Determination of 10-deacetylbaccatine III in *Taxus baccata* needles by micellar electrokinetic chromatography

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Summary

The optimization of the analytical buffer composition for micellar electrokinetic chromatography in order to make simple separations of 10-deacetylbaccatine III from sample matrices (other taxanes and other compounds) and to determine its concentration in the samples was performed. The samples were prepared from crude *Taxus baccata* L. needle extracts. The best results were obtained with the buffer containing 25 mmol/dm^3 Tris-HCl (pH 9.0), 40 mmol/dm^3 sodium dodecyl sulphate and 15% acetonitrile for a wavelength 230 nm (Diode Array Detector). Finally determination of 10-DAB III concentration in *T. baccata* needles collected from four different populations of Southern Poland was performed (mean concentration respectively: Cisy w Nowej Wsi – 0.135 mg/g d.w., Cisowa Góra – 0.185 mg/g d.w., Zadni Gaj – 0.143 mg/g d.w., Cisy nad Liswartą – 0.150 mg/g d.w.). There were no significant differences in the mean concentration of 10-deacetylbaccatine III among samples from different populations.

Key words: *10-deacetylbaccatine III; 10-DAB III; Taxus baccata; common yew, MEKC; micellar electrokinetic chromatography; taxanes*

INTRODUCTION

Paclitaxel is a diterpene amide derived from *Taxus* spp. known for its anticancer properties. It is an effective drug against an ovary [1] and breast [2] cancer. The most limiting factor for wide paclitaxel application in medicine is material availability. Extraction from wild living trees is extremely limited because of a large number of trees necessary for collecting enough amount of the active compound. Data collected in 1992 showed an annual request of 130 kg, meaning a necessity of collecting 1600 000 pounds of yew bark per year. As a consequence, there is a necessity to look for alternative sources of paclitaxel. One of them is hemisynthesis; it is a process of joining the side chains to natural paclitaxel precursors like baccatine III, or 10-deacetylbaccatine III [3, 4]. The second kind of paclitaxel (in meaning of its origin) in medicine is docetaxel – a hemisynthetic derivative of 10-deacetylbaccatine III (10-DAB III). This compound is one of several taxanes isolated from yew, particularly large concentration was found in the needles of the common yew (*Taxus baccata* L.) [5-7]. The taxanes concentration (also 10-DAB III) in yew tissues is very differentiated, and is related to many factors. These factors can be genetic (differences between species, origins, and specific trees), epigenetic (time of material collecting, kind of tissue) or environmental (an influence of soil type, climate, water).

The influences of all factor and relationships between them are not well known yet and need more research. It is important to find a simple and fast but precise method of taxanes analysis. The most frequently used analytical method is high performance liquid chromatography (HPLC) by separating in reverse phases on C18, phenyl, cyan or pentaflurophenyl columns [8-21]. Most researchers prefer detection by UV-Vis absorbance detectors.

Another method is micellar electrokinetic chromatography (MEKC). Three articles focused on this method of taxanes analysis were published so far: Chan et al. [22] used MEKC for analysis of 7 taxanes, subsequently, they modified it to the analysis of bark and needles extracts [23]. Shao and Locke [24] performed parallel separation of 14 taxanes.

Micellar electrokinetic chromatography has some advantages according to HPLC. Analyses are shorter and make possible separation of many compounds in the same run. Unfortunately, up to now there was only one developed method for determining 10-DAB III dissolved in pure taxanes solution. When a sample is made from a plant, there are some problems with organic impurities originating from the sample; this compound may interfere with taxanes. Works of Chan et al. [22, 23] only make it possible to determine a paclitaxel, cephalomannine and baccatine III in yew bark and needle extracts.

The aim of our study was to develop easy, fast and exact method to determine 10-DAB III concentration in the needles by MEKC for further research on 10-DAB III concentration differences in the wild growing yew populations.

MATERIALS AND METHODS

Reagents and standards

10-Deacetylbaccatine III was obtained from ICN Biomedicals and dissolved in methanol as a stock solution (5 mg/cm^3). Before analysis it was diluted with the running buffer (1:1 v/v) and then filtered. Other chemicals were reagent grade and purchased from Aldrich-Sigma. Double-distiled water was used in all experiments.

MEKC conditions

MEKC separations were performed with a Hewlett Packard HPCE3D capillary electrophoresis system equipped with a spectrophotometric UV-Vis diode array detector. 25 μm (i.d.) x 64.5 cm (total capillary length) fused-silica capillaries (CE Ext. Light Path Cap. from Agilent Technologies) were used for all experiments. The software used for instrument control, data collection and analysis was ChemStation 7.5 (Hewlett Packard).

The voltage applied for separation was 25 kV and the cassette temperature 30ºC. Samples were introduced by applying electrokinetic injection (30kV for 1s). Every day before the start of analysis the capillary was conditioned by flushing: water for 5 min., 1 mol/dm^3 NaOH for 60 min., water for 10 min., 0.2 mol/dm^3 NaOH for 30 min. Before each run the capillary was flushed with: water for 1 min., 0.2 mol/dm^3 NaOH for 2 min., water for 2 min. and running buffer for 5 min. The running buffer was consisted of: 25 mmol/dm^3 Tris-HCl (operational pH 8.0; 8.5 or 9.0), SDS (40 mmol/dm^3, 45 mmol/dm^3 or 50 mmol/dm^3) and ACN (15%, 20%, 25%, 30% or 35%(v/v)).

Sample preparation

Needles of *T. baccata* individuals were collected in July from four different populations of Southern Poland (Cisy w Nowej Wsi, Cisowa Góra, Zadni Gaj and Cisy nad Liswartą) from plants at ca. 150 cm from soil level and lyophilized (lyophilizer Labconco, USA) for about 96 hours (to reach a stable dry weight). Lyophilized needles were ground in the mortar and the obtained powder was transferred to closed tubes. For 10-DAB III analysis the powder from individuals growing in the same reservation was combined.

Using Ketchum et al. method [20] with some modifications, the 10-DAB III was extracted. 1.0 g of prepared powder was transferred to polypropylene tubes and extracted ultrasonically for 30 min. in 10 cm^3 of 40% (v/v) ethanol. Afterwards, the solution was centrifuged for 10 min. at a speed of 4000 rpm (about 2000 g). Supernatant was transferred to new tubes and the residue was extracted again. This step was repeated three times. Afterwards, the residue was discarded. The obtained combined extract was evaporated to dryness under the vacuum (Labconco-USA) at 35ºC. The residue was extracted in 600 μL dichloromethane (DCM),

centrifuged for 10 min. at the speed of 4000 rpm (about 2000 g) and supernatant was transferred to the Eppendorf tube. That was repeated twice, subsequently, the extract was combined and centrifuged for 20 min. at the speed of 14000 rpm (about 20000 g) to remove residue. The supernatant was transferred to a new Eppendorf tube and partitioned three times with deionized water (600 μL). Every time the sample was centrifuged for 15 s at the speed of 5000 rpm (about 7000 g). The water layer was discarded and the dichloromethane layer was dried under the vacuum. Next, the residue was dissolved in 20 μL of 100% methanol and shaken by a vortex. Subsequently, 180 μL of MEKC running buffer was added to obtain 200 μL samples with 10% (v/v) of methanol. For each analysis 100 μL of sample was used and the residue was frozen at –80°C.

Method validation

Identification of 10-DAB III was performed by migration time, UV spectrum in comparison to standard and by spiking with standard. Quantitation was by peak area relative to an external standard (10-DAB III). The calibration curve for standards at the expected concentration range (5-200 μg/sample) (five-point calibration) were linear with the correlation coefficient 0.998. Electropherograms were plotted at 230 nm. The recovery tests for the method were carried out by adding the standards to the raw materials. Three initial concentrations were prepared to estimate the recovery. Afterwards, the materials mixed according to standards were prepared using ultrasonic extraction and the samples were then analyzed by MEKC according to the procedure described above.

For each calibration level the precision of the method was calculated as %RSD: 3.83 for 200 μg/cm^3, 4.84 for 100 μg/cm^3, 3.19 for 50 μg/cm^3, 3.93 for 25 μg/cm^3 and 6.29 for 5 μg/cm^3.

RESULTS AND DISCUSSION

Preliminary experiments showed that the existing methods developed by Chan et al. [22, 23] as well as Shao and Locke [24] can not be used to determine 10-DAB III concentration in plant material due to impurity for which the retention time is very similar to 10-DAB III. Preliminary purification of crude extract by hexane according to Burkhin et al. [25] and Vanek et al. [26] and by mixture of dichloromethane:water (1:1) [27] did not give a positive effect. It was necessary to optimize the composition of the running buffer and MEKC conditions to separate 10-DAB III and impurity.

The time of a single run and resolution of method can be modified by a few factors: pH of the running buffer, concentration of compound that creates micelles (SDS) and addition of an organic modifier. Controlling these factors makes it possible to create proper analysis conditions to quickly separate 10-DAB III from other taxanes and impurities.

The used pH values of the running buffer (fig. 1) showed that when pH value increased from 8.0 to 9.0 the time of a single run was shorter and the resolution was a little worse. It is caused with strong pH dependence on the charge of the capillary wall. Its increase makes electro osmotic flow (EOF) faster. Recently, Chan et al. [22, 23] used the running buffer with pH value about 8.5 and Shao and Locke – pH 9.0 [24]. In order to shorten the analysis time, we choose pH 9.0 for the next step.

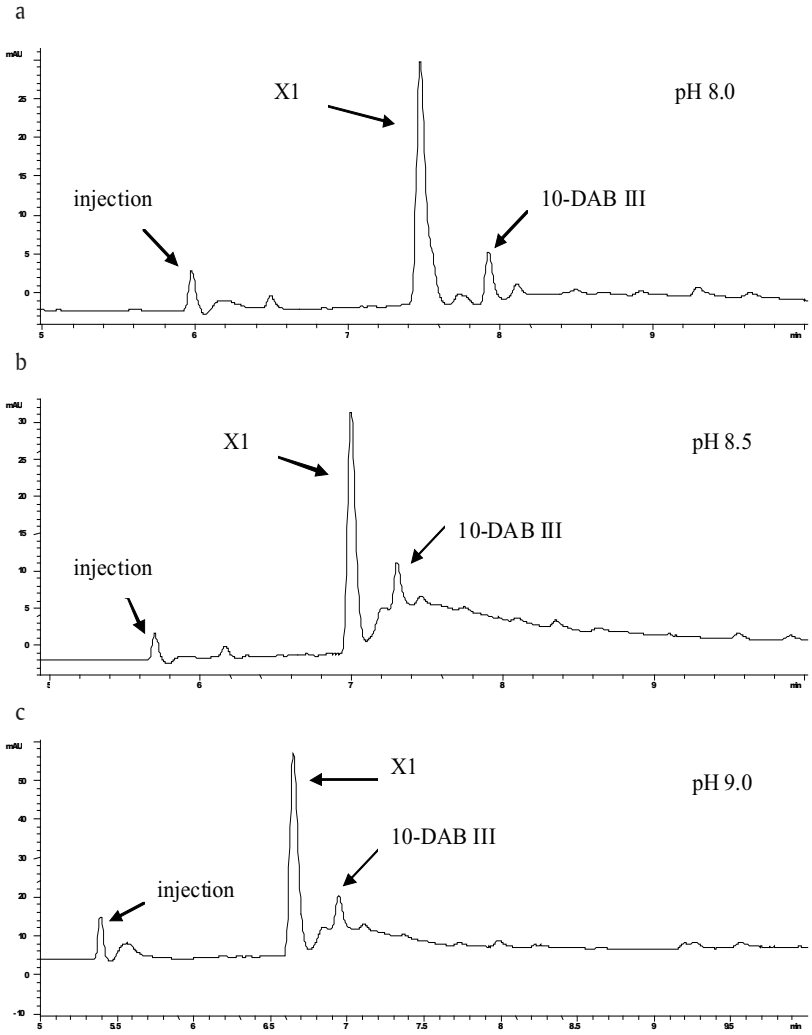

Figure 1. Electropherogram of *Taxus baccata* needles extract. MEKC conditions: 25 μm (i.d.) x 64.5 cm (the total capillary length) fused-silica capillary, background electrolyte, 25 mmol/dm^3 Tris-HCl (operational pH 8.0, 8.5 and 9.0), 40 mmol/dm^3 SDS and 20% ACN (v/v), applied voltage, 25 kV, UV detection at 230 nm, electro kinetic injection for 1 s.
10-DAB III – 10-deacetylbaccatine III
X1 – main impurity

Next factor that affects retention time and resolution of analyzed compounds in MEKC method is the concentration of surfactant (fig. 2). When the concentration is too low for micelles creation (CMC – critical micelle concentration), neutral molecules are running with EOF speed. Because SDS makes micelles with a negative charge, non-polar compounds move slower than EOF. That phenomena are analogical to reverse-phase HPLC (RP-HPLC) separation, so micelles are called the pseudostationary phase. When SDS concentration rises, the number of created micelles rises too. As a result, a single run is longer. In our research the best resolutions were obtained, when the SDS concentration was about 50 mmol/dm^3. This is according to Shao and Locke [24] whereas Chan et al. [22, 23] used SDS in concentration of 50 mmol/dm^3. But to short the analysis time we choose SDS concentration 40 mmol/dm^3 for the next step.

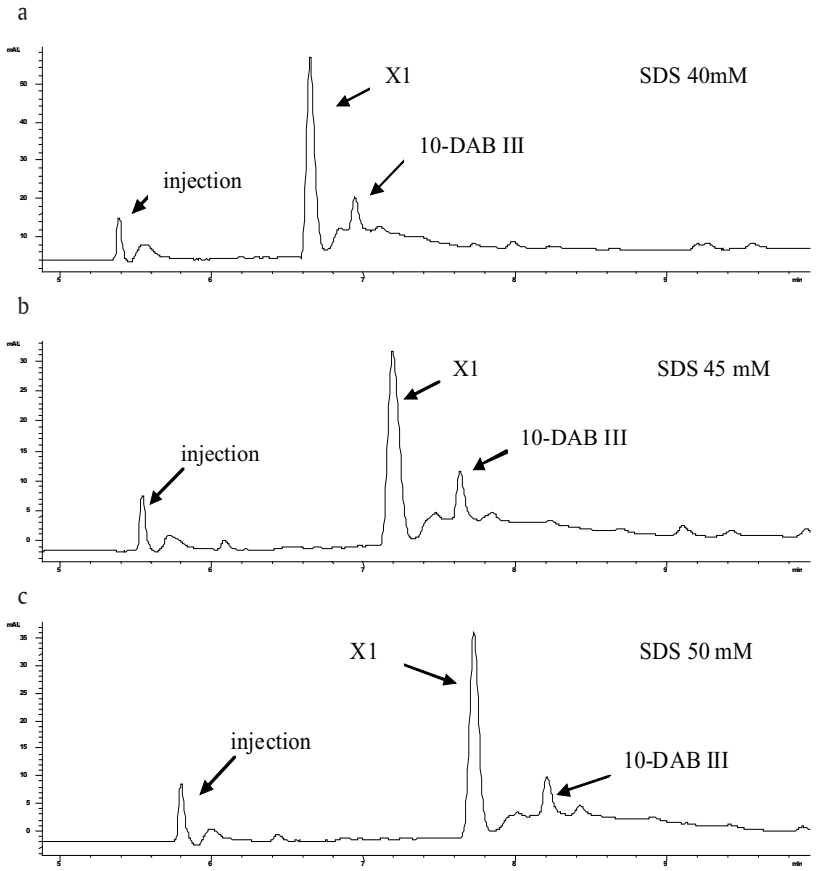

Figure 2. Electropherogram of *Taxus baccata* needles extract. MEKC conditions: 25 μm (i.d.) x 64.5 cm (the total capillary length) fused-silica capillary; background electrolyte, 25 mmol/dm^3 Tris-HCl, operational pH 9.0, different SDS concentration (40 mmol/dm^3, 45 mmol/dm^3 and 50 mmol/dm^3) and 20% ACN (v/v); applied voltage, 25 kV; UV detection at 230 nm; electro kinetic injection for 1 s.
10-DAB III – 10-deacetylbaccatine III
X1 – main impurity

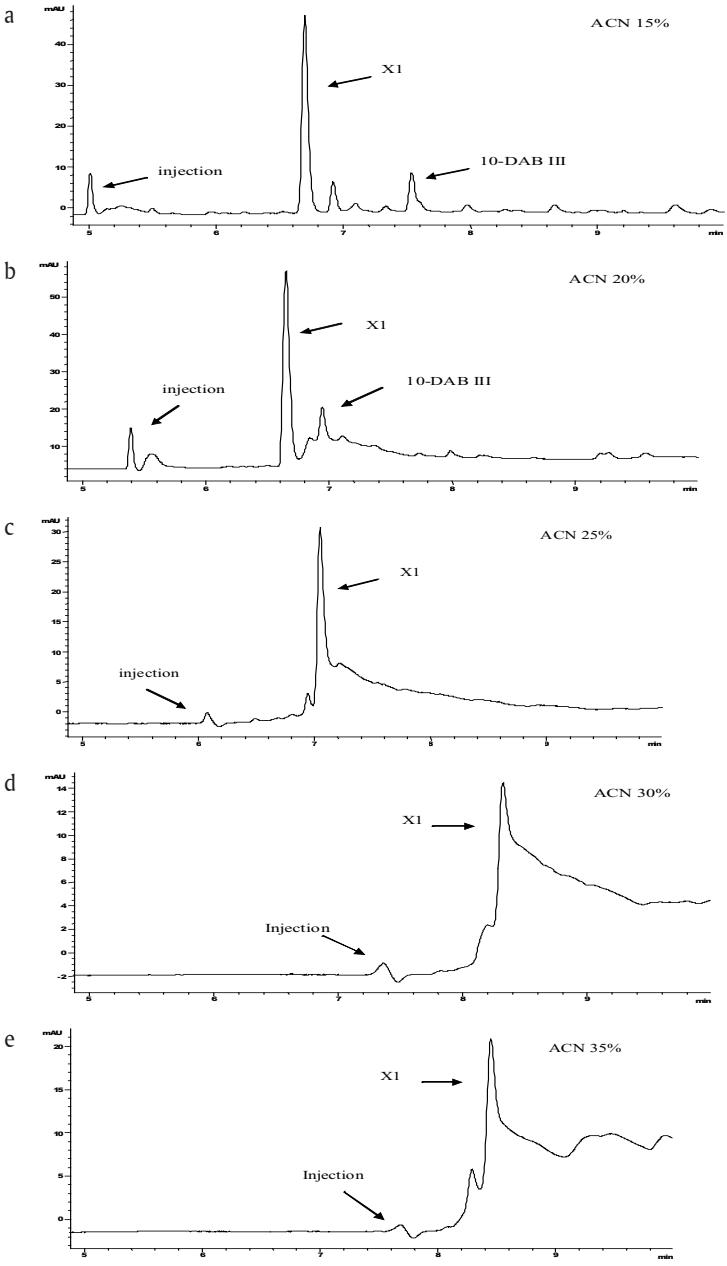

Figure 3. Electropherogram of *Taxus baccata* needles extract. MEKC conditions: 25 μm (i.d.) x 64.5 cm (the total capillary length) fused-silica capillary, background electrolyte, 25 mmol/dm^3 Tris-HCl, operational pH 9.0, SDS 40mmol/dm^3 and different acetonitrile concentration (15%, 20%, 25%, 30% and 35%(v/v)); applied voltage, 25 kV; UV detection at 230 nm; electro kinetic injection for 1 s.
10-DAB III – 10-deacetylbaccatine III
X1 – main impurity

The antagonistic effect of SDS has an organic modifier – acetonitrile (ACN). Addition of acetonitrile to running buffer increases both hydrophobicity of buffer and buffer affinity to more hydrophobic compounds so their retention time is shorter. Absence of acetonitrile makes separation of taxanes impossible using MEKC method because taxanes are hydrophobic compounds and their solubility in polar solvents is low. Addition of an organic modifier to the electrolyte increases solubility of taxanes in the buffer [24]. The oversized concentration of ACN creates changes in micelles size and structure and can lead to disintegration. This phenomenon was reported by Ahuja and Foley [28]; when the pH of the buffer was 7.0, SDS concentration was 50 mmol/dm^3 and ACN 50%. The form of surfactant molecules is not completely clear in the presence of high concentrations of organic modifier [24]. Although, using such a high concentrations of modifier makes possible analyses which are impossible when ACN is absent in the buffer. In our research best results were obtained when the concentration of ACN was 15% (fig. 3a). Increases in ACN concentration led method to lower separation ability and at a concentration of 25%, peaks were overlaid. According to Shao and Locke [24], best results could be obtained when acetonitrile concentration was 30%, but in our case that value was too high. The reason for this discrepancy was probably that Shao and Locke [24] made separation of pure standards of 14 taxanes. The problem appears when a sample originates from plants and is cleared only partially. Such analyses were performed by Chan et al. [23]. They found 25% concentration of ACN to be optimal. Unfortunately, they did not separate 10-DAB III so the results could not become compared.

Recovery was determined for the MEKC method. The average recovery rate for 10-DAB III was near 88%. That is a result similar to that of Li et al. [29] with ultrasonic extraction (about 94%) or by Mroczek and Glowniak [30] with SPE extraction step (about 82-98%)

Content of 10-DAB III in four *T. baccata* populations

The method was developed and applied to identify and determine of 10-DAB III in *T. baccata* needles collected from four different populations of Southern Poland in this assay. The calculated contents of the 10-DAB III are given in Table 1. Observed variations in concentrations of 10-DAB III, in the ranges of 0.055–0.343 mg/g, showed that the average contents of 10-DAB III were highest in Cisowa Góra, but analyses of test samples of four Southern Poland populations have shown that there were no significant differences in the mean concentration of 10-DAB III among analyzed populations. The contents of taxoids in the plants growing in one area may vary to a great extent from those growing in another area [31]. Yields of all taxoids show considerable variation and would appear to be dependent on many factors, e.g. *T. baccata* variety or cultivar, location, weather during the year, soil type and nutrition, etc. There is also no single month of the year which is universally ideal for harvesting yew shoots containing maximum amounts of all taxoids [32]. Ours research shows that it is impossible to select a population with highest level of 10-DAB III and analysis should be rather performed on a single individuals.

Table 1.

Concentration of 10-DAB III in *Taxus baccata* needles collected from four different populations of Southern Poland

reservation	concentration of 10-DAB III [μg/g d. w.]			standard deviation
	mean*	min	max	
Cisy w Nowej Wsi	135.07 a	67.45	298.87	65.56
Cisowa Góra	185.14 a	62.71	275.14	80.98
Zadni Gaj	143.32 a	54.99	342.78	86.55
Cisy nad Liswartą	150.09 a	57.36	253.18	61.06

10-DAB III = 10-deacetylbaccatine III

*The same letter indicates the absence of significant differences (α=0.05) among the concentration by the $\chi 2$ test.

CONCLUSIONS

Our research showed the possibility of using the MEKC method to determine 10-DAB III in the extract obtained from *T. baccata* needles. The used extraction method and optimized MEKC conditions (buffer containing 25 mmol/dm^3 Tris-HCl (pH 9.0), 40 mmol/dm^3 sodium dodecyl sulphate and 15% acetonitrile) allowed simple separation and determination of 10-DAB III from other taxanes and impurities within 8 min of single run and 10 min of capillary flushing (fig. 3a). In comparison with the fast and simple HPLC method described by Mroczek and Glowniak [30], MEKC needs lower amount of solvents so it is more economical and friendly to the environment, but extraction method could be improve. 10-DAB III content in wild growing trees determined by our method is comparable with results obtained by other authors.

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OZNACZANIE ZAWARTOŚCI 10-DEACETYLOBAKATYNY III W IGŁACH CISA POSPOLITEGO ZA POMOCĄ MICELARNEJ CHROMATOGRAFII ELEKTROKINETYCZNEJ

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S t r e s z c z e n i e

W prezentowanej pracy przeprowadzono optymalizację składu buforu umożliwiającego proste i szybkie rozdzielenie 10-deacetylobakatyny III od innych składników oraz oznaczenie jej ilości w analizowanych próbkach igieł cisa pospolitego za pomocą micelarnej chromatografii elektrokinetycznej. Najlepsze rezultaty uzyskano stosując bufor rozdziałowy zawierający: 25 mmol/dm^3 Tris-HCl (pH 9,0), 40 mmol/dm^3 dodecylosiarczanu sodu (SDS) i 15% acetonitrylu oraz dokonując odczytu przy długości fali 230 nm (Diode Array Detector). W celu sprawdzenia możliwości wykorzystania opracowanej metody przeprowadzono analizę zawartości 10-DAB III w igłach osobników rosnących w 4 rezerwatach w Polsce południowej. Średnie stężenie badanego związku wynosiło odpowiednio: Cisy w Nowej Wsi – 0,135 mg, Cisowa Góra – 0,185 mg, Zadni Gaj – 0,143 mg, Cisy nad Liswartą – 0,150 mg na gram suchej masy. Nie stwierdzono istotnych różnic między średnimi wartościami uzyskanymi dla poszczególnych populacji.

Słowa kluczowe: *10-deacetylobakatyna III, 10-DAB III, Taxus baccata, cis pospolity, MEKC, micelarna chromatografia elektrokinetyczna, taksany*