESE extraction step, to extract polar compounds and wherein mixtures of CO_2 with a co-solvent. It had been shown that a pre-treatment of raw material with supercritical CO_2 is required to efficiently remove lipophilic and nonpolar substances and thus making polyphenols more available for the second extraction (ESE) [12]. In this case, the objective of the "CO_2 extraction pre-treatment" was also to obtain a first fraction of lipids and to deactivate the enzyme myrosinase, a step which it is necessary in order to achieve a better extraction of the compounds [13].

Analysis of the extract

Quali-quantitative analysis of glucosinolates, phenols and lipids in the extracts was obtained by HPLC-MS. The measurements were obtained on a Varian 212 series chromatograph equipped with Prostar 430 autosampler and MS-500 Ion Trap as detector. MS spectra were recorded in positive and in negative ion mode (50–2000 Da). The APCI ion source was used for lipid analysis while the ESI was used for phenolic and glucosinolates. Fragmentation of the main ionic species were obtained during the HPLC run by the turbo data depending scanning (tdds) function, yielding the fragmentation pattern of eluted compounds. As stationary phase Agilent Zorbax C-18 (2.1 × 150 mm) 3.5 μm was used. As mobile phases solvent A (water 0.1% formic acid) and solvent B (methanol) were utilized. The solvent gradient started at 80% A then decreased to 0% A over 30 min.

RESULTS

Effect of the co-solvent on the extraction yield and composition of the extract

Preliminary extraction tests were performed with the aim of selecting the co-solvent that extracts better each compound of interest. First, $SCCO_2$ extraction without any modifier was carried out. Then three different co-solvents were tested: water, ethanol and methanol. All the runs were performed at the same conditions: 30 MPa, 45 °C, 0.3 kg/h of CO_2 and 8% of co-solvent with respect to the CO_2 flow rate. Two extractions tests were performed for each co-solvent. Table 1 shows the media and the standard deviation of the extraction percentage calculated from the two measures. Results indicate that a much higher quantity of extract is obtained when water is used as co-solvent (21.7 %), with respect to the extraction percentages obtained using ethanol and methanol as co-solvents and with pure supercritical CO_2. This could be due to the presence of water increases the density of the fluid mixture, causing swelling of the particles, and therefore with improving diffusion process and solubilisation of several compounds [9].

Table 1: Extraction percentage, total lipids conent (TLC) total phenolic content (TPC) and total glucosinolates content (TGC) from supercritical fluid extraction at 45°C and 300 bar using different co-solvents.

Co-solvent	Extraction percentage[a] (%)	TLC (mg/g)	TPC (mg/g)	TGC (mg/g)
Water	21.71±0.61	0.00±0.00	0.64±0.08	1.59±0.20
Ethanol	5.54±0.24	-	0.19±0.01	0.00±0.00
Methanol	5.56±0.06	-	0.28±0.01	0.30±0.02
None	1.5	338.01±12.65	0.00±0.00	0.00±0.00

[a] Extraction percentage= (mass of microalgae extract/mass of dried microalgae powder)×100

Regarding the composition of the extract, the fraction obtained by pure $SCCO_2$ contains 338.01 mg/g of lipids and phenolic and glucosinolate compounds were no identified, as shown in Table 1. The fraction obtained using water as co-solvent was the richest in polyphenols and glucosinolates content when compared with the other co-solvent or when the extraction was carried out without a modifier. Particularly, the extract obtained by $SCCO_2$+water contains 0.64 mg/g of phenolic compounds and 1.59 mg/g of glucosinolates. However, no lipids were found in this fraction.

Effect of the extraction conditions on the total yield

Once water was demonstrated to be the most efficient co-solvent to extract glucosinolates and polyphenols from *Eruca sativa*, the effect of the operative conditions on the extraction was studied. Figure 1a shows the effect of the temperature when the extraction is carried out at 30 MPa with 0.4 mL/min of co-solvent and a particle diameter lower than 0.5 mm. Figure 1b illustrates the influence of the extraction when the runs are performed at 65 °C, with 0.5 mL/min and a particle diameter lower than 0.5 mm. For the investigation of the influence of the co-solvent dosage, temperature and pressure of the tests were maintained at 65°C and 30 MPa, respectively, for a particle diameter less than 0.5 mm. Figure 4d shows how the milling influences the extraction at 65 °C, 30 MPa and 0.4 mL/min of water. All the runs were carried out with a flow rate of 0.3 kg/h during 60 minutes.

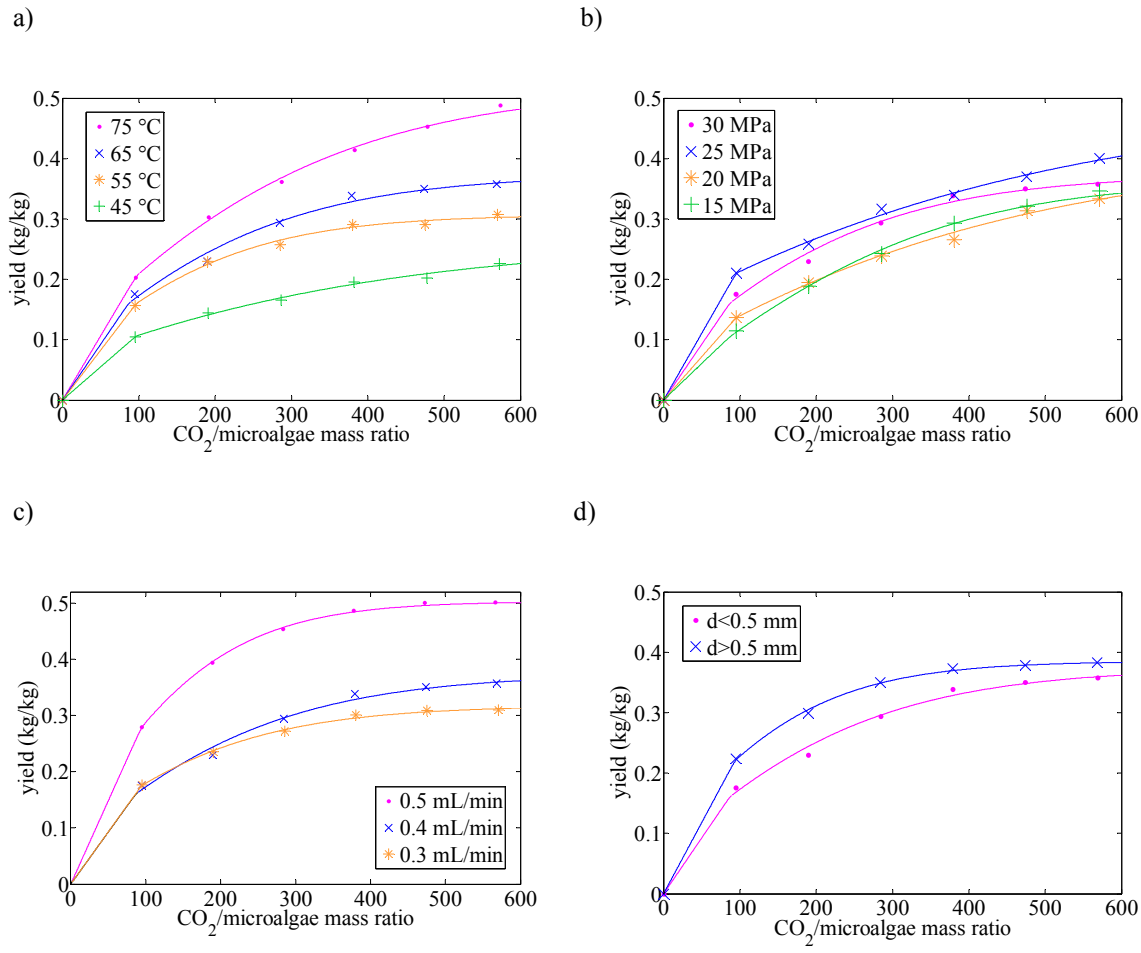

Figure 1: $SCCO_2$ extraction curves obtained at different operative conditions (temperature (a), pressure (b), co-solvent dosage (c) and particle diameter (d)), experimental and modelling. The curves represent the extraction yield ($kg_{extract}/kg_{insoluble\ matrix}$) as a function of the CO_2/microalgae mass ratio.

In kinetics evaluation, the curve modelling was performed using the model of broken and intact cells published by Sovová [14]. It is noted that, for the application of this model, the yield is calculated as the mass of extract collected divided by the insoluble *Eruca Sativa* powder. The model equations are described by Mouahid et al. [15]. As shown in Figure 1a, the effect of the temperature is very relevant, as the yield increases from 22.7% to 48.8%

when the temperature is varied from 45°C to 75°C. Apparently, when the temperature of water is increased, its polarity decreases and becomes similar to that of methanol, as reported by Min-Jung Ko et al. [8]. When the effect of the pressure is studied, no significant changes are found on the total yield of the extraction after 60 minutes. As represented in Figure 1b, four extraction tests were performed at four different pressures between 15 and 30 MPa. The lowest yield was obtained at 20 MPa and the highest one at 25 MPa, but there is not a clear tendency and indeed the results are very similar. Regarding the co-solvent dosage, a high increase on the extraction yield is found when the flow is raised from 0.4 to 0.5 mL/min. However, when such a quantity of water was tested, the system was blocked. This could be due to that the increase of co-solvent percentage may induce the saturation of CO_2 with water, with consequently formation of two phases for the specific conditions of the system [16]. Contrary to expectations, a very similar result was obtained when the tests were carried out at different particles diameters. It can be due to the fact that after the first milling and freeze-dried, the particles are small enough to allow the solvent and co-solvent to penetrate inside and extract the compounds.

First extract: Supercritical CO_2 extraction without the use of a co-solvent

Table 2 shows the lipid composition of the extract obtained in the first step of the extraction, that is carried out without the use of a co-solvent. The content of each compound is expressed as the weight measured in the analysis (mg) divided by initial mass of the freeze-dried and milled *Eruca Sativa* leaves loaded into the extractor (g). All analyses were made in duplicate. As Table 2 shows, the major lipid compounds, identified as TG(LnLnSt) and TG(LnLnLn), were found in an amount of 183.8 mg/g and 102.42 mg/g, respectively. They represent the 85% of the total lipid content of the extract.

Table 2: Lipid composition of the extract obtained by pure supercritical CO_2 extraction at 45°C and 300 bar.

	$SCCO_2$
MAG(Ln)	4.36±0.38
TG(LnLnSt)	183.81±2.10
TG(LnLnLn)	102.42±8.15
TG(LLnLn)	1.09±0.01
TG(linolenic)	9.56±0.04
TG(PLn)	0.57±0.04
TG(linolenic)	6.38±0.19
TG(LnLL)	0.94±0.01
TG(arachidic ac)	6.84±8.33
DG_PP	0.60±0.08
DG_(linolenic)	2.76±0.59
DG_CaCa	1.45±0.06
Not identified lipid	8.20±0.30
Not identified lipid	8.38±0.89
Not identified lipid	0.64±0.00
Total lipids content	338.01±12.65

Second extract: $SCCO_2$ using water as co-solvent

Table 3 shows the phenols composition of the fraction extracted by $SCCO_2$ using water as co-solvent. The measure was repeated four times. Table 2 reports the media and the standard deviation of the four measures. Leucodelphynidin was the major compound extracted by $SCCO_2$, and it was found in a quantity of 31.25 mg/100g. The presence of Quinic acid, Quercetin and its derivates was also significant, as shown Table 3.

Table 3: Phenols composition (mg/100g) of the extract obtained by supercritical extraction (CO_2+water) at 65°C and 250 bar.

	$SCCO_2$+water
Quinic acid	16.92±4.25
Quercetin-3-(2-feruloyl-glucoside)-3'-(6-feruloylglucoside)-4'glucoside	0.00±0.00
Quercetin-3,4'-diglucoside-3'(6-sinapoyl-glucoside)	14.40±2.12
Rutin	0.00±0.00
Leucodelphynidin	31.25±2.93
Q-acetil-sinapoil-diglucoside	8.91±1.64
Quercetin(Sinapoyl-glucoside)(sinapoyl-glucoside)-glucoside	8.38±0.61
Quercetin(Sinapoyl-glucoside)	11.46±1.04
Quercetin	16.28±1.53
Kaempferol	7.15±0.27
Isoramnetin	8.04±0.31
Procatequic acid glucoside	0.00±0.00
Quercitin diglucoside	0.00±0.00
Total	122.80±12.65

Table 4 shows the glucosinolate composition of the extract obtained by $SCCO_2$ using water as co-solvent. The measures were repeated twice. Table 3 reports the media and the standard deviation of the measures. It has been reported [17] that the most abundant GLS occurring in seedling and leaves extracts is DMB-GLS. Indeed, the major component extracted by $SCCO_2$+water was identified as DMB-GLS, as shown Table 4.

Table 4: Glucosinolate composition (mg/100g) of the extract obtained by supercritical extraction (CO_2+water) at 65°C and 250 bar.

	$SCCO_2$+water
Glucoerucin	22.02±3.96
Glucorafphanin	3.51±6.08
DMB-GLS	154.28±64.73
Glucocheirolin	5.70±9.88
Glucosativin	6.28±10.88
Total	191.79±68.12

CONCLUSION

In this study, supercritical fluid extraction from *Eruca sativa* leaves, using both pure CO_2 and CO_2 with co-solvents, was assessed. The extract obtained by pure supercritical CO_2 extraction contained 338.0 mg/g of lipids. For the extraction of a fraction rich in glucosinolate and phenolic compounds, water resulted to be the most efficient co-solvent. The extraction yield was favoured by high temperatures and high proportion of co-solvent dosage with respect to the CO_2 flow rate. The pressure and the milling of the particles did not affect significantly the extraction yield. The model of broken and intact cells developed by Sovová fitted well the experimental data. An extract containing a total phenolic content of 122.8 mg/100 g and a total glucosinolate content of 191.8 mg/100 g was obtained at 250 bar and 65°C. Thus in this work we report the application of a sequential extractive approach firstly using pure CO_2 for lipid extraction and then using water as co-solvent for phenolic and glucosinolate containing extract. The sequential extraction described can be attractive for the production of solvent-free extract containing health promoting constituents that can be useful as active ingredients in functional-foods or food supplements.

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