SUPERCRITICAL FLUID EXTRACTION OF TRITERPENOIDS FROM BIRCH BARK.

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The supercritical fluid extraction of birch bark was conducted. Among the triterpenoïds present in the matrix, we are particularly interested by betulinic acid and betulin. The goal of this work was to identify the optimal operating conditions in order to maximize the recovery of betulin and betulinic acid.

It was well established that betulinic acid is a chemotherapeutic agent in the treatment of neuroectodermal tumours and melanoma. Betulin is also an interesting compound, knowing it is a synthetic precursor of betulinic acid. Using binary and ternary mixtures, it was found that carbon dioxide – ethanol gives the maximum yield (29%) when the time extraction was six hours and 10% ethanol (on molar basis) was used.

INTRODUCTION

It was been well established that the outer bark of most white-barked birch species, such as *Betula papyrifera* March, are rich in pentacyclic triterpernoid compound, particularly betulin (Ekman, 1983; Eckerman, 1985). Betulin and other natural triterpenoids such betulinic acid have been extracted in good yield (20 to 30% of dry weight) from the bark of birch with organic solvents. The betulin is the major triterpenoid compound in bark of white birch and represents about 70% of the total triterpene.

The major interest in betulin lies in that it is a synthetic precursor of triterpenoid compounds having interesting pharmacological properties such as betulinic acid. A promising new development is the use of betulinic acid as a chemotherapeutic agent in the treatment of neuroectodermal tumours, covering neuroblastoma, medulloblastoma, glioblastoma and Ewing’s sarcoma cells (Fulda et al., 1999).

Conventional methods of extraction are deficient in several ways. The use of organic solvents at high temperatures may cause thermal degradation of some molecules. An other inconvenient is these organic solvents are hazardous to the environment and to the humans.

Conventional processes, such as solvent extraction, maceration, often require additional steps and are usually inferior to supercritical fluid extraction with respect to selectivity. The almost exclusive of supercritical carbon dioxide to extract natural compounds from plants destined for human nutrition and the pharmaceutical and perfume industries is due to its chemical and physical properties: it is safe, non-toxic, non-combustible, inexpensive and its critical temperature and pressure are not high (31°C, 73,8 bar).

Our goal is to use the supercritical carbon dioxide for extracting the natural compounds from the bark of birch.

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MATERIALS AND METHODS

1. Materials

The material was placed in the sample holder inside the reactor as shown in figure 1 below. The high pressure vessel is a SS 304 cylindrical vessel having approximately 500 cm^3 as internal volume (15.85 cm long, 12.70 cm o.d, 6.35 cm i.d). The holder is a rotating device with two cylinders. The inner cylinder (13.40 cm long, 2.54 diameter) holds a 400 mesh screen while the outer (13.40 cm long, 5.10 cm diameter) holds a 50 mesh screen. The rotating device is mounted on a stainless steel plate with an axis of rotation in which the upper end is housed in a teflon groove located on the cap of the autoclave. A magnetic bar is attached to the stainless steel plate which can girate around a spherical ball (0.3175 cm diameter), when an appropriate field is applied via a magnet (Alcomax II-814). The reactor ensemble is placed in a heated oven. Before entering the reactor, the fluid mixture passed through a preheater also located inside the oven. During these experiments, the agitation was not used.

A high pressure pump (Thar Technology, model P-50) with a maximum capacity of 50 g/min, pumped the liquid carbon dioxide at the desired flow rate. The CO_2 was then heated at the desired temperature in a prereactor located inside the oven. The liquid cosolvent (ethanol, methanol, and water) was pumped using a HPLC pump (Waters, Model 510A). The extraction was carried in a semi batch mode: batch charging of material and continuous flow of solvent. Thermocouples and Bourdon-tube test gauges measured temperature and pressures along the extraction apparatus, respectively. Pressure was regulated by high-pressure valves (Sno-Trik, model SS-410-FP), under manual control.

At the end of the experiment, the entire system was depressurized.. The material recovered after termination of the experiment was weighed, vacuum dried, reweighed and characterized by GC-MS analysis.

2. GC-MS Analysis

2. 1. Equipment

A Shimadzu GC-MS QP 5000 was used. It was equipped with a split-splitless injector and a DB-5MS fused silica column; 5% phenyl-methylpolysiloxane, 30m x 0.25 mm i.d., film thickness 0,25 µm. The injector was maintained at 280°C. Helium was the carrier gas at 1.2 ml/min: the sample (2 µl) was injected in a split mode (1:10). The GC17-A was fitted with a quadrupole mass spectrometer, model QP 5000. MS conditions were as follows: ionization energy, 70 eV; interface temperature, 325°C; scan speed, 1000 amu/s; mass range, 45-500. The software adopted to handle mass spectra and chromatograms was Shimadzu Class 5000. Mass Spectra Library (NIST 98) was used as a reference. The products of supercritical extraction were analyzed after methylation.

2. 2. Methylation

Methylation of carboxylic acids was done by using diazomethane. Knowing the risk associated with the manipulation of this reagent, a special preparation was elaborated as shown in Figure 2 below. Two tubes were filled with 20 ml of DCM. 1.5 g of KOH in 5 ml H_2O was added in central tube. Secondly, 0.5 g of Diazald in 5 ml DCM was also added in the central tube. A nitrogen gas flowed from the left tube at a flow rate of two to three bulls per second. With a syringe, 10 ml of methanol were added gently in the central tube. The reaction is terminated with the appearance of a yellow coloration of the solution of the right tube. A volume of 2 ml of solution of diazomethane is added to each 5 mg of product to derivatize. A reaction is completed in ten minutes. Then, the sample could be injected and analyzed by GC-MS.

2. 3. Results and discussion

We restricted our working field to pressures between 250 – 320 bar and temperatures varying between 40 and 80°C. All extracts were analyzed by GC-MS to monitor their composition. The maximum percentage of betulin and betulinic acid defined the optimum extraction conditions. Preliminary tests have shown that supercritical CO_2 was not efficient for extraction.

The first set of experiments was conducted using two cosolvents (ethanol and methanol) for an extraction time of two hours with a flow rate of CO_2 of 20 g/min, as shown in Table 1. From this table, it was clearly shown that the CO_2 doped with ethanol gives the better yield but is so far from the yield obtained using organic solvents.

As our goal is to obtain a betulinic acid and betulin enriched fractions, we have conducted a second of experiments, maintaining the same flow rate of CO_2 of 20 g/min. The extraction time was extended to six hours for the binary carbon dioxide - ethanol, and also by using a ternary system : carbon dioxide – ethanol – water. This ternary was suggested by authors who use it to extract taxol using supercritical fluids (Kim et coll., 2003).

The results are very promising compared to a previous work which has shown that 20% of triterpenoids could be obtained by subjecting them to supercritical fluid extraction with carbon dioxide at a temperature of about 80 to about 100°C and a pressure of about 540 bar to about 680 bar for a period time of about 3 hours to about 5 hours (Krasutsky et al., 2002).

Increasing extraction time from two to six hours has a beneficial effect since the yield was increased from the range 4 – 7% to 18% in the case of the experiments conducted by using carbon dioxide doped by 5% ethanol. At six hours extraction time and 5% ethanol, the yield varied very slightly from 18% at 40°C to 17.1% at 50°C. It is difficult to explain this decrease. From the thermodynamic point of view, the critical temperature is estimated to be 47°C for 5% wt ethanol (Pöhler et al., 1997).

From the results obtained during the second set of experiments, it could be seen that the maximum yield was around 29% when 90% carbon dioxide – 10% ethanol was used. In the case of the second set of experiments, we have observed that the quantity of the desired triterpenoids i.e betulinic acid and betulin were increased and reached near 4% for betulinic acid and above 55% for betulin.

CONCLUSION

It was shown that doping carbon dioxide with 10% ethanol maximize the recovery of triterpenoids. In this case, the composition if the extract was around 60% when we add betulinic acid and betulin. This future work will focus on developing methods able to concentrate more on these desired triterpenoids.

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Acknowledgements

This research has been made possible by the award of a research grant from Ministère Éducation du Québec (MEQ)

Table 1. Supercritical extraction of triterpenoids with an extraction time of two hours.

Experiment	Solvent (% mol.)	T (°C)	Pressure (bar)	Feed (g)	Yield (%) wt/wt
HUM1	CO_2 – EtOH (5%)	40	270	42.1	6.4
HUM2	CO_2 – EtOH (5%)	45	270	76.9	4.9
HUM3	CO_2 – EtOH (5%)	50	270	44.4	4.0
HUM4	CO_2 – MeOH (5%)	40	270	42.6	0.6
HUM5	CO_2 – MeOH (5%)	45	270	40.5	2.1
HUM6	CO_2 – EtOH (5%)	40	310	28.1	6.2

Table 2. Supercritical fluid extraction of triterpenoids with an extraction time of six hours.

Experiment	Solvent (% mol.)	T (°C)	Pressure (bar)	Feed (g)	Yield (%) wt/wt
HUM7	CO_2 – EtOH (16%) – water (4%)	60	320	30.0	14.0
HUM8	CO_2 – EtOH (16%) – water (4%)	80	320	29.2	25.5
HUM9	CO_2 – EtOH (5%)	40	320	28.7	18.0
HUM10	CO_2 – EtOH (5%)	50	320	28.7	17.1
HUM11	CO_2 – EtOH (10%)	60	320	28.5	29.0

Table 3. Compositions of extracts of selected triterpenoids.

		Extract composition (% wt/wt)		
Experiment	Solvent (% mol.)	Betulinic Acid	Betulin	Lupeol
HUM1	CO_2 – EtOH (5%)	2.7	39.1	4.0
HUM2	CO_2 – EtOH (5%)	2.2	31.6	3.8
HUM3	CO_2 – EtOH (5%)	2.4	33.3	4.1
HUM4	CO_2 – MeOH (5%)	2.9	14.5	6.8
HUM5	CO_2 – MeOH (5%)	2.7	26.0	8.4
HUM6	CO_2 – EtOH (5%)	2.6	27.9	6.2
HUM7	CO_2 – EtOH (16%) – water (4%)	3.4	44.9	0.8
HUM8	CO_2 – EtOH (16%) – water (4%)	2.5	55.1	1.4
HUM9	CO_2 – EtOH (5%)	3.9	56.0	1.1
HUM10	CO_2 – EtOH (5%)	3.8	56.0	1.2
HUM11	CO_2 – EtOH (10%)	3.8	56.0	1.0

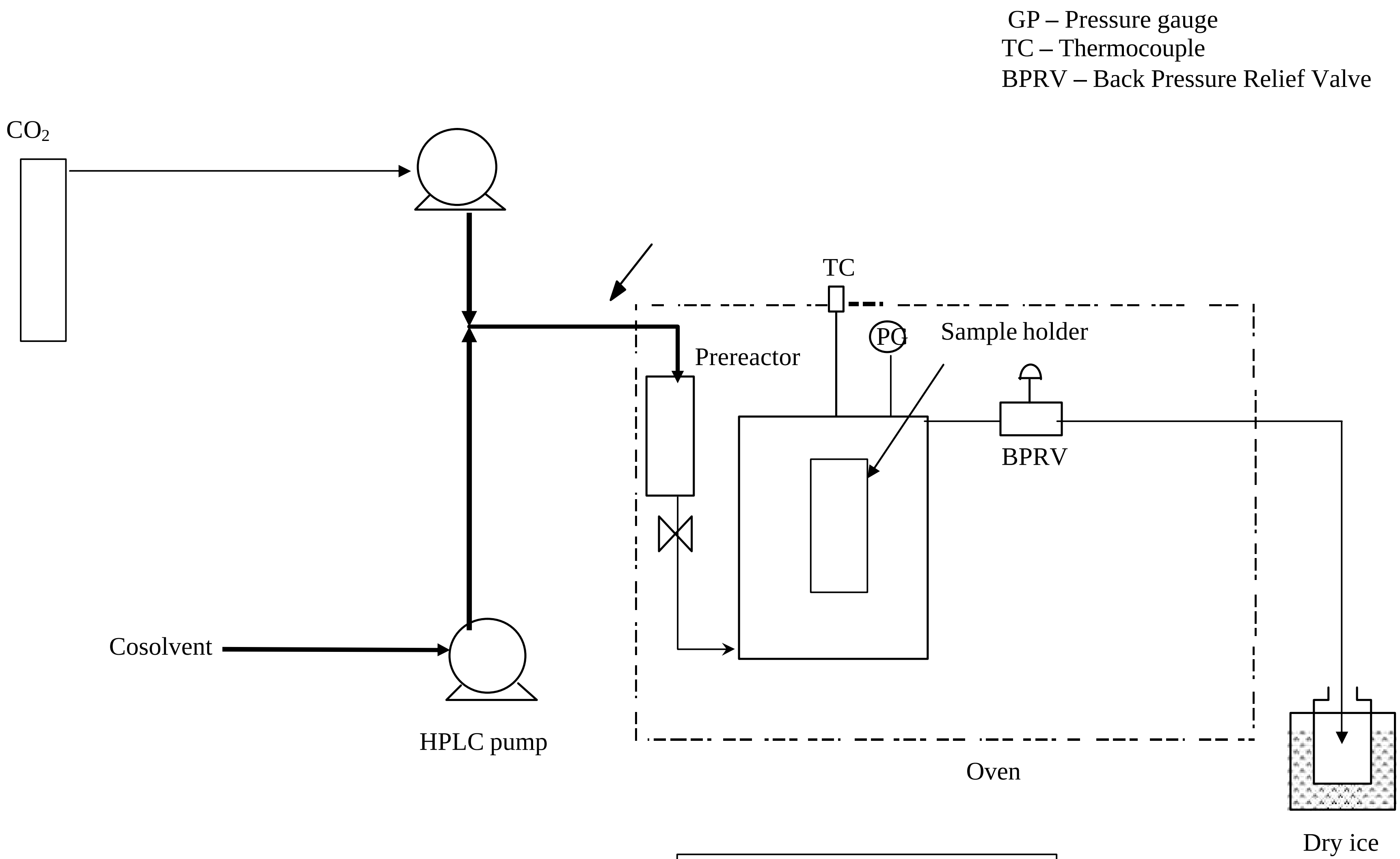

Figure 1. Experimental set-up

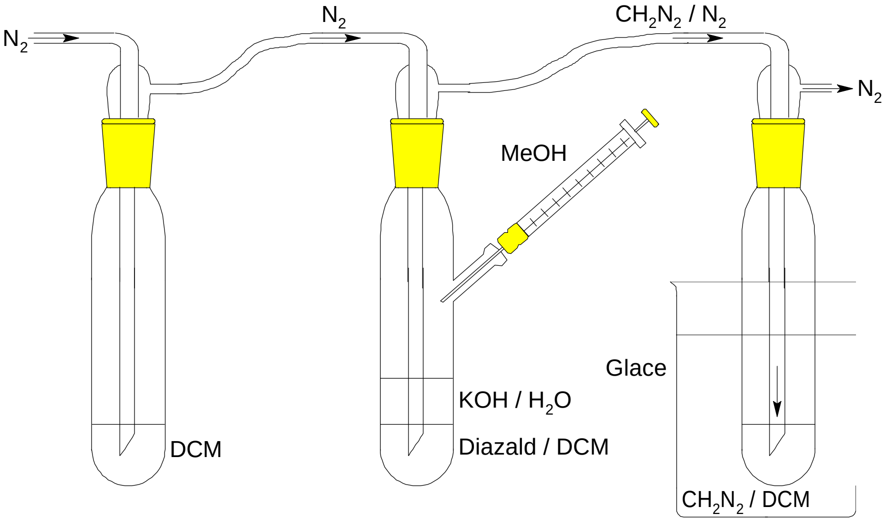

Figure 2. Set-up for the synthesis of diazomethane.