



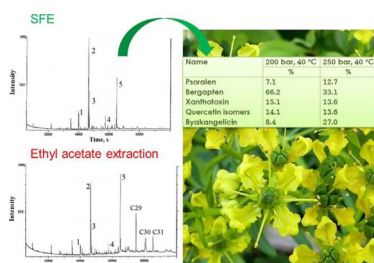
Concentration of *Ruta graveolens* active compounds using SC-CO₂ extraction coupled with fractional separation



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GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Ruta graveolens

Supercritical fluid extraction

Fractional separation

ABSTRACT

Supercritical CO₂ (SC-CO₂) extraction coupled with fractional separation of the extracts, has been used to concentrate the active principles of *Ruta graveolens*. Using 250 bar, 40 °C for the extraction process and cooling the first separator to −10 °C, an efficient extraction and fractionation was obtained. Different particle sizes of the vegetable matter were tested (from 0.20 to 0.80 mm); they indicated that the extraction process was faster when particle size was smaller; i.e., internal mass transfer controlled the process. Fractional separation allowed the selective elimination of cuticular waxes, that represented about 78% w/w of the total extract produced by SC-CO₂ processing. The extract collected in the second separator was concentrated in active compounds up to 87% w/w. For comparison purposes, extraction was also performed using an organic solvent: the extract obtained using ethyl-acetate, contained low percentages of active compounds, mainly due to the co-extraction of large quantities of waxes.

1. Introduction

Ruta graveolens extracts possess well known active properties, mainly connected to the presence of coumarins and furanocoumarins [1]; essential oil compounds have been also extracted from this vegetable matter [2,3]. Coumarinic compounds are produced by the plant as a defense from herbivores; therefore, they are mainly located in plant areas in which a loss would induce a serious decrease of plant fitness or in areas in which an attack is more possible, as fruits and leaves [4].

Recent scientific literature reports promising results of *R. graveolens*

extract in biomedical field, as anti-inflammatory, cytotoxic, antitumor, fungicide and bacteriostatic principle [5,6]. Indeed, it has been demonstrated that, due to the furanocoumarins action (e.g., psoralen, bergapten, isopimpinellin, xanthotoxin), in addition to the insecticide/antifeedant properties, these extracts are able to inhibit cell proliferation in colony formation assay, suggesting the presence of long-acting components that inhibit cancer cell proliferation [7].

R. graveolens extracts are commonly obtained by solvent extraction; methanol [5,6] and ethanol [1,8] are the organic solvents most frequently used. However, the traditional extraction techniques suffer

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several disadvantages, such as: low extraction selectivity, toxic solvent residues in the final extract and, generally speaking, environmental pollution.

Supercritical CO₂ (SC-CO₂) based techniques have been successfully proposed in the extraction of active compounds from vegetable matter [9–14]. However, at the best of our knowledge, only one paper related to SC-CO₂ extraction of *R. graveolens* leaves has been published [15]. It was focused on the use of *R. graveolens* extracts as model compounds in the mathematical modeling of supercritical extraction. Three compound families were indicated through key components: waxes, active principles and others; in particular, *n*-tritriacontane was selected among waxes and xanthotoxin was selected as the model compound for furanocoumarins. These compounds were extracted all together; i.e., no fractionation was operated among active and unwanted compounds.

Particularly, waxes (i.e., paraffins) give to the extract a solid consistency and are co-extracted also when organic solvents are used. Therefore, until now, no one attempted at fractionation/concentration of *R. graveolens* extracts, either using traditional techniques, or using SC-CO₂ processing.

Since the concentration of the active principles and the elimination of toxic organic solvents is a very relevant issue in the medical use of *R. graveolens* extracts, the scope of this work is to apply an opportune SC-CO₂ extraction processing, to produce concentrate and solventless *R. graveolens* active principles. Different CO₂ densities, CO₂ flow rates and vegetable matter particle sizes will be explored, adopting a process scheme that uses fractional separation in a first cooled separator, to divide the extracts in waxes and active compounds. A comparison with organic solvent extraction will be also performed.

2. Materials and methods

Ruta graveolens (leaves and fruits) was bought from “Erba Vita” (Chiesanuova, RSM, Italy) and it had a moisture content of about 10% w/w. It was dried and ground using a cooled milling apparatus; mean particle size was determined by mechanical sieving. Ethyl acetate (anhydrous, 99.8%) was bought from Sigma aldrich. Psoralen, bergapten, xanthotoxin (also called methoxsalen), quercetin and byakangelicin concentrations were calculated using calibration curves, obtained using standard solutions (Extrasynthese, Lyon, France). CO₂ (purity 99.9%) was supplied by Morlando Group S.R.L. (Sant’Antimo, NA – Italy).

2.1. Organic solvent extraction

10 g of ground *Ruta graveolens* (200 μm mean particle size) were added to 150 mL of ethyl acetate. After an overnight of magnetic mixing (about 15 h) at room temperature, the obtained solution was filtered and dried using a rotavapor. The extract yield was 7.0% w/w, corresponding to 0.7 g.

2.2. Supercritical CO₂ extraction apparatus

Supercritical CO₂ extraction experiments were performed in a home-made laboratory apparatus equipped with a 490 cm³ internal volume extractor. 130 g of the vegetable matter were mixed with 3 mm glass beads, to avoid caking and channeling of the fixed bed of particles. Extracted fractions were recovered using two separation vessels with an internal volume of 200 cm³ each, operated in series. The first separator was cooled using a thermostated bath (Julabo, mod. F38-EH); the second separator allowed the continuous discharge of the product using a valve located at the bottom of the vessel. A membrane high-pressure pump (Lewa, mod. LDB1 M210S), pumped liquid CO₂ at the desired flow rate. CO₂ was, then, heated to the extraction temperature in a thermostated oven. CO₂ flow rate was monitored by a calibrated rotameter (ASA, mod. N.5-2500, Serval 115022) located after the last separator. Temperatures and pressures along the extraction apparatus were measured by thermocouples and test gauges, respectively.

Pressure was controlled by high pressure valves. More details about the apparatus have been published elsewhere [16].

2.3. Characterizations

Gas chromatographic-mass spectrometric (GC–MS) apparatus was a Varian (mod. Saturn 2100T, San Fernando, CA); separation was achieved using a fused-silica capillary column (mod. DB-5, J & W, Folsom, CA) 30 m length, 0.25 mm of internal diameter, 0.25 μm film thickness. GC conditions were: oven temperature of 40 °C for 5 min; then, programmed heating from 40 to 250 °C at 2 °C/min and subsequent holding at 250 °C for 60 min. The injector was maintained at 280 °C (splitless 20 cm³/min) and helium was used as the carrier gas (1 cm³/min). Samples were run in dichloromethane with a dilution factor of 0.05% w/w. The selected parameters were: scan rate 110 amu/s, ionization energy 70 eV and EM voltage 3000 V. The content of psoralen, bergapten, xanthotoxin, quercetin and byakangelicin in the extracts was calculated converting gas chromatographic area traces into absolute values, using the ion trap relative response factors, that were calculated using external standards.

3. Results and discussion

In previous studies, coumarinic compounds were detected in the extracts of some vegetable matter, obtained using SC-CO₂ [17,18]; the solubility of coumarinic model compounds in SC-CO₂ was also studied [19]. Sovová and co-workers [11] measured xanthotoxin content in *R. graveolens* SC-CO₂ extracts and used this compound as the representative of furanocoumarins family in *R. graveolens* extracts showing that its solubility at 40 °C becomes relevant at pressures above 200 bar. It is also known that solid paraffins (waxes) located on vegetables surface are soluble in SC-CO₂ at all extraction conditions [20]. These preliminary considerations indicate that it is opportune to try *R. graveolens* active compounds SC-CO₂ extraction (SFE) operating at a minimum of 200 bar, 40 °C (CO₂ density 0.804 g/cm³), applying online fractional separation of the extract to eliminate waxes. To complete the set of processing parameters, in the first part of this work, CO₂ flow rate was fixed at 1.2 kg/h and mean particle size of the vegetable matter was 0.80 mm. The solution at the exit of the extraction vessel was sent to a first separator operated at 200 bar, –10 °C to selectively precipitate the waxes. This procedure has been successfully used for the separation of waxes from several vegetable matter, as previously described in the literature [14,21–23]. Then, the second separator, operated at 15 bar, 24 °C, was used to precipitate the remaining part of the extract. The second separator was discharged at fixed time intervals to recover and weight the extract, producing extraction kinetics curves. Applying the described process and operating conditions, after 420 min extraction, the yield of the extract recovered in the second separator, asymptotized at 1.05% w/w. Then, the extraction pressure was increased at 250 bar and the extraction was continued for further 220 min, to show if other SC-CO₂ extractable compounds were present in the vegetable matrix. An overall yield increase was obtained of about 0.30% w/w. At the end of the process, the system was depressurized and the content of the first separator was recovered. It was a white, odorless powder and was about 4.60% w/w (5.98 g) with respect to the starting material; i.e., more than 3 times by weight larger than the material recovered in the second separator (1.35% w/w, 1.75 g). This result confirms the relevance of the fractional separation procedure; if all the material extracted from the vegetable matrix was collected altogether in the second separator, about 77% w/w of the product would be represented by undesired, difficult to separate, solid waxes compounds. Considering all extract contributions (1.35 + 4.60% w/w), an overall extraction yield of 5.95% w/w was obtained.

The same process was repeated changing the particle size of the vegetable matter, using particles of 0.60 and 0.20 mm mean diameter; the extraction was performed at the same operative conditions

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