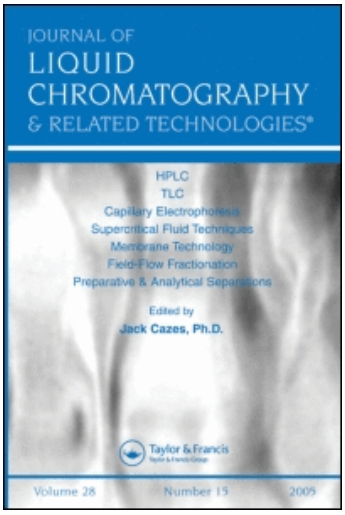


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# Seasons Study of Four Important Taxanes and Purification of 10-Deacetylbaccatin III from the Needles of *Taxus baccata* L. by Two-Dimensional Liquid Chromatography

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**Abstract:** Simultaneous seasonal variation of four analogous taxanes, namely taxol (paclitaxel), cephalomannine, 10-deacetyl baccatin III (10-DAB), and baccatin III, from the needle of Iranian yew (*Taxus baccata* L.) has been monitored. Powdered samples of needles have been subjected to microwave-assisted extraction (MAE) and the extracted sample has been purified by liquid-liquid extraction (LLE) and analysis by reverse phase high performance liquid chromatography (RP-HPLC). These samples were identified by the LC-MS/MS system. The results indicated that taxane concentrations depend on seasonal and location. Analyses of these samples showed that 10-DAB has higher concentration than other taxanes and its highest concentration has been obtained in August. Thus, a heart cutting two-dimensional liquid chromatography was used for obtaining better resolution of taxanes. The first column in the analytical scale chromatography was a Eurospher-100 $C_8$ and the second was Nucleosil-100 $C_{18}$ which has been eluted at a flow rate of 1 mL $min^{-1}$ with 70:30 v/v and 50:50 v/v water/acetonitrile, respectively. Optimized conditions in analytical chromatography

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have been extended to semipreparative scale in which the first column was a Eurospher-100 $C_8$ and the second was a Eurospher-100 $C_{18}$, both eluted with 70:30 v/v water/acetonitrile at a flow rate of 10 mL $min^{-1}$. Detection has been carried out at a wavelength of 227 nm throughout the analysis in both scales. Final purity of the fraction containing 10-DAB, collected from the semipreparative column, is 82% after the first semipreparative run. Results have been confirmed by spiking of the standard 10-DAB sample and analysis by nuclear magnetic resonance (NMR).

**Keywords:** 10-Deacetyl baccatin III semipreparative, Seasonal variation, Semipreparative chromatography, Taxol, *Taxus baccata* L., Two-dimensional liquid chromatography



## INTRODUCTION

There are some independently seasonal variation studies in the literature[1–5] showing a large variation in taxanes content between the different species, cultivars, tissues, and harvesting times. The content of paclitaxel (Taxol) and 10-deacetylbaccatin III (10-DAB) in different samples varied from 0 to 500 μg/g and 0 to 4800 μg/g dried needles, respectively. The concentration of 10-deacetylpaclitaxel, cephalomannine, and baccatin III were reported in the range of 0 to 500 μg/g dried needles.[6]

One of the most important compounds of natural origin, which has been employed in the pharmaceutical industry is 10-DAB. It belongs to taxoids extracted from different parts of yew trees (Family Taxaceae). 10-DAB has been used as precursor in the semi-synthesis of taxol during the last two decades[7–9] and has played an important role in its large scale production.[10] Also, 10-DAB has been used in the semi-synthesis of Taxotere,[7] showing anti-tumor activity two times more than that of taxol, as well as cephalomannine, another analogue of taxol showing anti-tumor activity.[11]

There were some reports indicating that 10-DAB content in the needles of *T. baccata* L. is comparable to that of Taxol in the bark of *T. brevifolia* Nutt.[7,12] Therefore, it could be extracted from the needles to be used in semi-synthesis instead of direct extraction of Taxol from the bark which kills the tree. Different extraction and separation methods have been introduced, each of which has its advantages and disadvantages.[13,14] Besides, the presence of many taxoids with a high structural similarity is an additional cause of difficulty in the separation of this class of natural compounds. Several purification stages are usually needed when dealing with these samples.

It is evident that microwave-assisted extraction (MAE) can be employed as a selective method, since the selection of proper irradiation

frequency results in extraction of certain compounds.[15,16] In our previous works, we showed that MAE can be used for preparation of purified samples of Taxol for analytical and preparative HPLC.[17,18]

In the situation that the purified sample is still complex and compounds of similar structure are co-eluted in the column, two-dimensional liquid chromatography (column-switching technique) can be used. Lots of unwanted impurities and co-extracted compounds are eliminated by switching the valve between columns at proper intervals. Bovanová and Brandšteterová[19] comprehensively reviewed column switching techniques and their applications in the analyses of food samples.

Along with our previous study for isolation and purification of taxol, we suggest a method for isolation and purification of the taxol precursor, namely, 10-DAB III from the needle of the Iranian yew (*Taxus baccata* L.).[17,18,20] In order to maximize the production of taxanes it will be necessary to understand the month or season in which the plants contain the highest level of taxanes. The main objective of the present study is to evaluate the effect of the harvest season on taxane contents, namely taxol, 10-DAB III, baccatin III, and cephalomannine. Microwave energy was used for preliminary extraction, which was followed by a cleanup stage for extraction of taxoids. Further separation of 10-DAB III was carried out by two-dimensional reversed phase high performance liquid chromatography (RP-HPLC). The obtained conditions in the analytical scale were extended to semipreparative chromatography.



## EXPERIMENTAL

### Apparatus

For commercial extraction, a National (Matsushita Electric Industrial Co. Japan) domestic microwave oven was used. Its power could be used at six different levels varying from 10 to 100% of the total power. Analytical HPLC consisted of a Knauer WellChrom (Germany) system with HPLC pump K-1001 and diode array detector K-2800. The first column was a Eurospher-100 $C_8$ (5 μm, 250 × 4 mm, Knauer, Germany) and the second was Nucleosil-100 $C_{18}$ (5 μm, 250 × 4 mm, Sorbtech, USA). A six-port two-position switching valve (Coati, California, USA) was employed for switching between columns. The semipreparative HPLC system included a WellChrom preparative pump K-1800, UV detector K-2501, and a Büchi fraction collector B-684 (Flawil, Switzerland). The first column in the prep HPLC was a Eurospher-100 $C_8$ (5 μm, 120 × 16 mm) and the second was a Eurospher-100 $C_{18}$ (5 μm, 120 × 16 mm). A 300 MHz NMR system from Bruker Company (Ettlingen, Germany) was used for analysis of 10-DAB III. Finally, the LC-MS/MS system included, a micro

column C18 (5 μm, 150 × 1 mm) and a ThermoFisher Scientific (Bremen, Germany) ion trap mass spectrometer (model LCQ, mass range m/z 10-2000). Instrument control, data acquisition, and processing were conducted by the Xcalibur software. Typical negative ESI-MS conditions were: capillary voltage −2.0 kV and skimmer cone voltage −20 V. The collision energy for MS/MS was 25 eV.

## Chemicals

Standards of 10-Dacetyl baccatin III, Bacctin III, Paclitaxel (Taxol) and Cephalomannine were purchased from Sigma (Mo, St Louis, USA). HPLC grade acetonitrile was purchased from Caledon Laboratories Ltd. (Ont., Canada). Methanol, dichloromethane, and n-hexane all more than 99.5% were obtained from Merck (Darmstadt, Germany). HPLC grade water was used throughout the analysis.



## Plant Material

Fresh intact clippings of *Taxus baccata* L. were obtained from the Botanical garden of the University of Tehran, Iran, in August 2006. After being dried for 5 days at room temperature, the needles were separated from the stems and both were ground to a particle size of 1–3 mm and stored in a refrigerator until analysis.

## Microwave-Assisted Extraction

A 1.5 g aliquot of ground needles was transferred into a test tube with a screw cap and placed in a container, which was transparent to the microwave energy. The collection was placed in the microwave oven and extracted with 90% aqueous methanol for 5 min at the lowest power. The conditions for MAE were adjusted with respect to our previous study on determination of taxoids in which the needles were extracted by MAE and the condition was optimized via experimental design.[17] Solvent composition, solvent to sample ratio, instrument power, and irradiation time were factors, which had been optimized.

## Liquid Liquid Extraction (LLE)

LLE was carried out according to Glowniak and Mroczek.[21] An aliquot of 10 mL of methanol extract was mixed with 10 mL water and extracted with *n*-hexane (2 × 10 mL). The hexane extract, which contained lipids,

waxes, and pigments, was discarded and the aqueous layer was extracted with dichloromethane ($5 \times 10$ mL). In this stage, polar compounds like carbohydrates remained in aqueous phase and taxoids were extracted in the organic phase. Dichloromethane extracts were combined, evaporated under reduced pressure, and dissolved in 50 mL acetonitrile.

## HPLC Method Development

Chromatographic analyses for monthly determination of four studied taxanes, according to their polarities were different. For determining 10-DAB and baccatin III, the perfectsil C8 column with water/acetonitrile (70:30 v/v) at flow rate of 1 mL $min^{-1}$ and detection wavelength of 227 nm was used, while for paclitaxel and cephalomannine, the same flow rate and detection wavelength was used, but the Eurospher-100 C18 column water/acetonitrile (51:49 v/v) was used. For semipreparative Separation of 10-deacetybaccatin III, the analytical scale was carried out on a two-dimensional system consisting of two HPLC columns, which were linked by a six-port two-position switching valve (Scheme 1). Several mobile phases such as methanol, acetonitrile, tetrahydrofuran, and different ratios of these organic solvents with water were examined. Also, several reversed phase columns (C8, C18, and phenyl stationary phases) were investigated. The best conditions were as follows: first column (Eurospher-100 C8) was eluted with water/acetonitrile (70:30) at a flow rate of 1 mL $min^{-1}$ and detection was carried out at a wavelength of 227 nm throughout the analysis. Switching time was determined by injection of a 10-DAB standard onto this column before performing the 2D system. Mobile phase for the second column was water/acetonitrile (50:50 v/v) pumped at a flow rate of 1 mL $min^{-1}$ and detection wavelength was 227 nm. There were two positions for the 2D system used in the present experiment, load position and injection position. In load position (Scheme 1a), the eluant from the first column was transferred to the waste, while in the injection position (Scheme 1b) the eluant from the first column was transferred onto the second column. At each run, the extract was injected onto the first column and the valve was switched to injection position in the beginning of the retention time of the standard, which had previously been determined. Then it returned back to load position at the end of the standard peak.

Semipreparative separation was carried out on a 2D system the same as the analytical scale, but with a considerable difference in the flow rate. Flow rate was calculated from the equation:

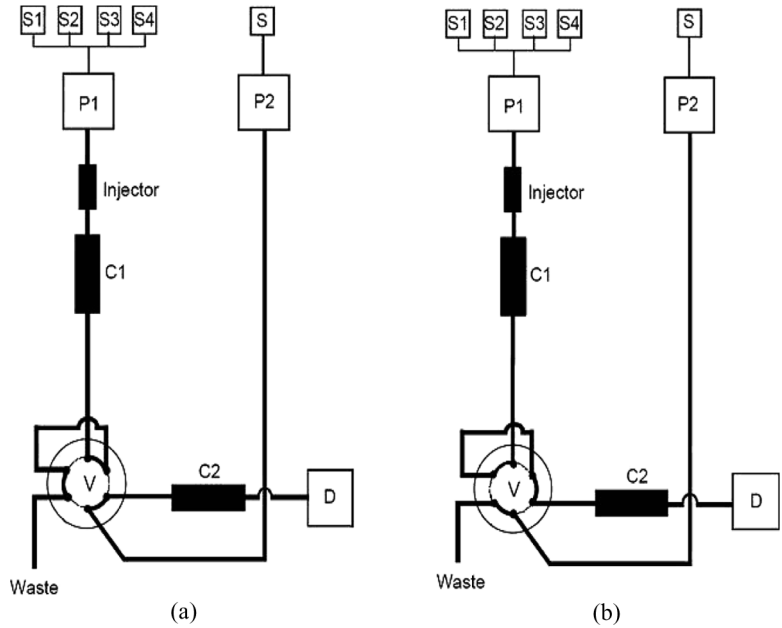
$$\frac{V_1}{V_2} = \frac{r_1^2}{r_2^2} \tag{1}$$

where $V_1$ and $V_2$ are flow rates of the mobile phase and $r_1$ and $r_2$ are inner diameters of the columns in analytical and preparative scale, respectively.[22] The purified sample was injected onto the first column and the fraction containing 10-DAB was transferred onto the second column by switching the valve to injection position during the beginning and the end of the 10-DAB standard peak.

## RESULTS AND DISCUSSION

Although MAE prepares simpler taxane samples than LLE from the needles of *Taxus baccata*,[17] the injection of the sample after MAE and then LLE onto the first column in analytical HPLC did not show complete resolution (Figure 1). In spite of using a polar mobile phase to increase the interactions of taxoids with the stationary phase, overlapping was observed in the separated peaks. On the other hand, gradient elution could not improve these results.[17] The study of UV-VIS spectra of these signals by photodiode array detection in HPLC indicated that a majority






***Scheme 1.*** Schematic diagram of two-dimensional chromatographic system. P1: High pressure quaternary solvent delivery system, P2: Feeding pump, V: 6-port 2-position valve, C1: Column in first separation dimension, C2: Column in second separation dimension, D: Detector, (a): system configuration for load position, (b): System configuration for inject position (elution of fraction from C1 onto C2).

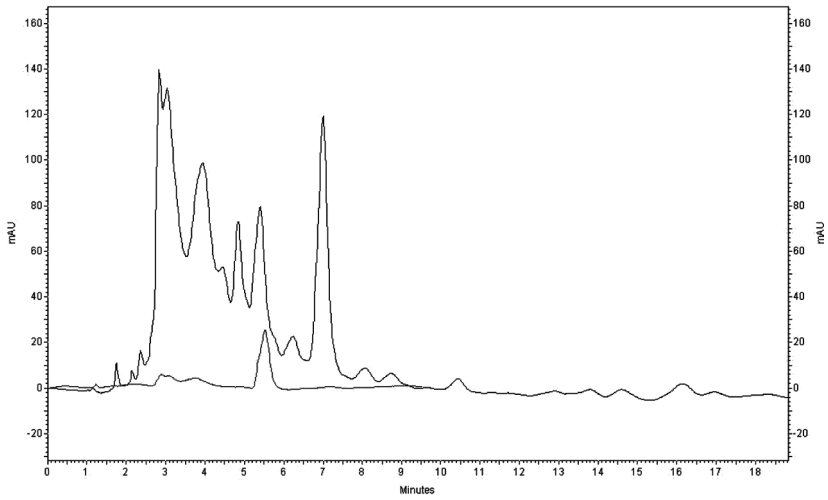



***Figure 1.*** Chromatogram illustrating the separation of the extract after LLE as cleanup step of standard taxol and extracted sample. Separation conditions involved a mobile phase composition of water/acetonitrile (70:30). The flow rate was 1.0 mL $min^{-1}$, with an injection volume of 20 μL. Detected at 227 nm.



of these compounds belong to taxoids. The overlapping pattern of the profile can be attributed to the chemical structural similarity of this class of natural compounds. To obtain better identification results, a mass spectrometry detector was used.

## MS Analyses of Taxanes

For identification, liquid chromatography-electrospray-mass spectrometry/mass spectrometry (LC-ESI-MS/MS) analyses were carried out for taxanes profiling of purified liquid-liquid extracts of the needles of *Taxaus bacata* L. Several reports have been cited in the literature demonstrating the applicability of MS and MS/MS coupled with liquid chromatography in the analyses of taxanes.[23] In this work, $[M + NH_4]^+$, $[M + H]^+$, and $[M + Na]^+$ ions of these taxanes were determined. The interpretation of fragmentation and MS/MS results were performed according to Talebi et al.[17] and Kerns et al.[24] reports. According to Figure 2, the observed mass at 871 Dalton corresponds to $[M + NH_4]^+$ ion of taxol and the observed mass of 854 Dalton is related to $[M + H]^+$ ion taxol. For the other peaks at mass of 832, 587, and 545, Daltons corresponds to the $[M + H]^+$ ions of cephalomannine, baccatin III, and 10-DAB III, respectively. These results are confirmed by spikes of standards of these taxanes to extracted samples.

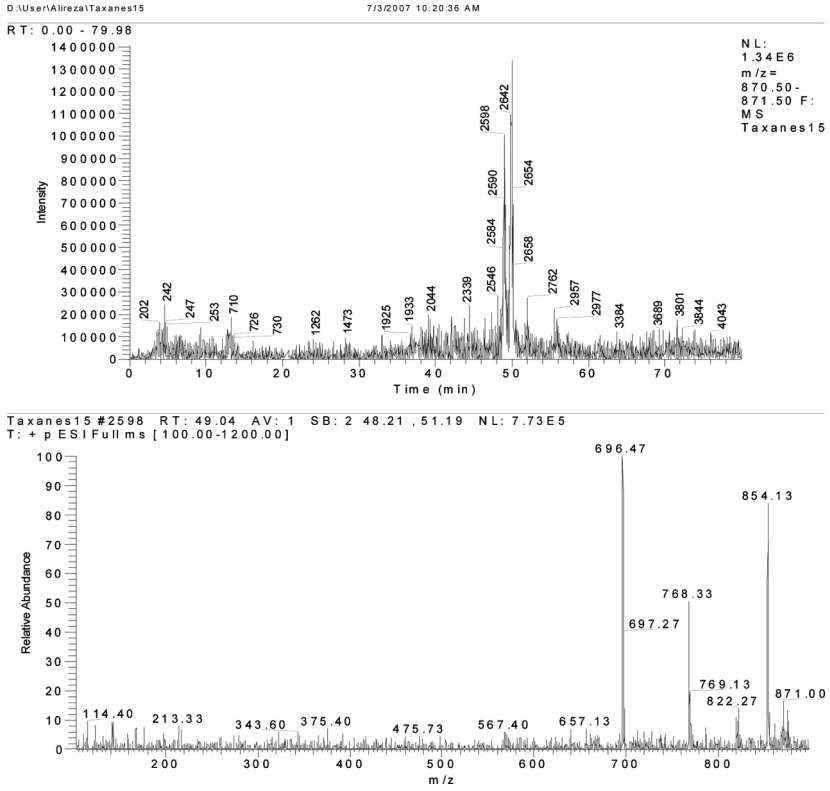



*Figure 2.* Selected molecular ion (871 m/z) in TIC (up) and mass spectrum of Taxol (down).

## Seasonal (Monthly) Variation Study of *Taxus baccata* L.

In this study, regular collection of needle samples started in August 2006 and analytical determination of taxanes from the needle of *Taxus baccata* L. indicates that the taxane content depends significantly on the type of plant and the harvest month of the year. It appears that choice of the right time for harvesting plant material can lead to the availability of taxane in highest yields (Figure 3). The concentration of 10-DAB was achieved higher than other studied taxanes and its highest concentration was obtained in August.

## 2D-Analytical HPLC

For further separation, two-dimensional RP-HPLC was used. The obtained conditions in the analytical scale were extended to semipreparative

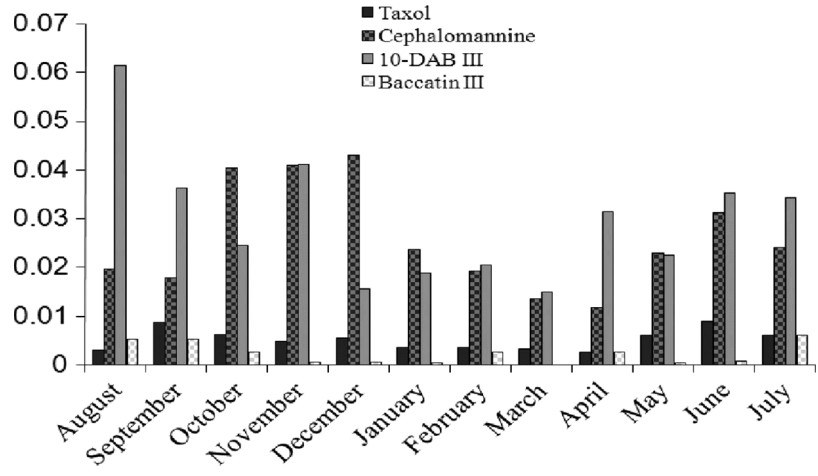



***Figure 3.*** Seasonal variation result for studied taxane (dry weight in percent versus month).



chromatography. After evaluation of several chromatographic conditions to achieve final resolution, 2D HPLC facilitated the isolation of 10-DAB from other compounds in the analytical column, Figure 4. There is a

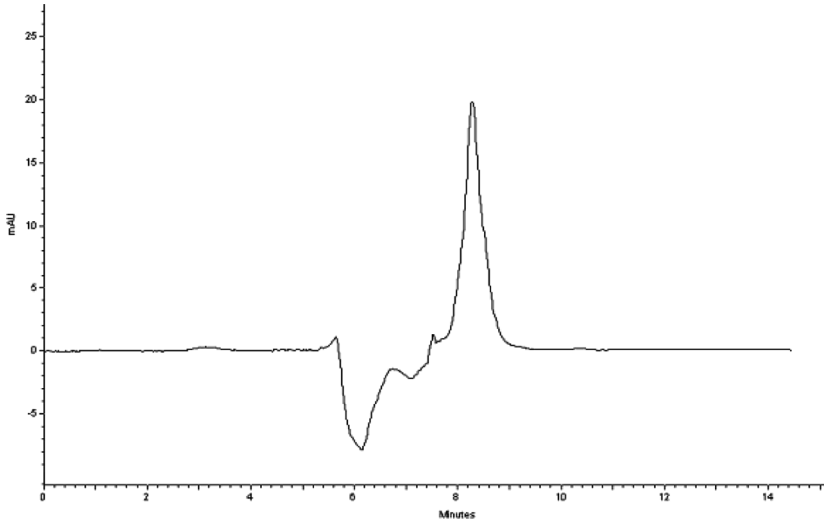



***Figure 4.*** Chromatogram of the isolation of a standard sample of 10-DAB by analytical column-switching technique for finding suitable semi-preparative chromatographic conditions.

negative peak in the chromatogram, which belongs to the variations in the system pressure when switching between columns.

Column switching processes should have a high repeatability, which can be achieved by switching the valve at the same rates and the same intervals during repetitions. To retain the first column highly efficient, it can be eluted overnight and regenerated occasionally.

## 2D-Semi-Preparative HPLC

Poor resolution in the first column can be a result of several factors such as chemical structural similarities of the taxane compounds under study, high complex samples, and maybe low efficiency of the employed column. Thus, a second column is required for better resolution.

Equation 1 predicts 16 mL $min^{-1}$ for flow rate in preparative chromatography, based on the optimal condition of analytical chromatography. But, this flow rate reduced resolution of the preparative column due to a lower theoretical plate of a preparative than an analytical column. Therefore, the flow rate was reduced to 10 mL $min^{-1}$ for good separation, which was adopted all through this study. Although reducing the flow rate 10 mL $min^{-1}$ modifies the profile, the resolution problem remains unresolved. This can be explained by the fact that the presence of contaminants in preparative use of chromatography has strong effects on the column efficiency, as well as obedience of the separation process from a certain isotherm.[25] Gradient elution may be helpful in this case where several fractions are to



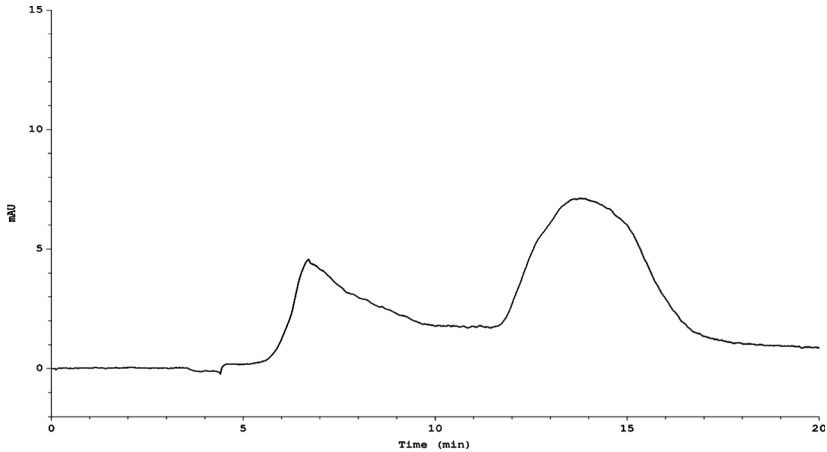



***Figure 5.*** Chromatogram of the separation of 10-DAB after column-switching by semi-preparative HPLC. Separation condition involved a mobile phase composition of water/acetonitrile (70:30), flow rate: 10.0 mL $min^{-1}$, injection volume: 4 mL.

be collected, but it will impose high costs, since the recycling process will not be possible. Using a second column for the analysis of a fraction containing only a few components seems to be more useful. As a result of overloaded injection (2 mL), the chromatogram has a rectangular shape. To use highest capacity of the column, volume overload and mass overload should

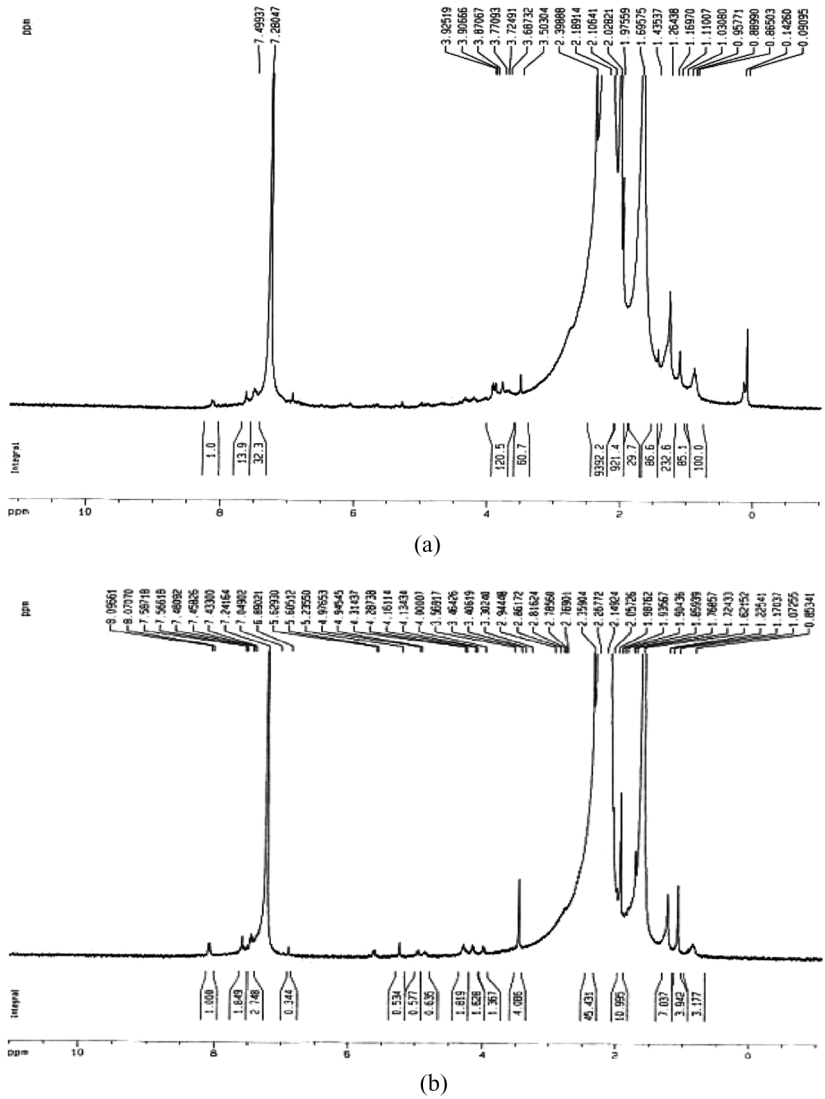



*Figure 6.* NMR spectrum of (a) 10-DAB III standards and (b) collected 10-DAB III from semi-preparative HPLC.

coincide. As presented in Figure 5, injection of 4 mL sample results in separated peaks showing both mass and volume overloads.

The fraction belonging to the 10-DAB (second peak in Figure 5) was collected and reanalyzed by analytical HPLC to determine its purity, which was found to be 82%. Since the 10-DAB peak appears between the peaks relating to contaminants, its purity can be increased by reducing the width of the collection window.

The fraction containing 10-DAB, collected from the second semi-preparative column, was lyophilized, dissolved in $CDCl_3$, and subjected to NMR spectroscopy. Comparison of the NMR spectra of the sample with that of the standard verified the result of the final HPLC analysis (Figure 6a and b).

## CONCLUSION

The main advantage of the proposed method is the application of a two-dimensional system, which not only resulted in separation of the compound of interest in a high yield, but also facilitated the recycling process. When the process of 2D chromatography is preceded by a suitable cleanup stage, it works much better, as shown when using LLE in the present study. The use of optimized conditions in MAE was another manipulation to reduce extraction time and minimize the presence of contaminants in the extracts. In spite of using ordinary stationary phases, results were satisfactory owing to the suitability of selective methods employed in both extraction and purification stages. The same approach can be followed to optimize the conditions for separation of other valuable taxoids. Moreover, the proposed method has the capability of being extended to preparative scale chromatography, which is frequently employed in the pharmaceutical industry.



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