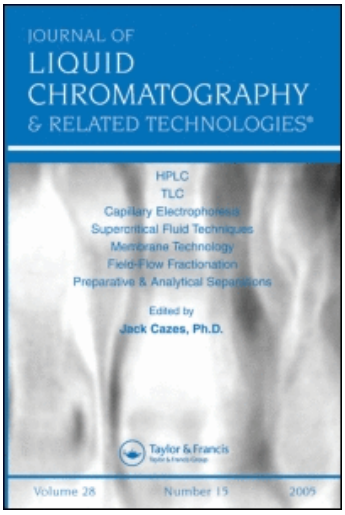

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Extraction Methods of 10-Deacetylbaccatin III, Paclitaxel, and Cephalomannine from *Taxus baccata* L. Twigs: A Comparison

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Abstract: Four types of solvent extraction methods (ultrasound and microwave assisted extraction, pressurized liquid extraction, and extraction in the Soxhlet apparatus) for paclitaxel, cephalomannine, and 10-deacetylbaccatin, taxoids recovered from common yew twigs, were compared. By use of pressurised liquid extraction (PLE), the most effective extractant of taxoids was determined. HPLC was used for the analysis of the extracts. Comparison of the obtained results revealed differences in the extraction power of the applied methods. The greatest yields were obtained by multiple PLE, which can be recommended as the best sample preparation method for taxoids analysis in yew twigs.

Keywords: 10-Deacetylbaccatin, Cephalomannine, Extraction methods, Paclitaxel, Taxoids, Taxus

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INTRODUCTION

The common yew (*Taxus baccata* L.) and its varieties are known for their content of taxoids, diterpenoid compounds which exhibit anticancer properties.[1,2] Analysis of taxoids in yew species is a difficult, multistep, time- and energy-consuming procedure due to a very low concentration of these compounds and the presence of numerous ballast substances such as nonpolar chlorophylls and waxes, polar tannins, and phenolics.[3,4]

It is well known that the quality of the final result of plant material analysis strongly depends on the isolation degree of analytes from their matrix. The lower the level of an analyte in a matrix, the more essential is its recovery and the more important is the development of effective isolation procedures for its accurate analysis. In the case of plant analysis, extraction techniques are generally used. Most frequently, exhaustive extraction in the Soxhlet apparatus, maceration, digestion, extraction under reflux, etc., are applied.[5–8]

Although they are relatively simple methods, they suffer from such disadvantages as long extraction times, relatively high solvent consumption, and often unsatisfactory reproducibility.[9]

The recently developed isolation techniques, such as ultrasound assisted extraction (USAE), microwave assisted solvent extraction (MASE), or pressurized liquid extraction (PLE) eliminate most of the above mentioned drawbacks of the traditional extraction methods. However, they may differ in their extraction effectiveness.

The application of ultrasound waves and higher temperature in USAE results in a better penetration of the matrix by the extracting solvent and better solubility, diffusivity, and transport of the isolated compound. The advantages of MASE result from the performance of the heating source, which relies on the solvent dipole movement (so-called volume heating). Microwave extraction efficiency increases with the increase of analytes' polarity and with solvents' dielectric constant. Moreover, the potentialities of the technique can be enlarged by the variation of pressure in closed microwave-based extractors. More recently, special attention has been addressed to PLE, sometimes called accelerated solvent extraction. This technique is based on the conventional heating of sample with extractant at elevated pressure.[10,11] The application of such conditions makes possible extraction at a temperature exceeding the extractant boiling temperature under atmospheric pressure, leading to the effective isolation of the analyte in a short time and with a small amount of solvent.

The purpose of this paper is to investigate the effect of four extraction methods on the yield of chosen taxoids (paclitaxel, cephalomannine, and 10-deacetylbaccatin) from common yew twigs. The following

extraction techniques are compared: Soxhlet extraction, ultrasonic extraction with solvent at ambient temperature and at 60°C, microwave assisted solvent extraction in open and closed systems, and pressurised liquid extraction.

There are some papers concerning the application of modern extraction methods in taxoids analysis from yew.[12–14] However, they deal with different diterpenoid compounds examined in different matrices (bark, seeds). Thus, it is impossible to compare the efficiencies of different extraction methods from the results reported in the cited works.

EXPERIMENTAL

Materials

Plant Material

Twigs of *Taxus baccata* L. were collected in the Botanical Garden of Maria Curie Skłodowska University in Lublin, (voucher specimen AR 379). Plant material was dried at 45°C for about 12 h in an oven with passive ventilation. Dried material was milled in a laboratory grinder, sieved to obtain fraction 0.2–1.5 mm and divided into 5.00 g portions.

Standards

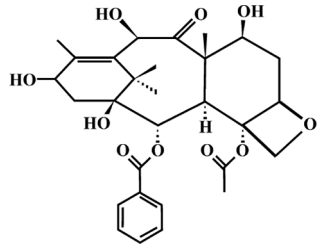
10-Deacetylbaccatin III and paclitaxel (Taxol®) were purchased from Sigma (St. Louis, MO, USA), whereas cephalomannine was supplied by the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD, USA). Chemical structures of these taxoids are presented in Figure 1.

Sample Preparation Methods

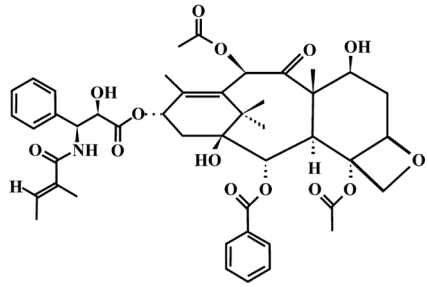
For statistical purposes, each sample preparation procedure was repeated three times in given experimental conditions.

Exhaustive Extraction in Soxhlet Apparatus

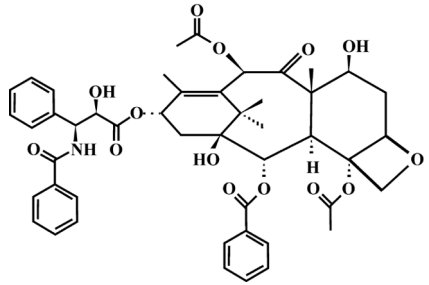
A paper thimble with a sample of the plant material was placed in a Soxhlet apparatus and extracted with pure methanol over 21 h. The obtained extract was subjected to the analytical procedure described further.



10-deacetylbaccatin III



Cephalomannine



Paclitaxel

Figure 1. Chemical structures of investigated taxoids: 10-deacetylbaccatin III, cephalomannine and paclitaxel.

Ultrasound Assisted Solvent Extraction

An Erlenmeyer flask containing a sample of the plant material and methanol (100 mL) was placed in an ultrasonic bath (Unimasz UM-4 Koszalin, Poland). The same sample of the plant material was extracted three times for 30 min at 25°C or 60°C using a fresh portion of methanol in each case. The combined extracts were filtered and subjected to analytical procedure.

Microwave Assisted Solvent Extraction in Open and in Closed System

The sample of plant material was mixed with 100 mL of methanol and irradiated with microwaves using a Plazmotronika UniClever BMZ I

bath (Wrocław, Poland) equipped with a 600 W generator. The extraction was performed for 30 min in an open and in a closed system, applying 60% of the generator power.[15–18]

Pressurised Liquid Extraction (One-Cycle PLE)

PLE was performed with a Dionex ASE 200 instrument (Dionex, Sunnyvale, CA, USA). An exactly weighed portion of the plant material was mixed with neutral glass to reduce the volume of the solvent used for the extraction,[19] and placed into a 22 mL stainless steel extraction cell. The influence of the following factors on the taxoids' extraction was examined:

- Extracting solvent type (methanol, n-hexane, toluene, dichloromethane, ethyl acetate, n-propanol, chloroform, water);
- Extraction temperature (using the extractant giving the best yield);
- Extraction time (for the best extractant and temperature).

All extractions were performed at the same pressure, equal to 60 bars. After the extraction process, the extraction cell content was flushed using the same solvent in the amount equal to 60% of the extraction cell volume, and purged for 120 s applying pressurized nitrogen (150 p.s.i). The whole volume of collected extracts was between 25–31 mL, depending on the packing density of the extraction cells. Between runs, the ASE system was washed with the extraction solvent.

Exhaustive Pressurised Liquid Extraction

Exhaustive (multiple) PLE was performed in optimised conditions with methanol at 115°C under the pressure of 60 bar during 15 minutes. The procedure was repeated four times on the same sample of plant material, i.e. until no taxoids were detected by an HPLC method.

Sample Preparation for HPLC Analysis

Each of the obtained extracts was evaporated to dryness in a vacuum rotary evaporator under reduced pressure (type 350P Unipan, Poland), dissolved in methanol, and transferred to a 50 mL measured flask and filled up to volume with methanol.

To purify the crude extracts from hydrophobic ballast substances, the SPE method described in Ref. [20] with some modification was applied. The SPE-cartridge was filled with 1 g of silanized silica (E. Merck, Darmstadt, Germany) and conditioned with pure methanol (10 mL), distilled water (10 mL), and 75% solution of methanol in water (10 mL).

Then, the sample of crude extract in 75% methanol (5 mL of extract dissolved in methanol plus 1.7 mL of distilled water) was introduced to the SPE-cartridges and eluted with a 10 mL portion of 75% solution of methanol in water. The eluate was evaporated to dryness and dissolved in 2 mL of methanol before chromatographic analysis.

HPLC Analysis of Extracts

Chromatographic analysis was performed using an HP 1100 liquid chromatograph with a DAD detector (Hewlett-Packard Palo Alto, CA, USA). A stainless-steel column C_{18} (150×4.6 mm) Symmetry Shield 5 μm (Waters, USA), thermostated at 25°C, was applied.

The following linear-complex gradient of acetonitrile (gradient grade, E. Merck, Darmstadt, Germany) in milliQ water was used for elution (flow rate $1\,mL \times min^{-1}$): gradient from 20 to 45% of ACN during 10 min, next increase from 45 to 50% in 10 min, and final increase to 100% for 2 min. This type of gradient was optimized using the DryLab G computer program.[21]

The external standard method was applied for taxoids quantification. Calibration curves were constructed in the following concentration ranges:

0.009–0.05 mg/mL ($R^2 = 0.9999$)
for 10-deacetylbaccatin III;

0.003–0.02 mg/mL ($R^2 = 0.9963$)
for cephalomannine; and

0.009–0.05 mg/mL ($R^2 = 0.9997$)
for paclitaxel.

RESULTS AND DISCUSSION

As mentioned in the Introduction, the present investigation concerns a comparison of extraction yields of paclitaxel, cephalomannine, and 10-deacetylbaccatin from yew twigs using various extraction techniques. The evaluation of the influence of extraction method type on the extraction yield requires the application of the same solvent in all the compared extraction methods. Therefore, the experiments started with the selection of the most effective solvent for the extraction of examined taxoids. PLE in default conditions (at temperature 100°C and pressure 60 bar during 10 minutes) was chosen for this purpose because of its effectiveness and short extraction time.

According to the literature, methanol, dichloromethane, and ethyl acetate are usually applied as taxoid extractants.[22–27] To remove ballast

substances disturbing the chromatographic analysis, some extraction procedures of taxoids from plant material involve a preliminary extraction process in which toluene, n-hexane, or chloroform is used.[28–31] Although the literature reports that the last two solvents do not extract taxoids, it was, nevertheless, decided to apply in the PLE experiments not only the most popular extractants but, additionally, the non-polar and medium polar ones recommended for preliminary extraction. The results of these experiments are presented in Table 1.

As seen from the collected data, all the applied solvents exhibit the ability to extract taxoids from yew twigs under PLE conditions. The highest extraction yield is obtained using methanol as the extracting medium. Weaker, but similar in their extraction strength, are such solvents as dichloromethane, ethyl acetate, chloroform, toluene, and n-propanol. Even n-hexane extracts small amounts of taxoids in the PLE process. The presented results prove that the solvents not extracting taxoids in traditional extraction techniques[28–31] do exhibit some extraction ability under PLE conditions. Hence, the removal of ballast substances from yew twigs by preliminary PLE extraction using n-hexane or toluene could lead to wrong analytical results.

It is well known that the extraction of analytes from plant matrices strongly depends on temperature. Hence, the subsequent step of the experiments was to verify how the extraction temperature changes affect the extraction yield of the analysed compounds. This examination is justified and desirable due to the fact that taxoids are thermolabile compounds,[3,25] and that PLE can be carried out at temperatures markedly exceeding the boiling point of extractant due to high pressure in the PLE process. The above mentioned experiments were extended to include

Table 1. Comparison of the PLE yield of the selected taxoids extracted from twigs of *Taxus baccata* with various solvents – mean values (n = 3) (±SD)

	Taxoids content (in mg/g of dry weight sample)		
Solvent type	10-Deacetylbaccatin III	Cephalomannine	Paclitaxel
n-Hexane	0.0051 ($\pm 1.5 \cdot 10^{-4}$)	0.0131 ($\pm 5.9 \cdot 10^{-4}$)	0.0030 ($\pm 1.6 \cdot 10^{-4}$)
Toluene	0.0607 ($\pm 1.2 \cdot 10^{-3}$)	0.0440 ($\pm 1.5 \cdot 10^{-3}$)	0.0206 ($\pm 8.3 \cdot 10^{-4}$)
Dichloromethane	0.0777 ($\pm 1.8 \cdot 10^{-3}$)	0.0424 ($\pm 1.8 \cdot 10^{-3}$)	0.0201 ($\pm 8.8 \cdot 10^{-4}$)
Chloroform	0.0769 ($\pm 1.8 \cdot 10^{-3}$)	0.0349 ($\pm 1.2 \cdot 10^{-3}$)	0.0195 ($\pm 7.1 \cdot 10^{-4}$)
Ethyl acetate	0.0742 ($\pm 1.8 \cdot 10^{-3}$)	0.0408 ($\pm 1.3 \cdot 10^{-3}$)	0.0238 ($\pm 8.9 \cdot 10^{-4}$)
n-Propanol	0.0982 ($\pm 2.4 \cdot 10^{-3}$)	0.0646 ($\pm 2.7 \cdot 10^{-3}$)	0.0316 ($\pm 1.1 \cdot 10^{-3}$)
Methanol	0.1470 ($\pm 3.6 \cdot 10^{-3}$)	0.0831 ($\pm 2.3 \cdot 10^{-3}$)	0.0360 ($\pm 1.4 \cdot 10^{-3}$)
Water	0.0499 ($\pm 1.2 \cdot 10^{-3}$)	0.0477 ($\pm 2.0 \cdot 10^{-3}$)	0.0056 ($\pm 3.2 \cdot 10^{-4}$)

the influence of extraction time changes on the taxoids' extraction efficiency. They were all carried out using methanol, which was found to have the highest extraction power in the PLE process under default conditions (see Table 1). The obtained data are shown in Figures 2 and 3.

As can be seen from the graphs, the highest extraction yield of the analysed taxoids is obtained during 10–15 min of the PLE procedure at 130°C. Further increase of extraction temperature (150–200°C) leads to a gradual decrease of extraction yield for all the quantified taxoids. An increase of extraction time to 20 min (at 130°C) also causes a small decrease of the taxoids extraction yield. It can be concluded that a short operation time (10–15 min), even at relatively high extraction temperature (100–130°C), does not cause noticeable degradation of the thermolabile taxoids. Similar conclusions were drawn by Kawamura et al., who investigated the isolation of taxoids from *Taxus cuspidata* bark.[12]

The results of the experiments performed to compare the yield of the chosen taxoids from yew tissue using selected extraction techniques are gathered in Table 2. The table is divided into two parts. Part "A" collects the taxoid yields obtained by a single extraction performed under conditions most frequently applied for a given method (USAE under atmospheric pressure over 30 min at ambient temperature or at 60°C, MASE employing 60% of generator power over 30 min in open or in closed system, and PLE in default conditions). Part "B" contains the taxoid yields obtained using exhaustive Soxhlet extraction (the extraction

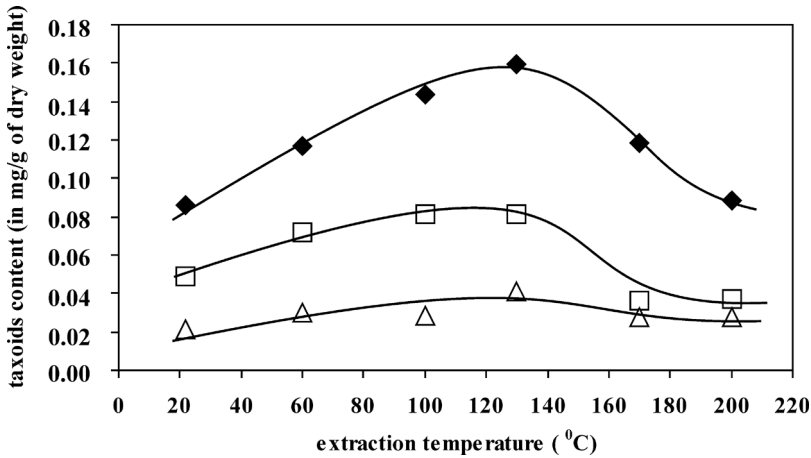


Figure 2. Dependence of the PLE yield of taxoids (10-deacetylbaccatin III – black diamonds, cephalomannine – squares and paclitaxel – triangles) from the twigs of *Taxus baccata* "Aurea" against the extraction temperature. PLE conditions: extraction time–15 min, extractant–methanol. For clarity of figure SD bars were omitted.

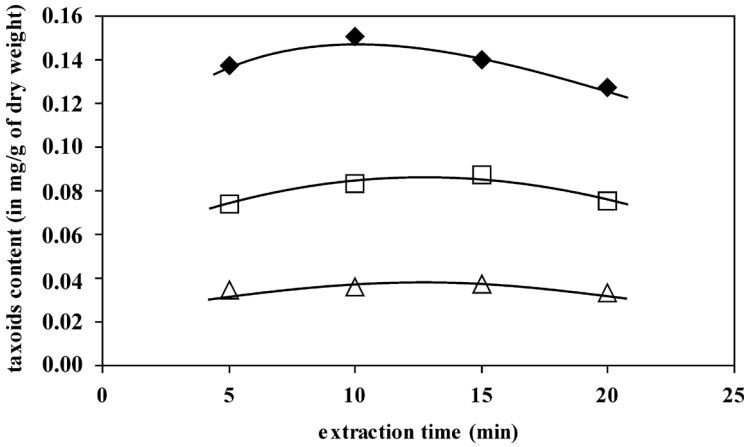


Figure 3. Dependence of the PLE taxoids yield against the extraction time. PLE conditions: extraction temperature 100°C, extractant–methanol. For symbols–see Figure 2. For clarity of figure SD bars were omitted.

lasted for 21 h) and multiple PLE extraction under the optimal conditions (4 extraction cycles of the same portion of plant material carried out at 130°C at 60 bars; each cycle lasted 15 min). Multiple PLE extraction is treated as exhaustive. The multiple (exhaustive) USAE and MASE were not performed due to the complexity of this type of procedure. Methanol was chosen as the extractant in all the experiments.

As results from the data corresponding to the single extraction process (see Table 2 part "A"), the highest yield of the examined taxoids is

Table 2. Yield of taxoids extracted from twigs of Taxus baccata with methanol by different extraction methods – mean values (n = 3) (±SD)

		Taxoids content (in mg/g of dry weight sample)		
Part	Extraction method	10-Deacetylbaccatin III	Cephalomannine	Paclitaxel
A	USAE (20°C)	0.1114 ($\pm 5.0 \cdot 10^{-3}$)	0.0795 ($\pm 5.4 \cdot 10^{-3}$)	0.0249 ($\pm 2.4 \cdot 10^{-3}$)
	USAE (60°C)	0.1229 ($\pm 5.0 \cdot 10^{-3}$)	0.0755 ($\pm 5.1 \cdot 10^{-3}$)	0.0246 ($\pm 2.4 \cdot 10^{-3}$)
	MASE closed	0.0341 ($\pm 2.1 \cdot 10^{-3}$)	0.0181 ($\pm 1.5 \cdot 10^{-3}$)	0.0008 ($\pm 1.0 \cdot 10^{-4}$)
	MASE open	0.1454 ($\pm 6.0 \cdot 10^{-3}$)	0.0429 ($\pm 3.0 \cdot 10^{-3}$)	0.0136 ($\pm 1.6 \cdot 10^{-3}$)
	PLE*	0.1470 ($\pm 4.2 \cdot 10^{-3}$)	0.0831 ($\pm 3.4 \cdot 10^{-3}$)	0.0360 ($\pm 1.4 \cdot 10^{-3}$)
B	Soxhlet	0.1766 ($\pm 1.4 \cdot 10^{-2}$)	0.0890 ($\pm 1.2 \cdot 10^{-2}$)	0.0104 ($\pm 1.9 \cdot 10^{-3}$)
	PLE**	0.2059 ($\pm 5.6 \cdot 10^{-3}$)	0.1175 ($\pm 4.8 \cdot 10^{-3}$)	0.0518 ($\pm 3.0 \cdot 10^{-3}$)

*One-cycle PLE – values repeated from Table 1.

**Exhaustive PLE.

obtained by PLE in default conditions. In relation to PLE, USAE reveals slightly smaller amounts of cephalomannine, and significantly smaller amounts of 10-deacetylbaccatin III and paclitaxel. It is worth noting that the increase of USAE temperature from 20 to 60°C does not affect the yield of the examined compounds. MASE performed in an open system gives the result comparable with PLE only for 10-deacetylbaccatin III. The yields of the two other compounds in open MASE are significantly lower. The lowest amounts of examined taxoids are revealed in yew twigs when MASE performed in a closed system is applied.

The comparison of the results from single PLE in default conditions with the results obtained using Soxhlet extraction may require a revision of the opinion on PLE superiority over the classical extraction method when applied for isolation of some taxoids from yew twigs. The application of the Soxhlet method allows for extracting greater amounts of 10-deacetylbaccatin III and cephalomannine than the single PLE method. However, it should be noted that the applied Soxhlet method is recognised as an exhaustive extraction procedure, in general. The results obtained with this method could not be directly compared with the results from the single PLE procedure (static extraction) but, with the results from the multiple PLE, which can be treated as exhaustive extraction. The yield of analytes obtained by the multiple PLE (four consecutive extractions of the same sample in optimal conditions) is presented in the last line of Table 2. It shows that the multiple PLE, in comparison to Soxhlet extraction, allows isolation of considerably greater amounts of 10-deacetylbaccatin III and cephalomannine and even a 5-times greater amount of paclitaxel. Moreover, the multiple PLE process is significantly shorter than the Soxhlet extraction procedure.

The dissimilarities in the yields of the examined compounds isolated by means of various extraction methods can result, not only from different extraction power of the applied techniques, but also from the compounds' degradation. In general, the degradation degree depends on the extraction temperature and time. An extremely short extraction time in the PLE process guarantees a low (if any) degradation degree of taxoids, in spite of the two times greater extraction temperature in PLE than in the Soxhlet apparatus – compare 4×15 min at 130°C *vs.* 21 hrs at 65°C. All this additionally emphasizes the importance of extraction time on the extraction process of thermolabile compounds, and further supports PLE superiority over the classical extraction method for taxoids from taxus baccata twigs.

CONCLUSIONS

The consideration of the presented results reveals differences in the extraction power of the methods used for the isolation of 10-deacetylbaccatin III,

cephalomannine, and paclitaxel from twigs of *Taxus baccata* L. One-cycle PLE in default conditions lasting 10 min. gives extraction yields comparable with those obtained by the 21-hour Soxhlet extraction for 10-deacetylbaccatin III and cephalomannine, while, the PLE yield for paclitaxel is considerably greater. MASE and USAE isolate smaller amounts of the compounds in comparison to one-cycle PLE and Soxhlet extraction, although USAE at ambient temperature and 60°C, and MASE in the open system give higher yields of paclitaxel than Soxhlet extraction. The greatest yields of the investigated taxoids are obtained by multiple PLE, which should be recommended as the best sample preparation method for taxoids analysis in yew twigs. In the optimal temperature and time of the PLE process, taxoids degradation is not noticeable. It needs to be stressed that solvents used for preliminary extraction of ballast substances (which are not considered as taxoids extractants in classical extraction) extract small amounts of taxoids in PLE conditions.

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