HYDROTHERMAL AND SUPERCRITICAL COMBINED EXTRACTION PROCESS OF FRACTIONS WITH ANTIOXIDANT ACTIVITY FROM *Cytisus scoparius*

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Abstract. A combined process using biorenewable solvents was proposed to extract the phenolic fraction from *Cytisus scoparius* branches (CsB). Conventional solvent extraction using ethanol, pressurized hot water extraction (autohydrolysis) and supercritical CO_2 extraction under selected operational conditions were performed to obtain soluble fractions with antioxidant properties. Both the phenolic content and composition were analyzed. The combination of autohydrolysis and supercritical CO_2 processing provided extraction yields of 25% of the raw material and extracts with phenolic content and ABTS radical scavenging capacity comparable to ethanolic extracts.

Keywords: *Cytisus scoparius*, combined process, supercritical CO_2, antioxidant, flavonoids

1. Introduction

Cytisus scoparius L. is a perennial shrub of the Leguminosae family, native to western and central Europe. The flowers, leaves and branches are traditionally used for the diuretic, sedative, antidiabetic and hepatoprotective properties [1-5], although caution with use and dosage has been claimed due to the presence of alkaloids. *C. scoparius* contains flavones, isoflavones, flavonols and carotenoids. The anti-stress and ansiolytic action has been associated with the antioxidant action of the phenolic components [2, 6-9].

The extracts from *C. scoparius* were active as radical scavengers against nitric oxide and DPPH, superoxide anion and hydroxyl radicals. Protection against peroxidation of β-carotene-linoleic acid in emulsion and lipids in rat liver microsomes was also reported [1, 8, 10]. *C. scoparius* extract protects liver from oxidative stress in Wistar albino rats by lowering serum glutamate oxaloacetate transaminases, serum glutamate pyruvate transaminases, lactate dehydrogenase and thiobarbituric acid reactive substances levels. Both reduced glutathione and hepatic antioxidant enzymes were increased by treatment with the plant extract [2]. The anti-stress action has been associated with the antioxidant action of the phenolic components [9, 10].

Autohydrolysis processing of *C. scoparius* branches with hot compressed water has been proposed to produce a fiber-containing solid fraction mainly made up of cellulose and lignin and a liquid phase containing sugar oligomers derived from hemicelluloses. The solid phase was used for manufacturing polylactic acid based biodegradable composites, and in the soluble fraction the sugar oligomers could be suitable as prebiotic agents [11]. However, in this soluble phase also a soluble phenolic fraction is present. The extraction and

recovery of the soluble phenolic fraction would improve the utilization of sugars and contribute to the integral utilization of the raw material.

In the present study the combined use of biorrenewable non toxic solvents was proposed to obtain fractions with antioxidant activity from *C. scoparius*. Conventional solvent extraction using ethanol and extraction with pressurized hot water (autohydrolysis) or with supercritical CO_2 was proposed. Supercritical CO_2 was used to extract the initial ground CsB and to purify the freeze-dried autohydrolysis liquors. The composition and radical scavenging properties of the extracts obtained under selected conditions are reported.

2. Materials and methods

2.1 Materials

Branches from *Cytisus scoparius* (CsB) were collected in December 2010 in Lugo (Spain) and dried at room temperature in a dry and dark place. The dried CsB were ground and sieved to select the fraction of particle size smaller than 1 cm. Samples were homogeneized and stored in closed plastic bags until use.

2.2 Conventional solvent extraction

Ground CSB were extracted at 25ºC with 96% ethanol, using a liquid to solid ratio 10 (v/w) in an orbital shaker at 175 rpm during 30 minutes. The suspension was filtered to separate the extract and the solid residue, which was further subjected to a second extraction stage under the same conditions. The suspension from the second extraction stage was filtered to separate the extract and the solid residue. The extracts from the two stages were combined and the solvent was vaccuum evaporated in rotavapor at 60ºC. The extract was resuspended in distilled water, freezed at -80ºC and liophylized. Extracts were characterized for solubles extraction yield, total phenolics and radical scavenging activity.

2.3 Autohydrolysis

Ground CsB were contacted with water in a batch 3.75 L reactor (model 4551) (Parr Instrument Company– Moline, Illinois). Experiments were performed using a liquid to solid ratio 8 kg:kg (db). Previous studies have shown that this variable has a slight effect on the process performance. A non isothermal heating profile was established (Figure 1), the final temperature studied was 190°C. Autohydrolysis liquors, separated from the solids by filtration, were lyophilized and the powdered extract was further characterized with regard to soluble solids, total phenolics solubilisation and antioxidant activity.

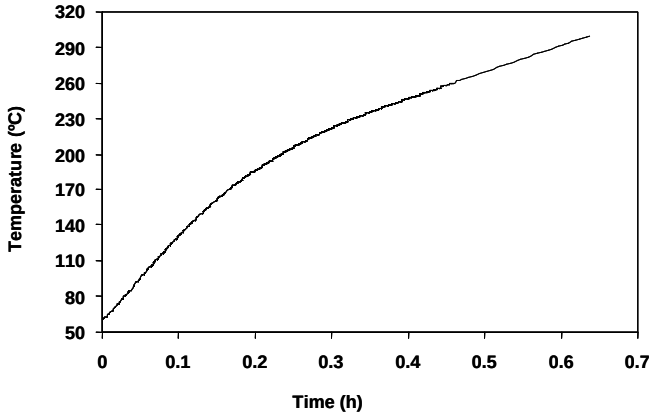

Figure 1. Heating profile of the Parr reactor during non isothermal heating.

2.4 Supercritical CO_2 extraction

Extractions were performed using a supercritical fluid extractor (Thar Process, Inc.) with a 1000-mL extraction cell, which was filled with 20 g ground CsB or the freeze-dried autohydrolysis liquors. Solvent mass flow was fixed at 25 g/min and the extraction pressure was controlled by micrometering valves. The

carbon dioxide used was Premier-X50S from Carburos Metálicos S. A. (Ourense, Spain). Dynamic extractions were performed when the experimental conditions (25 MPa and 45 °C) in the extractor were achieved. 10% ethanol modified CO_2 was used as solvent to extract the ground CsB or to purify the autohydrolysis extract.

2.5 Combined extraction process

The flow diagram of the proposed combined extraction process is shown in Figure 2. Several alternatives have been considered, including the direct extraction with supercritical CO_2 to obtain Extract 1 and the direct solvent extraction of the ground branches with 96% ethanol to obtain Extract 2. The exhausted solids after the ethanolic extraction were processed under selected conditions in a pressurized reactor. The liquid phase from the autohydrolysis process was freeze-dried to yield Extract 3, which was also extracted with supercritical CO_2 to yield Extract 4.

2.6 Determination of the total phenolic content

The phenolic content was measured by the Folin-Ciocalteu method [12] and expressed as grams of gallic acid equivalents (GAE) (Sigma-Aldrich Química S.A.)

2.7 Trolox equivalent antioxidant capacity (TEAC)

This assay, based on the scavenging of ABTS radical (2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonate)), was performed according to the procedure suggested by Re *et al*. [13]. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate allowing the mixture to stand in the dark at room temperature for 12–16 h before use. In the next step $ABTS^{\bullet+}$ solution was diluted with PBS (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30°C. After addition of 1.0 mL of diluted $ABTS^{\bullet+}$ solution to 10 μL of antioxidant compounds or Trolox standards in ethanol or PBS the absorbance reading was taken at 30°C exactly 1 min after initial mixing and up to 6 min. Appropriate solvent blanks were run in each assay. The percentage inhibition of absorbance at 734 nm was calculated as a function of the concentration of extracts and Trolox. The extract concentration leading to 50% drop of the initial ABTS concentration was defined as EC_{50}.
All tests and analyses were run in triplicate, and the average values are presented.

2.8 Chromatographic analysis of the phenolic components

HPLC analyses were carried out in an Agilent HPLC 1100 chromatograph equipped with a DAD detector and a Supelcosil LC18 column. The injection volume was 10 and 5 μL for SE and LHW samples respectively, with a flow rate of 1 mL/min. A non-linear gradient of the solvent mixture MeOH:H_2O:CH_3COOH (9:90:1, v:v:v) (solvent A) and MeOH:H_2O:CH_3COOH (90:9:1, v:v:v) (solvent B) was used. Elution gradients were used as follows: 0 min, 100% A, 0% B; 30 min, 60% A, 40% B; 42 min, 100% A, 0% B. The phenolic compounds were identified according to their retention times and absorption spectra using commercial standards: gallic acid, hydroxymethylfurfural, chlorogenic acid, kaempferol, naringine, 2- furfuraldehyde, 3,4-dihydroxybenzaldehyde, syringic acid, acetovanillone, vanillin, siringaldehyde, rutin, isoquercetin, hesperidin, quercetin (Sigma-Aldrich Química S.A).

3. Results and discussion

Cytisus scoparius, possess a yearly flowering cycle and seasonal variations in extractives yield and compositions were observed. From a previous study the seasonal chemical characterization of the ethanolic extracts revealed that maximum extraction yields were attained in Winter. The maximum phenolic content was observed in February-April, the most abundant in decreasing order being rutin, isoquercetin, kaempferol, quercetin, syringic acid, 3,4-dihydroxybenzaldehyde, acetovanillone, vanilline, naringin and hesperidin. From the studies processing the solid residue obtained after the ethanolic extraction under optimized conditions in a pressurized reactor at temperatures between 150 and 240 °C, it was observed that the maximum solubles and phenolics extraction yields could be attained at 190-210 °C.

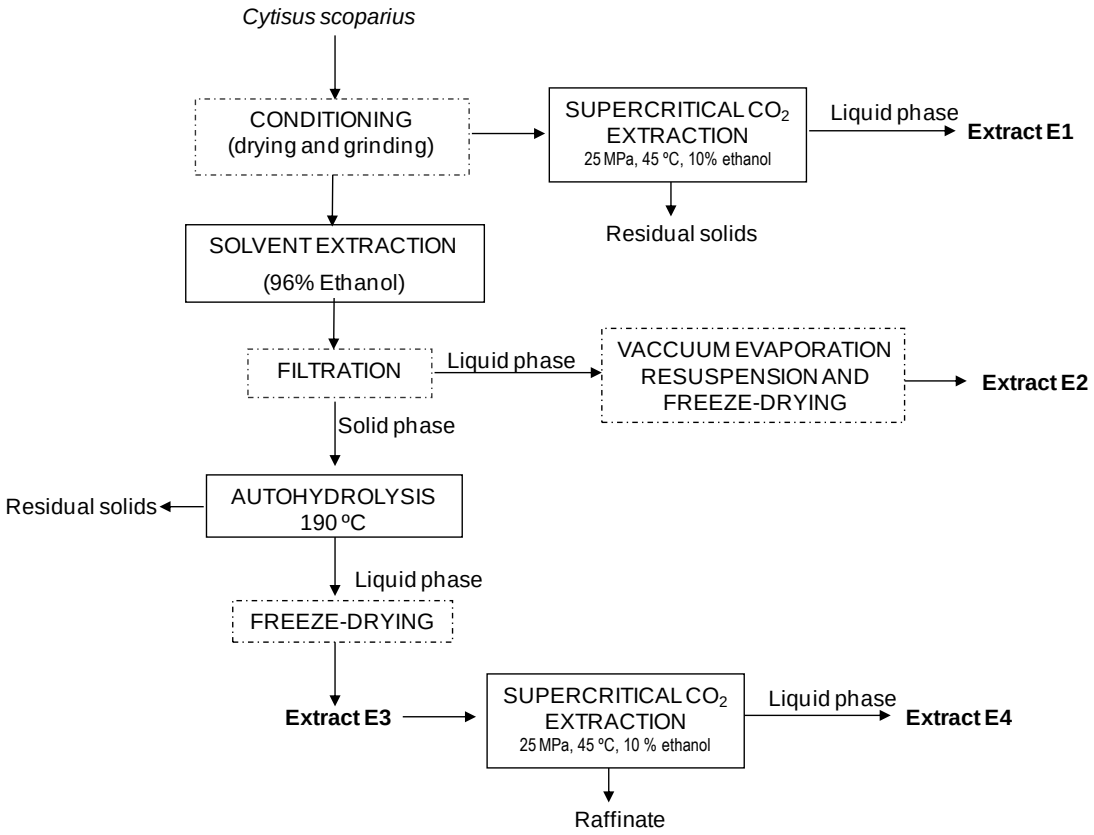

Figure 2. Flow diagramm of the combined process to extract soluble fractions with antioxidant properties from *Cytisus scoparius* branches

Samples of *Cytisus scoparius* branches collected in Winter were processed according to the scheme in Figure 2, in each stage operation under previously selected contitions was established. The phenolic extraction yield and the phenolic content and ABTS radical scavenging of the products obtained in each of the proposed stage are summarized in Table 1. The proposed integrated process is aimed at recovering both the free extractibles and the phenolic fraction susceptible of being released during mild acid hydrolysis of the cell wall. The direct supercritical CO_2 extraction of the *Cytisus scoparius* ground raw material allowed a yield of 1.4% and yielded a product with 5% of phenolic compounds and ABTS radical scavenging lower than BHT. Ethanol extraction of CsB led to higher yields than the direct supercritical CO_2 extraction, but was less selective with regard to phenolics. However, the ABTS radical scavenging capacity was improved respect to that of the supercritical extracts.

Extraction with pressurized hot water of the exhausted solids after the ethanolic extraction provided an extraction yield of 15.9% of the raw material and an extract with phenolic content and ABTS radical scavenging capacity (0.15 g Trolox/g extract) similar to that observed for the supercritical extract 1.

The combination of autohydrolysis and supercritical fluid processing allowed the extraction of 25% of the initial *Cytisus scoparius* material, producing extracts with relatively low phenolic content and moderate radical scavenging capacity when compared to synthetic antioxidants. The supercritical CO_2 extraction of the extract resulting from the autohydrolysis process provided a more selective extraction of phenolic compounds, with lower total yield but higher phenolic purity and ABTS radical scavenging capacity (one fourth of the radical scavenging capactity of Trolox).

Table 1. Extraction yields and ABTS radical scavenging capacity of the extracts from *C. scoparius* branches

	Extraction yield (g/100 g CsB)	**Total phenolics extraction yield** (g GAE/100 g extract)	**TEAC EC_{50}** (g/L)
Extract 1	1.42	4.95	5.94
Extract 2	9.10	1.20	1.12
Extract 3	15.91	6.94	5.40
Extract 4	0.55	8.87	1.92
BHT			0.167

4. Conclusions

Cytisus scoparius branches collected in Winter presented higher content of radical scavenging compounds. A combination of extraction stages using biorenewable clean solvents was proposed for the production of enriched phenolic extracts with antioxidant properties from *Cytisus scoparius*. The extracts were comparable to those obtained with conventional solvent extraction with ethanol.

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