

Purification of taxol from chloroform extracts of *taxus wallichiana* on solid matrix by supercritical CO₂ extraction

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Taxol, proven to be an effective drug for the treatment of various types of cancer, occurs in small amount in the bark *Taxus brevifolia* (0.01 – 0.05 %). Supercritical extraction with carbon dioxide + cosolvents was proved to be effective in extracting taxol and baccatin III. However the concentration of the two compounds is very low.

In the present study dried plant material of *Taxus wallichiana* was first extracted with chloroform to get a crude extract with a taxol and 10 becetyl–baccatin III concentration of 0.17 and 0.61 % respectively. This extract was further purified after adsorbing on a solid surface using supercritical CO₂. Different desorption conditions were used with temperature 313, 323 and 333 K and pressure from 150 to 250 bar. The purified extract yield varied from 15 to 25 % with increased purity depending from the experimental conditions.

INTRODUCTION

Paclitaxel (Taxol®) is one of over 100 naturally occurring taxoids that has been proven effective in treating ovarian carcinomas, metastatic breast cancer, non small-cell-lung cancer, adenocarcinoma and squamous cell carcinoma of the oesophagus. Taxoids are produced in the needles, roots, and bark of all species of yew and may be isolated from extracts of regenerable parts of the plant and their cell cultures. Its natural abundance in the bark of *Taxus brevifolia* is very low (0.01-0.05%) and the tree is slow growing. For this reason other four species of *Taxus* family and needles of the plants were considered as source of paclitaxel. In the extraction process attention is also focused on the possibility of recover of closely-related taxoids compounds: baccatin III and 10-deacetylbaccatrin III.

The large-scale extraction of paclitaxel and baccatin III using organic solvents and their purification using column chromatography is tedious and time consuming. The use of supercritical fluids for the extraction of sensitive components under thermally stable conditions without decomposition represents a valid alternative. The solubility of paclitaxel in pure carbon dioxide is very low [1, 2] due to his polar nature; nitrous oxide shown relatively higher solubility. Therefore the quantity of Paclitaxel and baccatin III extracted using pure supercritical carbon dioxide is limited [3], and only adding 3 wt % of ethanol as cosolvent is possible to obtain results similar to those obtained with normal organic solvents [3 - 5].

In the present study dried plant material of *Taxus wallichiana* was first extracted with methanol to get a crude extract and this extract was further purified after adsorbing on a solid surface using supercritical CO₂. Different desorption conditions were used with temperature 313, 323 and 333K and pressure from 150 to 250 bar. The purified extract yield varied from 15 to 25 % with increased purity depending from the experimental conditions.

MATERIALS AND METHODS

Taxus wallichiana is a hill region plant of India and is available in Jammu & Kashmir, Arunachal Pradesh, Himachal Pradesh and Uttarakhand. We obtained the plant from Jageshwar (Uttarakhand, India).

The adsorbent used in the desorption process was silica gel (Merck). Organic solvent used for the extraction was chloroform pro analysis grade. Carbon dioxide with a purity of 99.98 % was obtained from SIAD.

Organic solvent extraction and adsorption

The organic solvent extraction was carried out at central Institute of Medicinal and Aromatic Plants using methanol as a solvent. Raw material was grounded and contacted with the solvent in a conventional Soxhlet apparatus. The ratio (wt/wt) between the feed of raw material and solvent was 1:3. The extracts were mixed with fixed amount of silica gel and treated in a rotary evaporator under vacuum at 40°C to eliminate the solvent.

Supercritical fluid desorption

The supercritical fluid equipment used in the present investigation is the LAB SFE LAB SFE 100 ml from Separex: is composed of a CO₂ heater, an extraction vessel with extraction cells of different volumes, a temperature and pressure control system, and an extract collector.

The extraction unit is designed for operation up to 500 bar and 150°C.

Liquefied gas from the reservoir is cooled and pumped by a dual-piston pump. Pressure is controlled very precisely with a heated back-pressure regulator.

The extractor was filled in the bottom with glass particles in order to homogeneously distribute the carbon dioxide flow and in the upper part with the adsorbent particle coated with the raw organic extracts (20 % of extract over the adsorbent).

Quantification of the extracts

The equipment consisted of a Spectra System P2000 pump with an SCM 100 vacuum membrane degasser and an UV/VIS detector (UV/VIS – 151 GILSON) operating at 230 nm. Samples were injected with a model 7725i sample valve equipped with a 100 µL loop (Rheodyne, Rohnert Park, CA, USA); the volume injected was 20 µL. Separations were carried out with a Supelco Discovery, RP-C18 (25cm x 4.2 mm; 5µm i.d.) reversed phase column (Supelco, Bellefonte, PA, USA) equipped with a Supelco Pelliguard LC-18 2cm precolumn (Supelco). The eluents were acetonitrile (A) and water (W). Each solvent was prepared fresh before use. The mobile phase was isocratic, using 40% A and 60% W for 15 min at a flow rate of 1ml/min. The system was operated with oven temperature 30°C.

Calibration curves were obtained from an ethanolic dilution series of the reference standards containing Taxol. Concentrations ranging between 10-100 mg/mL of Paclitaxel and 100-1000 mg/ml of baccatin III were prepared from the standard stock dilution by serial dilution with methanol for the calibration data. Calibration levels (n=5) were measured six times. Linear regression was used to establish the calibration curve. Results were calculated using the peak areas were calculated by means of Chromcard software (Thermoquest).

RESULTS AND DISCUSSION

The analysis of methanol extract, used as starting material for the desorption, reports a taxol and 10-deacyl baccatin III (DAB) of 0.17 and 0.61 % respectively. 12 g of supported material were introduced in the LAB unit and a flow rate of 6 ml/min, measured at the pump

conditions, was used in the different desorption tests. The influence of desorption temperature and pressure was investigated in the range 313 – 333 K and 150 – 250 bar. Samples were collected at different interval times and analyzed. From the variation of the quantity desorbed with time (reported in Figures 1 and 2 for 313 and 323 K) it is possible also to evaluate, by extrapolation, the maximum extracted yield. In Figure 1 the yields obtained at 150 bar and at the different temperatures are reported.

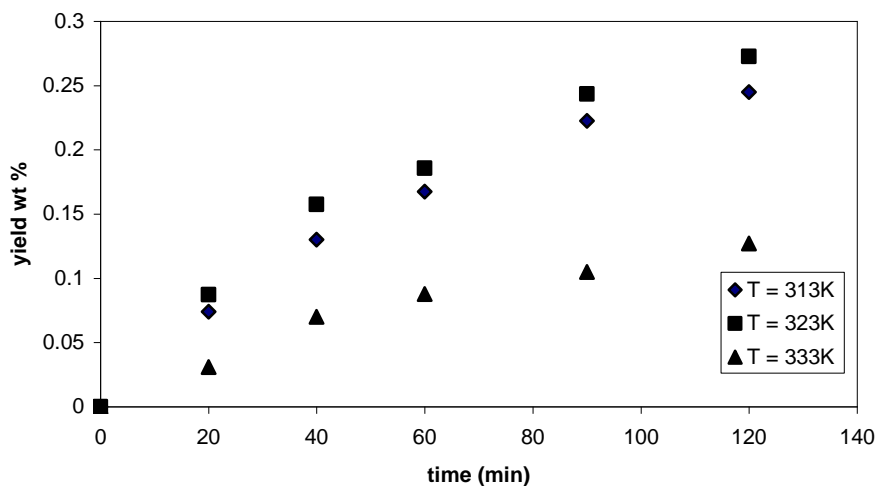


Figure 1: Desorption yields obtained at 150 bar.

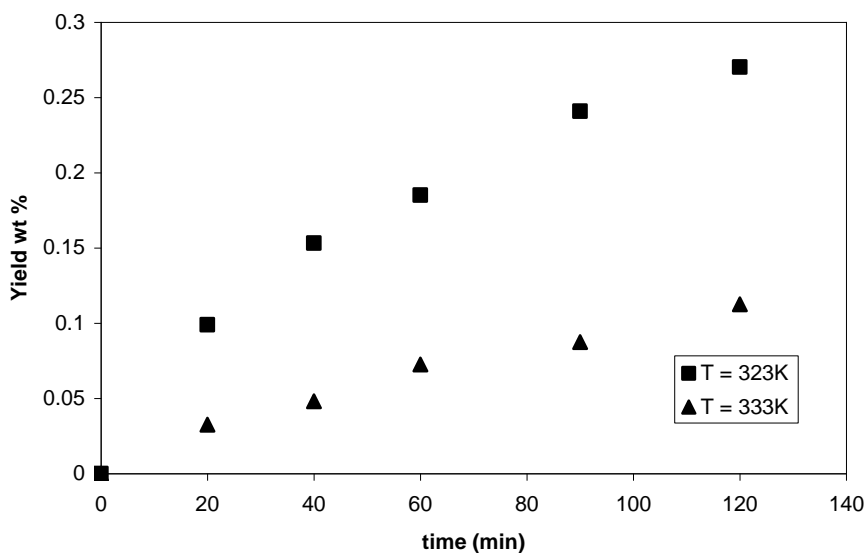


Figure 2: Desorption yields obtained at 200 bar.

It is interesting to observe an inversion on the effect of temperature at pressures between 200 and 250 bar. At 200 bar as previously observed at 150 bar the temperature has negative effect

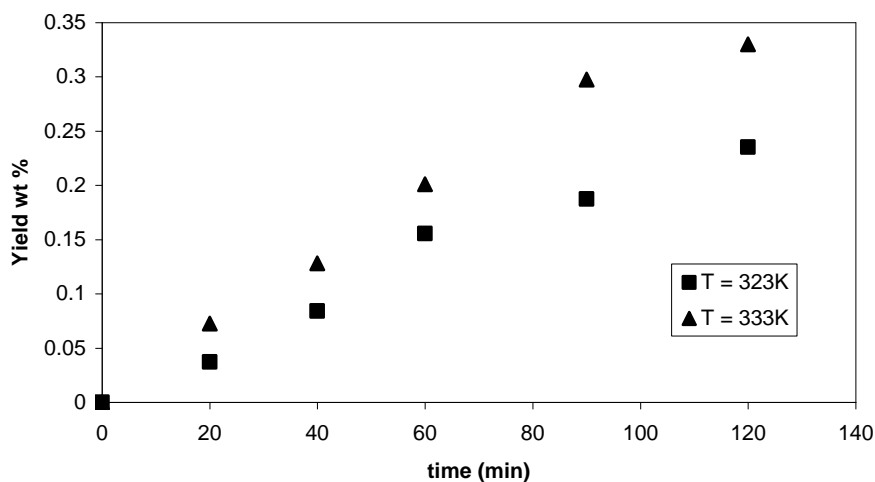


Figure 3: Desorption yields obtained at 250 bar.

whereas has a positive effect at the higher pressure. It is also interesting to observe the effect of pressure at constant temperature. At the lower temperature, 313K, the yield decreases dramatically with increasing pressure, at 323K the yield remains constant between 150 and 200 bar (24.3 %) and slowly decreases at 250 bar (18.7 %), at 333K the yield increases with pressure and reaches the maximum value at 250 bar: 29.75 %.

The composition of the different fractions is practically constant with the time. In Figure 4 the compositions obtained at 323K and 250 bar are reported.

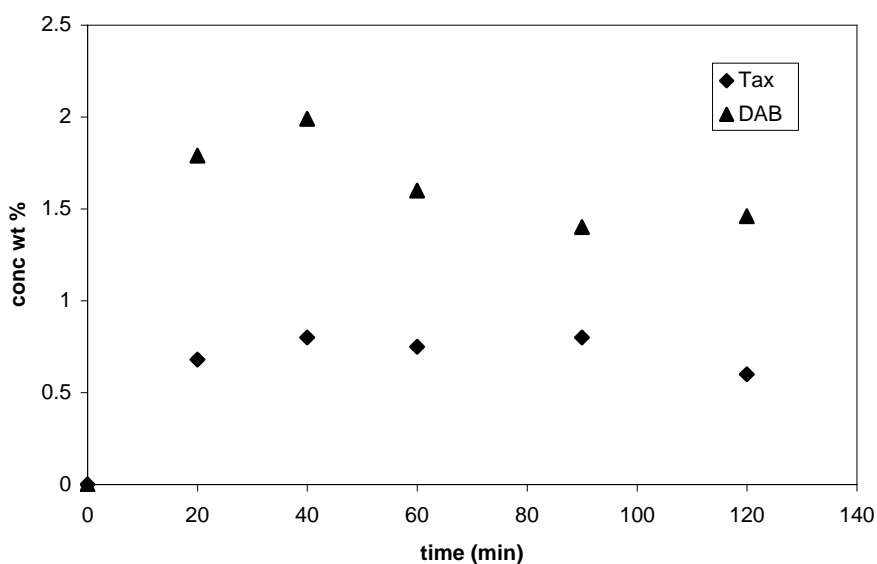


Figure 4: Composition of the fractions obtained at 323K and 250 bar.

The temperature and pressure influence the composition. At 150 bar at all the temperatures investigated there is not enrichment in the solute concentration: practically the fractions have the same content of paclitaxel and DAB as the original organic extracts used as feed material.

At 200 and 250 bar there is a net increment of the solute concentrations and the maximum purity is reached for the desorption performed at 323 K at 200 bar (Taxol 0.95 %, DAB 2.3 %) and 250 bar (Taxol 1.49 %, DAB 3.69 %). Vice versa the experimental conditions that assure the maximum desorption yield (T = 323K and P= 250 bar) gives desorption fractions with a lower taxol and DAB content: Taxol 0.38 % and DAB 0.81%.

Comparing the overall results, yield and fraction compositions, the better desorption conditions are temperature 323K and pressure 200 or 250 bar.

In these conditions the yields of both taxol and DAB increase with time (Figure 5)

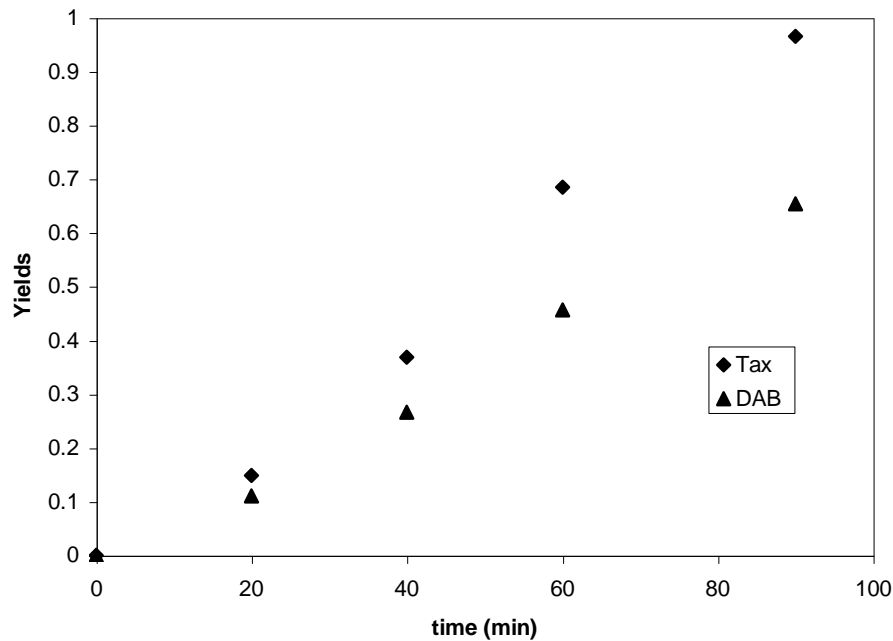


Figure 5: Desorption yields of Taxol and DAB obtained at 323K and 200 bar.

Many approaches have been used to modeling supercritical desorption processes [6 - 13] also due the increasing interest in using this technique for the improved purification of natural materials [14 - 20].

A pure empirical approach [19] for the correlation of desorption data obtained in the study of the recovery of onion flavours suggest using the equation:

$$Y = b_1 \left[1 - \exp(-b_2 t) \right] \quad (1)$$

where b_1 and b_2 The other models are developed considering the adsorption equilibrium and mass transfer resistance. Equilibrium theory, which assumes that mass transfer resistance are negligible, is the simplest method of describing the measured desorption data and have been used by several authors [8, 9, 12]. When equilibrium theory is applied to isothermal, fixed bed, plug flow desorption, the desorption profile will be a function of the adsorption equilibrium relationship. The behavior of an adsorber can be described by equation 1 [21] when equilibrium is assumed:

$$t = \frac{L}{u} \left[1 + \left(\frac{1-\varepsilon}{\varepsilon} \right) \frac{dq}{dc} \right] \quad (2)$$

where dq/dc is the slope of the adsorption isotherm, L is the length of the bed, u the velocity and ε is the void fraction in the packed column.

This equation is used to develop an expression for the breakthrough curve for the desorption of a uniformly saturated bed with a pure carrier. The derivative term dq/dc is obtained from the relevant adsorption isotherm expression and after integration an equation describing the desorption profile can be obtained:

$$\left(\frac{\partial z}{\partial t} \right)_c = \frac{v}{1 + \left(\frac{1-\varepsilon}{\varepsilon} \right) \frac{dq}{dc}} \quad (3)$$

where z is the axial position in the column. Using, as an example, the Freundlich isotherm:

$$q = Kc^{\frac{1}{n}} \quad (4)$$

and the derivative of this expression and integrating an expression relating the effluent concentration to the desorption time is obtained. In the case of the above mentioned Freundlich isotherm:

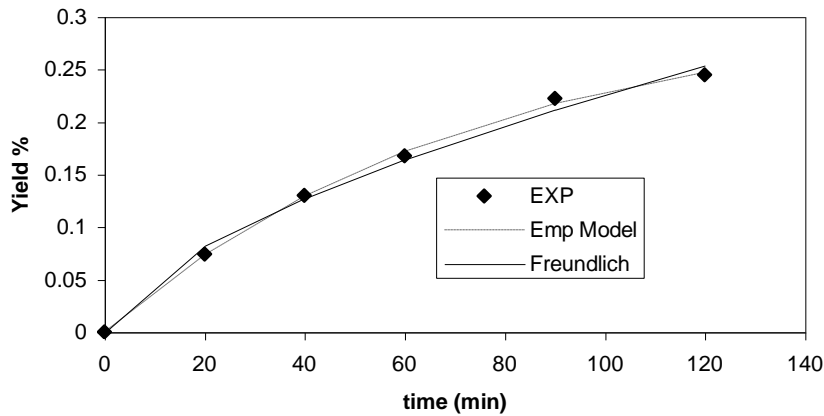


Figure 6: Comparison between empirical and Freundlich models (experimental data at 313K and 150 bar).

$$c = \left[\left(\frac{n\varepsilon}{K(1-\varepsilon)} \right) \left(\frac{ut}{L} - 1 \right) \right]^{\frac{n}{1-n}} \quad (5)$$

In order to obtain the fraction desorbed as a function of time the concentration profile given by the equation (4) must be integrated.

As an example in Figure 6 a comparison between the results obtained with the empirical and Freundlich models are presented for the data obtained at 313K and 150 bar. It is evident that the empirical approach gives results very close to those obtained using the equilibrium theory.

Using the Langmuir isotherm:

$$q = \frac{KQc}{1 + Kc} \quad (6)$$

where K is a constant and Q (mass of adsorbate for a complete monolayer covering) is often considered as a second empirical constant.

The concentration profile is given by:

$$c = \frac{1}{K} \left\{ \left(\frac{KQL(1-\varepsilon)}{\varepsilon(t-L)} \right)^{\frac{1}{2}} - 1 \right\} \quad (7)$$

The assumption of local equilibrium is probably not fully justified and a different approach is that suggested by Tan and Liou [10-12].

Neglecting the axial dispersion effect the mass balance in the bulk phase in the column is given by:

$$\varepsilon \frac{\partial c}{\partial t} + u \frac{\partial c}{\partial z} = -(1-\varepsilon) \frac{\partial q}{\partial t} \quad (8)$$

And the mass balance for the solid is expressed in term of a linear adsorption expression:

$$\frac{\partial q}{\partial c} = -kq \quad (9)$$

The concentration profile is at the exit of the column is expressed by:

$$c = \frac{1-\varepsilon}{\varepsilon} q_0 \left[\exp \left[-k \left(t - \frac{\varepsilon L}{u} \right) \right] - \exp(-kt) \right] \quad (10)$$

The three approaches were applied to model the experimental desorption profiles.

As an example in Figure 6 the comparison between the fraction desorbed determined experimentally and that obtained by correlation with the three models is presented.

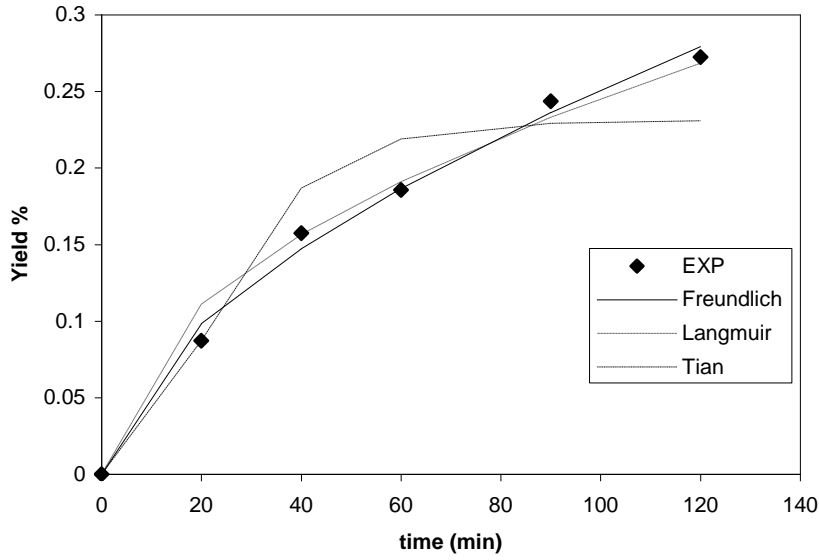


Figure 6: Comparison between desorption profiles calculated with the different models. (experimental fat obtained at 323K and 150 bar).

The profiles obtained using the Freundlich and Langmuir isotherms are similar and larger deviations are showed by the Tian and Liou equation.

The comparison between the three models can be done calculating for the different experimental data sets the Akaike number AIC [22]:

$$AIC = N \ln \left(\frac{\chi^2}{N} \right) + 2 \frac{(M+1)(N-2)}{N-M-2} \quad (11)$$

where N and M are, respectively, the number of experimental points and the model fitting parameters. The model showing the smallest AIC is the one preferred. In order to estimate how much the model showing the smallest AIC is more likely it is necessary to define the probability p_{AIC} :

$$p_{AIC} = \frac{e^{-0.5\Delta}}{1 + e^{-0.5\Delta}} \quad (12)$$

where $\Delta = AIC_{OM} - AIC_{smallest}$ is the difference between the AIC number relative to the more likely model and the AIC number relative to the other models.

In the case of the desorption data the Freundlich model show the best fitting performance and therefore it was choose as reference or model with smallest AIC number.

In table 1 the probability that the Freundlich model must be correct respect to the the Langmuir and Tian and Liou models are reported. From the values reported it is evident that the Freundlich approach is the most suitable for the correlation of the experimental data obtained.

		313K	323K	333K
150 bar	Langmuir	0.958949	0.878103	0.911206
	Tian and Liou	0.998114	0.998278	0.909793
200 bar	Langmuir	0.990785	0.999776	0.995782
	Tian and Liou	0.992847	0.999941	0.998867

Table 1: Probability of Freundlich respect to the Langmuir and Tian and Liou models approach.

CONCLUSION

Taxol recovery from *taxus wallichiana* is preformed coupling the organic solvent extraction and desorption. In this way fractions with enhanced concentrations of taxol and 10-deacetylbaicatin III are obtained.

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