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Co-solvent-Modified Supercritical Carbon Dioxide Extraction of 10-Deacetylbaccatin III from Needles of *Taxus baccata* L

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Abstract: 10-Deacetylbaccatin III was extracted from the ground needles of *Taxus baccata* L. growing in Turkey using sub- and supercritical carbon dioxide with and without co-solvents by using two different methods (as an entrainer and modifier) and compared to Soxhlet extraction. SFE applications were carried out in the pressure range between 10 to 40 MPa and temperature ranges between 35 to 45°C. Recovery of the target compound increased with increasing temperature and pressure. The highest quantity of 10-deacetylbaccatin III obtained from needles of *Taxus baccata* L. was about 718 mg/kg when 200 μL of methanol was used as an entrainer at 45°C.

Keywords: Co-solvent, 10-deacetylbaccatin III (10-DAB), entrainer, Paclitaxel (Taxol), soxhlet extraction, supercritical carbon dioxide extraction, taxotere, *Taxus baccata* L.

INTRODUCTION

Recently, the extraction of medicines from natural plants has received considerable attention from research groups and pharmaceutical companies for the treatment of various diseases, especially cancer (1–4). Many compounds have been isolated from natural plants and clinically tested. Taxol® (Paclitaxel) has been shown to be useful as an anticancer agent

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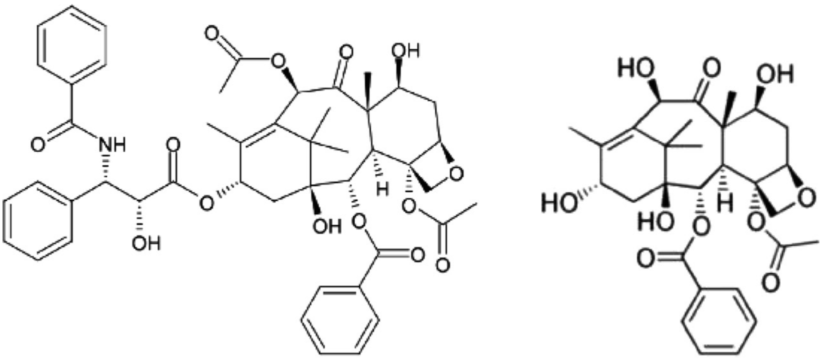
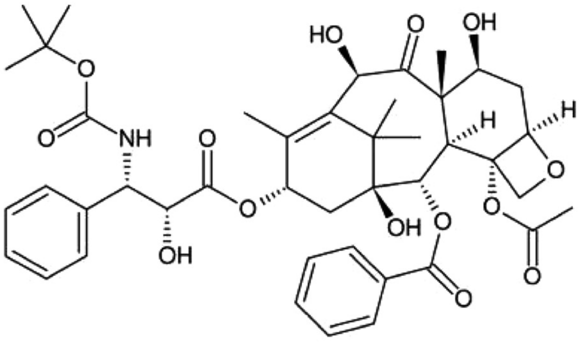
(2,3). The first studies concerning Taxol's antimitotic property started in the late 1950's in connection with the National Cancer Institute (NCI) cancer screening program. Taxol® (Fig. 1a) was first isolated from the stem bark of the Pacific yew (*Taxus brevifolia*) (1) and it has been isolated from several *Taxus* species, such as *T. brevifolia*, *T. cuspidata*, *T. baccata*, and *T. yunnanesis*. It is applied against a variety of ovarian, breast, and skin cancers because it inhibits the depolymerization of tubulin (2,3).

Taxol exists in a very low concentration in the bark of *Taxus* species (4). Some research groups studied different methods to obtain Taxol from *Taxus* species. Chun et al. extracted paclitaxel and baccatin III from the needles of *Taxus cuspidata* by supercritical fluid extraction (SFE) and they obtained the highest recovery of target compounds in the extracts



(a) Paclitaxel (b) 10-Deacetylbaccatin III



(c) Taxotere (Docetaxel)

Figure 1. (a) Paclitaxel; (b) 10-Deacetylbaccatin III; (c) Taxotere (Docetaxel).

during the first four hours of SFE, i.e. about 0.262 and 0.644 mass% for paclitaxel and baccatin III, respectively (5).

Heaton et al. extracted taxicin effectively from *Taxus baccata* L. by using SFE with 10% methanol as co-solvent (6). Vandana et al. studied Taxol extraction from *Taxus brevifolia* by using SC-nitrous oxide as the main solvent with ethanol and obtained high amounts of taxanes compared to SC-CO_2 extraction (7).

As Taxol has very active properties for some cancer treatments but is found in low amounts in the bark, some research groups have attempted to obtain the Taxol molecule synthetically. Several approaches for obtaining Taxol, such as total synthesis, tissue culture processes, production by a Taxol-producing fungus, extraction and isolation from the *Taxus* species, and semi-synthesis from 10-deacetylbaccatin III (Fig. 1b), have been used (2).

Among these methods, a semi-synthesis from 10-DAB is the major method for commercial production of Taxol and its semi-synthetic analog docetaxel (Taxotere-Fig. 1c). Although different research groups have already reported the total synthesis of Taxol on a lab scale, all these procedures are very complex and not commercially suitable (8).

The semi-synthetic procedure consists of only a few steps, and results using this procedure to obtain Taxol from 10-DAB are very favorable (9). 10-DAB III, a diterpenoid, can be derived from the renewable leaves, which are known to be more abundant in *Taxus baccata* L. (2). Different analytical methods have been developed for the isolation of taxanes from different *Taxus* species. Denis et al. succeeded in recovering 0.1% (w/w) of 10-DAB III from fresh needles of *Taxus baccata* L. (3,10). To date various studies have been reported in the literature including those on the applications of supercritical fluid extractions because of their very useful advantages that selectivity or solvent power can easily be changed by changing the temperature and pressure applied to the fluid (11). Supercritical carbon dioxide (SC-CO_2), in particular, is an attractive supercritical solvent due to its low critical pressure (7.4 MPa), low critical temperature (31.1°C), and the fact that it is both non-toxic and inert. The disadvantage to using pure CO_2 for extractions is that, with no net dipole moment, CO_2 is an ineffective solvent for materials with higher polarity (12). Different polar co-solvents have been used to increase extraction efficiencies because the co-solvent can increase the strong analyte-matrix interactions and remarkably increase the polarity of the pure solvent (13).

Carbon dioxide was chosen as the primary solvent and four co-solvents, as an entrainer (MeOH, EtOH, DMSO, THF), were tested for their effect on the total extract yields obtained. Methanol and ethanol were chosen because they are highly soluble in CO_2 and have been shown to be effective co-solvents in terms of both polarity and ability to disrupt plant matrix bonding (12). Tetrahydrofuran (THF) was chosen because it is a highly volatile liquid used mostly as a solvent due to its excellent solvating power (14). Dimethylsulfoxide (DMSO) was chosen because it is a low toxicity solvent frequently used in the pharmaceutical industry. DMSO also exhibits high solubility in carbon dioxide, due to Lewis-acid base interaction of its sulfonyl group with the carbonyl groups of carbon dioxide (15,16).

The main effect of a co-solvent is the solubility enhancement that results from an increase in solvent density and/or intermolecular interactions between the co-solvent and the solute. Selectivity of a separation can be improved by co-solvent addition if there are specific intermolecular interactions between the co-solvent and one or more of the mixture components (17).

According to the Peng-Robinson equation, CO_2 density is maximum under low temperature and high pressure conditions. The solvating power of supercritical fluid increased with increasing supercritical fluid density (18). Therefore, we tried to investigate the co-solvent effect at moderate temperature and high pressure when using methanol and ethanol as a modifier.

In this study we focused on the extraction of 10-deacetylbaccatin III from *Taxus baccata* L. needles using SFE with different co-solvent addition techniques. SFE has not been used for direct extraction of 10-deacetylbaccatin III from *Taxus baccata* L. growing in Turkey.

EXPERIMENTAL

Samples

Needles of *Taxus baccata* L. were collected on Uludag Mountain, 30 km south of Bursa (Turkey). They were dried at 30°C and processed in a rotary mill to obtain efficient mass transfer between the samples and supercritical fluids. The powdered plant material (d_p; i.e., 3×10^{-4} m) was stored in a glass flask, protected from light and humidity. The dried needles of *Taxus baccata* L. were pretreated with n-hexane three times in order to remove waxes and non-polar structures. Dewaxed sample has $9.95 \pm 0.25\%$ water content. The needle powders were then collected using filter paper and dried for SFE experiments.

SFE Apparatus and Procedure

The supercritical fluid extraction system consisted of two ISCO 260D syringe pumps (ISCO, Lincoln, NE, USA), an isotemp oven (Teknosem, Turkey), and high pressure valves (HIP, Erie, PA, USA). Carbon dioxide was supplied by BOS A.S (Turkey) in high purity (99.95%).

Supercritical Extraction with Entrainer (CO_2 + Entrainer)

10-Deacetylbaccatin III was extracted from the needles of *Taxus baccata* L. using a supercritical fluid extraction system. A 0.3 g plant sample was placed inside a 0.8 mL extraction cell (50 mm × 4.6 mm i.d., Alltech). Then a co-solvent was added to the cell by injecting 100 and 200 µL (12.5% and 25% v/v regarding cell volume) into the sample in the extraction cell. The vessel was closed tightly and placed in a Teknosem Isotemp oven for SFE. Both ends were screwed to tubing and leaks were checked in the constant pressure mode. When the leak check was being performed, valves V2 and V3 were open and valve V4 was closed. After the leak checks the oven was heated to the extraction temperature depending on the desired temperature of that run. The system consisted of a three-way shut-off valve, V3, and three two-way shut-off valves, V1, V2, and V4. Schematic diagrams of the extraction system are given in Fig. 2. It was described in detail in our previous work (19). The first stage of the extraction was carried out at a different pressure range (10 to 40 MPa) and a different dynamic extraction time (5 to 30 min). As was to be expected, an overall increase in extraction yield with increasing pressure and dynamic extraction time (Figs. 4,5). Therefore we set the extraction pressure as 40 MPa and dynamic extraction time 30 min for all continuous extraction experiments. Static and dynamic extraction was performed for 30 min. The flow rate of supercritical fluids was

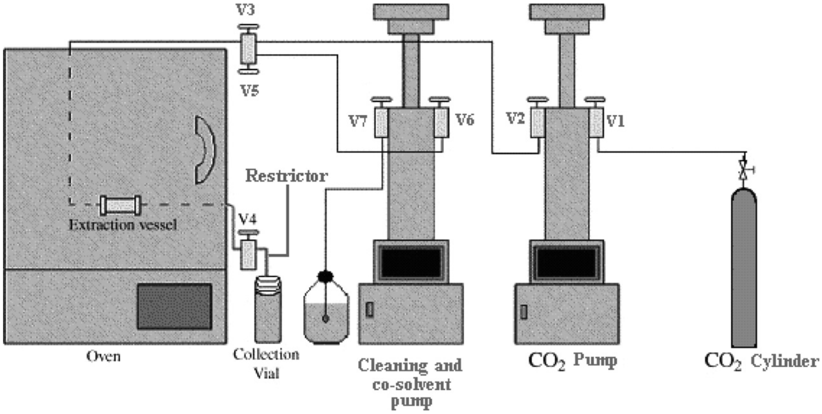


Figure 2. Schematic diagram of the supercritical fluid extraction system.

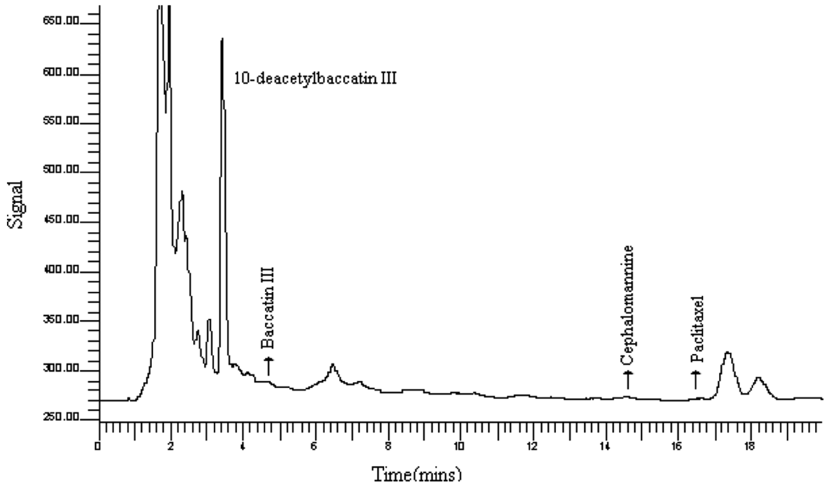


Figure 3. Extract chromatogram for SFE of taxanes at 40°C and 40 MPa with methanol used as a co-solvent.

approximately 0.5 ml/min. Extracted analytes were collected by a restrictor into a 6 ml vial (Agilent) containing 5 ml of methylene chloride. After the extract was collected, the methylene chloride was vaporized to near dryness in nitrogen atmosphere. After each experiment the line and valves were washed with methanol and dichloromethane by a second pump (cleaning pump).

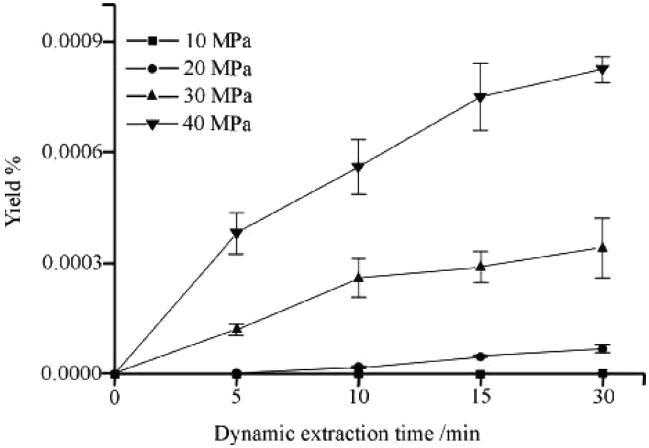


Figure 4. Effect of pressure and dynamic extraction time at 35°C by using pure carbon dioxide.

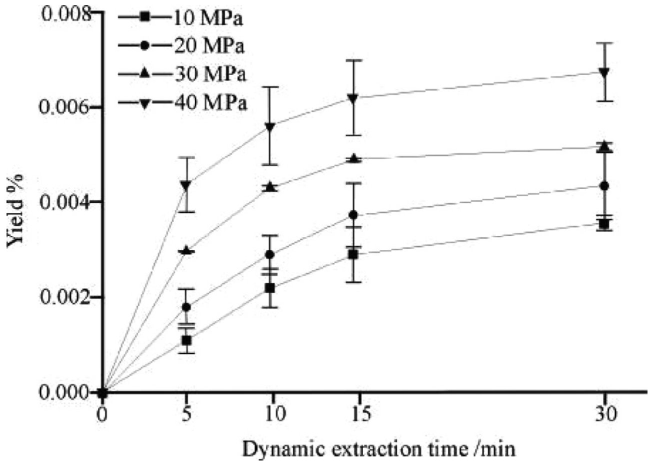


Figure 5. Effect of pressure and dynamic extraction time at 45°C by using pure carbon dioxide.

Supercritical Extraction with Modifiers (CO_2 + Methanol and CO_2 + Ethanol)

When a co-solvent is added to CO_2, the critical temperature of the resulting mixture is elevated, limiting the amount of co-solvent that may be added if the extraction is to occur in the supercritical region at 35–45°C (20,21). It should be mentioned that under the operating temperatures and pressure of interest, the binary CO_2 + methanol and CO_2 + ethanol solvent mixture are one-phase in supercritical state, where the critical temperature and pressure for CO_2 + methanol and CO_2 + ethanol mixture (at 5.0 mol% co-solvent) are 41.3°C and 7.4 MPa, and 42.5°C and 7.3 MPa, respectively (22) (Table 1). Supercritical CO_2 + methanol and CO_2 + ethanol experiments were performed in the temperature range 35–45°C and at 40 MPa constant pressure with 2, 4, and 6 mass% methanol and ethanol as co-solvents.

Subcritical Extraction with Modifiers (CO_2 + Methanol and CO_2 + Ethanol)

When a co-solvent is added to pure CO_2 as a modifier by a second pump, the critical temperature of the mixture increases (20). Addition of 6% (w/w) methanol or ethanol as co-solvent makes the critical temperature 45°C (CO_2 + methanol or ethanol) but between 35 and 45°C addition

Table 1. Critical temperature and pressure of CO_2 + methanol and CO_2 + ethanol mixtures (calculated using SF-Solver Software, ISCO Inc., Lincoln, NE, USA)

Methanol and ethanol concentration (wt.%)	Tc (°C) $CO_2 + MeOH$	Pc (MPa) $CO_2 + MeOH$	Tc (°C) $CO_2 + EtOH$	Pc (MPa) $CO_2 + EtOH$
0	31.1	7.3	31.1	7.3
3	35.8	7.9	37.8	8.1
5	41.3	7.4	42.5	7.3
10	51.8	7.4	53.7	7.2
15	61.8	7.6	63.3	7.2
20	72.7	7.5	76.1	7.1
100	239.4	8.1	243.3	6.1

of 7% (w/w) methanol and ethanol as co-solvent makes the medium subcritical (or near critical) at 40 MPa pressure. Therefore, keeping the pressure at 40 MPa and temperature range between 35 and 45°C adding 8–20% (w/w) co-solvents by syringe pump makes the extraction subcritical (Table 1).

Soxhlet Extractions

In order to compare the extraction efficiency with SFE, 5 g of *Taxus baccata* L. needles were weighed into a cellulose extraction thimble and a sample was extracted with methanol, ethanol, and petroleum ether for 9 hours at the rate of 2 cycles/h. After the Soxhlet extractions, the solvent was evaporated by reduced pressure evaporation and the extract was dried. It was dissolved in calculated stoichiometric ratio volume of methanol and then filtered using nylon membranes of 0.45 μm pore size for HPLC analysis.

Extract Analysis

The amount of 10-DAB present in the needles extract was determined by HPLC analysis of the methanol soluble portion of the extracts. The SFE crude extract was dissolved in a small volume of methanol (300 μL) and then filtered using nylon membranes of 0.45 μm pore size (Whatman Int. Ltd.).

A Perkin Elmer Series 200 Model liquid chromatography system was used for the quantitative determination of taxanes, which was analyzed on a reverse-phase column (Phenomenex Luna, 250 × 4,6 mm, 5 μ Torrance, CA, USA) at ambient temperature. All standards were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The samples were eluted by the solvent mixture of MeOH:MeCN:H_2O (20:32:48, v/v) at a flow rate of 1.4 ml.min^{-1} (23). Chromatograms (Fig. 3) from the standard and extract were detected at 227 nm with a UV-DAD detector. The volume of the injection into the HPLC was 20 µL and the sample injection into the HPLC was performed three times for each sample and quantities of 10-DAB were then obtained by averaging these HPLC results. A calibration curve was produced for 10-DAB over the concentration range 2.5–40 mg/L and showed a linear response with a correlation coefficient of 0.9997 ($n = 3$). Detection limit was 0,25 µg/ mL (24).

RESULT AND DISCUSSION

The extraction of 10-deacetylbaccatin from needles of *Taxus baccata* L. growing in Turkey was investigated using a supercritical carbon dioxide extraction system with and without co-solvent. In order to increase the polarity of CO_2, various co-solvents were used to enhance recoveries of the target compound by two different methods. SFE was performed over the temperature range 35 to 45°C and at a pressure of 40 MPa to avoid degradation of the target compound.

Supercritical Fluid Extraction with Pure Carbon Dioxide

The first stage of SFE of *Taxus baccata* L. needles was carried out with pure carbon dioxide to investigate its solvating power. When extraction was carried out with carbon dioxide at 35°C and 10 MPa, the amount of target compound obtained was negligible; the values were close to the lower detection limit of HPLC. By increasing the temperature to 45°C and pressure to 40 MPa, 0.007 mass% yield of 10-deacetylbaccatin III was observed (Fig. 6 – yield% based on g 10-DAB/100 g needles).

After Soxhlet extraction of needles using ethanol, methanol, and petroleum ether, 0.0396%, 0.055%, and 0.0113% (% w/w), respectively, of 10-deacetylbaccatin III were obtained.

Table 2 clearly shows that Soxhlet extraction using ethanol, methanol, and petroleum ether for 10-deacetylbaccatin III was better than pure supercritical carbon dioxide extraction. Because of these results we decided to use some solvents as a co-solvent during supercritical carbon dioxide extraction of needles of *Taxus baccata* L.

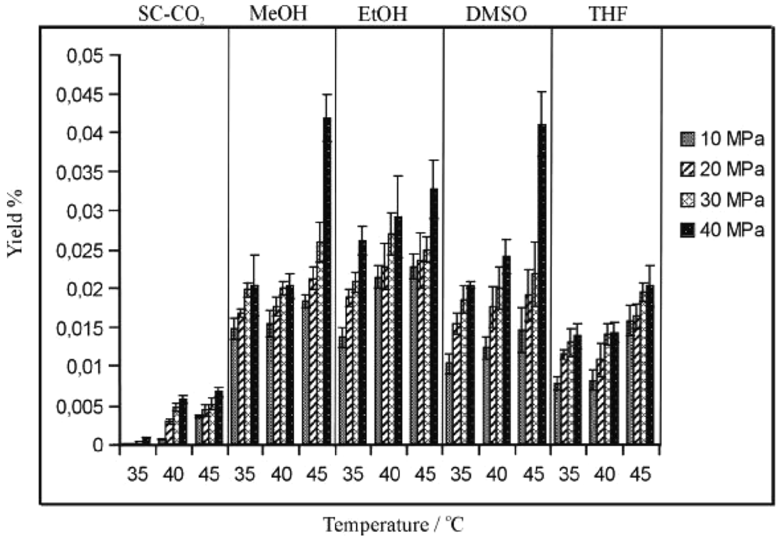


Figure 6. Effect of pressure and temperature of extraction of 10-DAB in supercritical solvent for different entrainer type. (Yield % based on g 10-DAB/100 g needles).

Supercritical Carbon Dioxide Extraction by Adding Co-solvents as Entrainer

Several co-solvents, namely methanol, ethanol, tetrahydrofuran, and dimethyl sulfoxide, were used as an entrainer with carbon dioxide under different extraction conditions in order to examine the influence of co-solvents on extraction capability. Two different amounts of co-solvent, 100 µL and 200 µL (12.5% and 25% (v/v) regarding cell volume), were investigated at 35 to 45°C. Table 2 shows the comparison of co-solvent effect on extraction efficiencies of 10-DAB from *Taxus baccata* L. (Table 2 results based on mg 10-DAB/kg needles). Extraction results of pure carbon dioxide and Soxhlet extraction are also presented for comparison purposes. All co-solvents showed better results than pure carbon dioxide when using either 100 µL or 200 µL volume. In general, the extraction efficiency of 10-DAB increased with the use of co-solvents. The best results were obtained with a CO_2-MeOH mixture (718 mg/kg needles) for 10-DAB. Furthermore, yield of extraction was influenced by co-solvent volume. Figure 7 shows that moles of consumed carbon dioxide for different entrainer type.

Table 2. Comparison of co-solvent effect on extraction efficiencies of 10-deacetylbaccatin III from *Taxus baccata* L. at 35–45°C and 40 MPa using 100 μL and 200 μL co-solvent

Co-solvent	Temperature (°C)	Co-solvent volume (μL)	*10-DAB(mg)
MeOH	35	100	147(18)
EtOH	35	100	136(21)
DMSO	35	100	156(9)
THF	35	100	78(21)
MeOH	40	100	154(30)
EtOH	40	100	213(22)
DMSO	40	100	172(32)
THF	40	100	140(30)
MeOH	45	100	418(22)
EtOH	45	100	235(11)
DMSO	45	100	189(24)
THF	45	100	203(23)
MeOH	35	200	483(13)
EtOH	35	200	387(32)
DMSO	35	200	436(25)
THF	35	200	224(14)
MeOH	40	200	581(21)
EtOH	40	200	562(13)
DMSO	40	200	537(16)
THF	40	200	245(24)
MeOH	45	200	718(16)
EtOH	45	200	605(18)
DMSO	45	200	626(7)
THF	45	200	414(25)

Solvent	*10-DAB(mg)
Soxhlet Extraction	
Methanol	396(25)
Ethanol	555(11)
Petroleum ether	113(12)

*10-DAB concentrations in mg/kg *Taxus baccata* L. (RSD% based on triplicate extractions)..

Supercritical Carbon Dioxide Extraction by Adding Co-solvents as Modifier

We examined the co-solvent effects by changing the co-solvent content in the mixed solvent from 2 to 6 mass% in methanol and ethanol. The

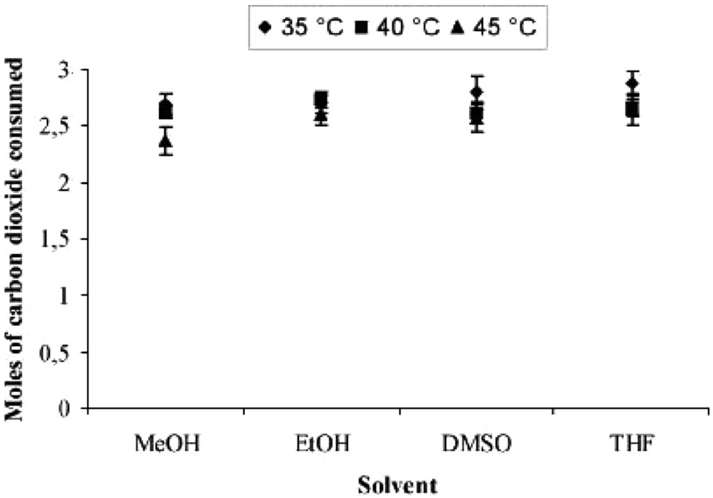


Figure 7. Moles of carbon dioxide consumed for used different entrainer type (200 μL).

amount of 10-DAB obtained using methanol is shown in Fig. 8. The variation in the methanol content in the mixed supercritical medium did affect largely the extraction efficiency until (6 mass% of methanol) a certain ratio of modifier. Between 2 and 6 mass% of methanol solvent medium is supercritical but between 8 and 20 mass% of methanol the medium is subcritical. Considering these changing properties of the extraction medium, in the supercritical region the yield of 10-DAB is of 0.022 mass% in 35°C and 40 MPa with 2 mass% methanol; this value doubled and reached 0.055 mass% at 45°C and 40 MPa using 6 mass% of methanol. In the subcritical region a sharp increase (Fig. 8) was not observed (8–20 mass% of methanol) in the yield of 10-DAB; furthermore, the yield of 10-DAB decreased when using 20 mass% of methanol. Palma et al. observed the same modifier effect for extraction of polyphenolic compounds from grape seeds. They were obtained using with 10% modifier produced 12% more extract than using with 40% modifier (25). Bicchi et al. indicated that methanol is miscible with CO_2 as high as 20% (26).

A similar trend was observed for ethanol during extraction under supercritical conditions (2–6 mass%). As seen from Fig. 9, in the supercritical region the maximum amount of 10-DAB was 0.044 mass% at 40 MPa and 45°C. Unlike methanol in the subcritical region, when the amount of ethanol was increased, the yield of 10-DAB increased; the maximum amount of 10-DAB was 0.056 mass% at 45°C and 40 MPa using 20 mass% ethanol. The same co-solvent effect was observed with the extraction of β-carotene using supercritical carbon dioxide. The maximum extraction yield was obtained under near critical conditions (27). When added the ratio of 20 mass% co-solvents, methanol and ethanol showed different behavior. This different behavior may be indicative of higher order interactions between the solute and the co-solvent. The type of interactions can be discussed qualitatively on the basis of pure component properties as, for instance, solubility parameters (28).

10-DAB Recovery after SFE

Previously, the best recovery of 10-deacetylbaccatin III was obtained by Denis et al., i.e., 1 g/kg of *Taxus baccata* L. needles, using a conventional solvent extraction technique (3). We succeeded in obtaining a comparable quantity, 718 mg/kg 10-DAB, by only adding 200 μL of methanol co-solvent in SFE. 10-Deacetylbaccatin III was already known to be the predominant taxane in *Taxus baccata* L., and this was confirmed by our study results as shown in Table 2. Recovery of taxane can change with changing co-solvent volume and co-solvent addition techniques to the matrix. Solvent power of SC-CO_2 can be increased by using small amounts of co-solvents. The co-solvent's effect is dependent on the concentration of the co-solvent in the supercritical phase, which is determined by the phase behavior of the mixture under operating conditions. Solvent–co-solvent mixtures should be completely miscible in the supercritical state under the operating conditions to achieve the desired effects. Increase in solvent density and intermolecular interactions are the major factors that contribute to the co-solvent effect. Physical interactions between the solute and co-solvent such as dipole–dipole, dipole–induced dipole, or induced dipole–induced dipole (dispersion) interactions and specific interactions such as H-bonding and charge transfer complexes are important contributors to the co-solvent effect. Therefore, an accurate interpretation of the co-solvent effect requires knowledge of the intermolecular interactions between the solutes and solvents of interest, which is considered by the change of physical properties in the supercritical conditions (17,18,29).

Figures 8 and 9 express recovery of 10-DAB at different temperatures and pressures at fixed co-solvent volume. The best recovery was 0.068 mass% for 10-DAB using methanol (10 mass%) as co-solvent introduced to the matrix as a modifier.

In general, SFE studies have been done at temperatures lower than at 100°C to prevent decreasing analyte solubility by lowering CO_2 density because of increasing temperature at constant pressure (13) so, we maintained the temperature range carefully.

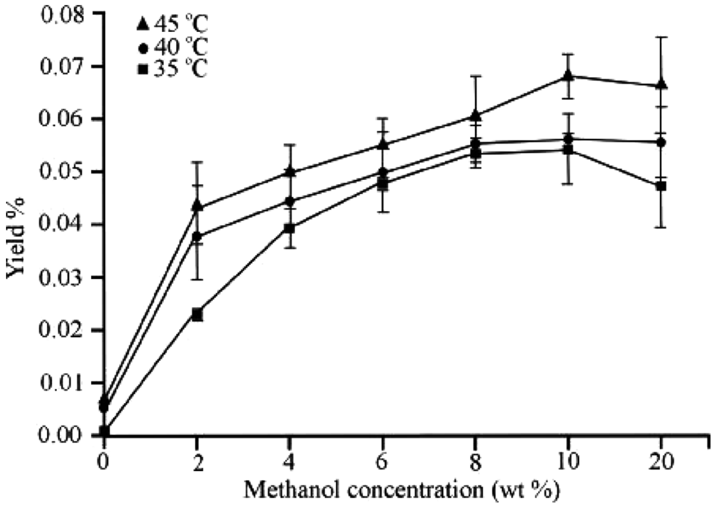


Figure 8. Yield (%) of 10-deacetylbaccatin III in CO_2 + methanol (2–20 mol%) at 40 MPa and different temperatures. 0(%) – pure CO_2. CO_2 + methanol (2–6 mol%) supercritical region. CO_2 +methanol (8–20 mol%) subcritical region.

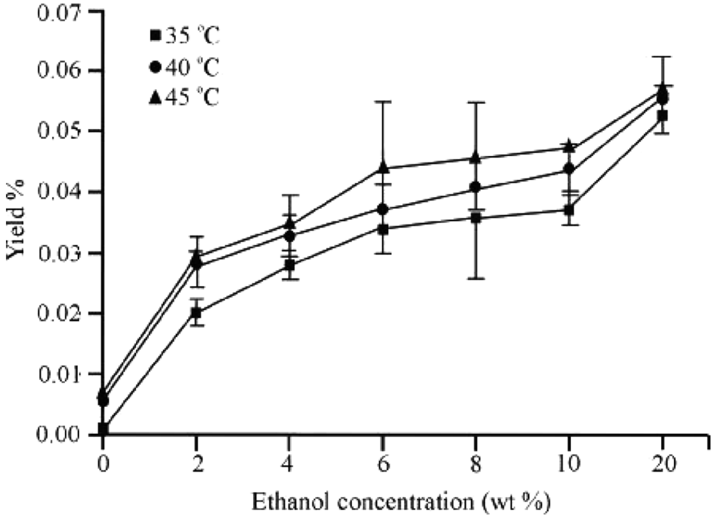


Figure 9. Yield (%) of 10-deacetylbaccatin III in CO_2 + ethanol (2–20 mol%) at 40 MPa and different temperatures. 0(%) – pure CO_2. CO_2 + ethanol (2–6 mol%) supercritical region. CO_2 + ethanol (8–20 mol%) subcritical region.

When looking at temperature and pressure effects in SFE, recovery of 10-DAB increased with increasing temperature (Fig. 6). According to the Peng-Robinson equation CO_2 density is maximum at low temperature and high pressure conditions. The solvating power of supercritical fluid increased with increasing supercritical fluid density (18). Therefore, we examined the co-solvent effect at moderate temperature and high pressure.

10-DAB Recovery on the SFE

The determination of recovery was performed on the SFE system. Sea sand was cleaned using acetone and dried. The pre-cleaned sand was added the extraction cell (50 mm × 4.6 mm i.d., Alltech). Then 10-DAB standard solution (40 ppm) was added to the cell by injecting 1 ml into the pre-cleaned sand in the extraction cell. The vessel was closed tightly and placed in a Teknosem Isotemp oven for SFE. Supercritical carbon dioxide extraction of the 10-DAB from the needles using methanol, ethanol, DMSO, and THF gave quantitative recoveries and were >90%. However, the recoveries using pure carbon dioxide were less quantitative and averaged only about 13%. Methanol and DMSO functioned well as extractants in SFE and the recoveries were >95%, but the recovery levels in the other two solvents were <95%.

CONCLUSION

A tremendous amount of attention has focused on Taxol and its analog docetaxel, antitumor agents. 10-Deacetylbaccatin III has been used for the semi-synthesis of Taxol and its analogs, which can be very attractive for recovery enhancement from *Taxus baccata* L.

Supercritical fluid extraction of 10-deacetylbaccatin III from *Taxus baccata* L. growing in Turkey has not yet been announced by any research groups. In this study the extraction of 10-deacetylbaccatin III from the leaves of *Taxus baccata* L. was performed using supercritical fluid extraction. The best recovery of 10-deacetylbaccatin III was obtained by Denis et al. at quantity of i.e. 1 g/kg of *Taxus baccata* L. needles using a conventional solvent extraction technique (3). We succeeded in obtaining a comparable quantity, 718 mg/kg 10-DAB, by only adding 200 μL of methanol co-solvent in SFE. Paclitaxel, cephalomannine, and baccatin III were detected in HPLC analysis as well, but we disregarded these as they were only in trace concentrations. The objective of this study was to investigate the effect of temperature, pressure, co-solvents, and co-solvent addition techniques to the matrix in supercritical carbon dioxide extraction of 10-DAB from *Taxus baccata* L. It was known that various co-solvents increase the extraction efficiency of 10-DAB.

In conclusion, supercritical fluid extraction is a useful method for the extraction of natural compounds from matrix. In addition to solvating power and solubility, wasting of co-solvent is avoided because of the short extraction time in SFE compared to conventional extraction methods. Therefore SFE also can be more effective, fast, green, and high-tech separation process compared to other conventional extraction methods for 10-DAB recovery.

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