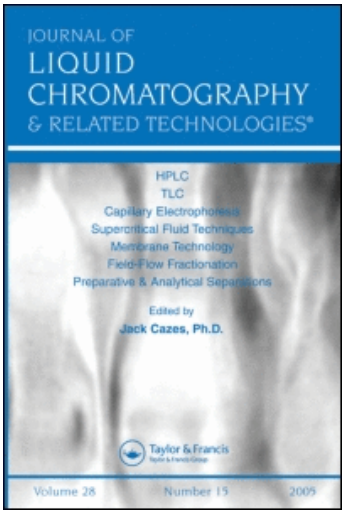

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Purification of Paclitaxel Isolated from *Taxus baccata* L. Cell Culture by Microwave-Assisted Extraction and Two-Dimensional Liquid Chromatography

Alireza Ghassempour[ab]; Masume Noruzi[c]; Mehdi Zandehzaban[c]; Zahra Talebpour[d]; Ahmad Yari Khosroshahi[e]; Nahid M. Najafi[c]; Mostafa Valizadeh[e]; Tahereh Poursaberi[d]; Hamid Hekmati; Hassanali Naghdibadi[f]; Hassan Y. Aboul-Enein[g]

[a] Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, Tehran, Iran [b] Inorganic and Analytical Chemistry Institute, Giessen, Germany [c] Faculty of Science, Department of Chemistry, Shahid Beheshti University, Tehran, Iran [d] Faculty of Science, Department of Chemistry, Alzahra University, Vanak, Tehran, Iran [e] Faculty of Agricultural, Department of Biotechnology, Tabriz University, Tabriz, Iran [f] Institute of Medicinal Plants, Iranian Academic Center for Education, Culture & Research, Iran [g] Pharmaceutical and Medicinal Chemistry Department, National Research Centre, Dokki, Cairo, Egypt

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Purification of Paclitaxel Isolated from *Taxus baccata* L. Cell Culture by Microwave-Assisted Extraction and Two-Dimensional Liquid Chromatography

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[1]Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, Tehran, Iran
[2]Faculty of Science, Department of Chemistry, Shahid Beheshti University, Tehran, Iran
[3]Faculty of Science, Department of Chemistry, Alzahra University, Vanak, Tehran, Iran
[4]Faculty of Agricultural, Department of Biotechnology, Tabriz University, Tabriz, Iran
[5]Behan Chemical Co., Tehran, Iran
[6]Institute of Medicinal Plants, Iranian Academic Center for Education, Culture & Research, Iran
[7]Pharmaceutical and Medicinal Chemistry Department, National Research Centre, Dokki, Cairo, Egypt

Abstract: Microwave-assisted extraction (MAE) has been applied for extraction of paclitaxel from a cell culture. Solid phase C_{18} has been used for clean up of the sample obtained from the optimized condition of MAE. The factorial design shows that the temperature and ratio of methanol to water (as an extraction solvent) are effective and extraction time is not an essential parameter for MAE of paclitaxel

Correspondence: Professor Hassan Y. Aboul-Enein, National Research Centre, Tahrir Street, Dokki, Cairo 12311, Egypt. E-mail: enein@gawab.com. and Dr. A. Ghassempour, present address: Inorganic and Analytical Chemistry Institute, Schubertstr. 60, Haus, 16D-35392, Giessen, Germany. E-mail: aghassempour@scientist.com; alireza.ghassempour@anorg.chemie.uni-giessen.de

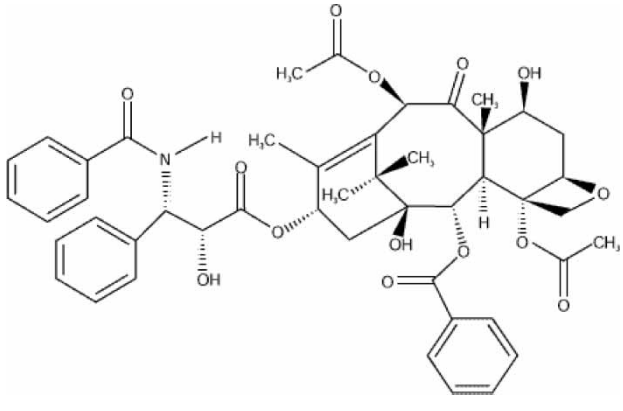
from the cell culture. Recently, we introduced MAE for extraction of paclitaxel from the needle of *Taxus baccata* L. Paclitaxel was extracted from the cell culture and the needle of *Taxus baccata* L, have been purified by heart-cutting two dimensional high performance liquid chromatography (2D-HPLC) in analytical and semi-preparative scales. The columns used in the analytical chromatography were a Eurospher-100 C_8 and Nucleosil-100 C_{18}, respectively, and paclitaxel was eluted at a flow rate of 1 mL min^{-1} with 40:60 v/v and 45:55 v/v water/acetonitrile, respectively. These conditions used in analytical chromatography, have been extended to a semi-preparative scale in which the first column was a Eurospher-100 C_8 and the second was a Eurospher-100 C_{18}, but the mobile phase used consisted of 40:60 v/v water/acetonitrile eluted at a flow rate of 11 mL min^{-1}.

Detection has been carried out at a wavelength of 227 nm throughout the analysis in both methods. The yield and purity of paclitaxel obtained by this method were 89 and 89% for the cell culture and 85 and 83% for the needle of *Taxus baccata* L., respectively.

Keywords: Paclitaxel, Cell culture, *Taxus baccata*, Microwave-assisted extraction, Two-dimensional liquid chromatography, Semi-preparative HPLC

INTRODUCTION

Paclitaxel (Taxol, Scheme 1) is one of the most important anticancer agents in the treatment of refractory, ovarian, breast, and other cancers.[1,2] Supplies of paclitaxel are limited and not affordable from the environmental point of view, because the original and major source of the drug is the bark of *Taxus brevifolia*.[3] The yield of purification of paclitaxel from *T. brevifolia* is about 0.01% of the dry weight of bark.[4] Also, there are significant differences in paclitaxel amounts according to location, season, and tissue variation. There



Scheme 1. Paclitaxel structure.

are four alternative means of obtaining paclitaxel, namely: semi-synthesis from its natural precursor (10-deacetylbaccatin III), total synthesis, production by fungi or bacteria, and cell culture. In recent years, many publications were reported on the production of taxanes in plant tissue and cell cultures[5–9] and their large-scale separation from different media.[10–13]

Microwave-assisted extraction (MAE) has been advantageously used for years for the extraction of biologically active compounds from different matrices. The main advantage of MAE is the rapid heating of the solvent, in closed vessels, at a temperature above its boiling point.[14–16] This high temperature and resulting pressure allow the active ingredient(s) to be effectively extracted from the sample. Also, processing time is decreased (usually no more than 10 min); furthermore, MAE requires smaller volume of organic solvents than the conventional techniques, which render it an economically and environmentally friendly technique. The combination of solid phase and MAE produces an effective method for extraction and clean up samples from a complex media.[17–20]

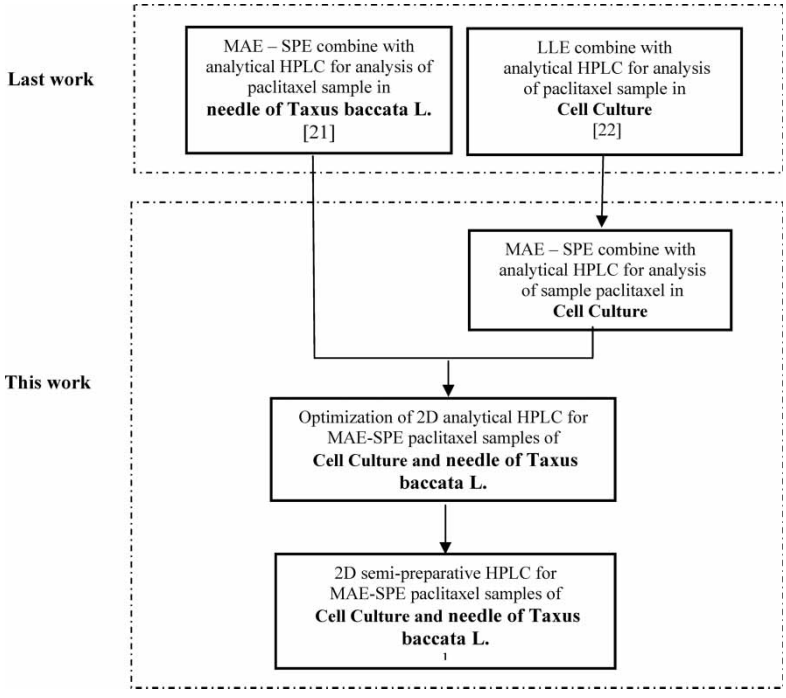
There are very few reports of procedures for extraction, isolation, and purification of paclitaxel that are directly applicable to commercial scales. Existing purification methods using solvent extraction and chromatography procedures primarily aim to obtain crude paclitaxel and related compounds, such as terpenoids, lipids, chlorophyll, and phenols.[13]

Scheme 2 shows a profile of the work presented in this paper. Talebi et al.[21] reported the MAE of paclitaxel from *Taxus baccata* L. needles by methanol-water as an extracting solvent, prepurified by a solid phase of a C_{18} disk and determined the influence of extraction parameters by factorial design. Also, monitoring of paclitaxel in the cell culture was reported by Yari-Khosroushahi et al.[22] This paper describes the use of MAE in the extraction of paclitaxel from Taxus cell culture followed by solid phase extraction as a clean-up step. The analysis of samples has been carried out by reversed phase HPLC. There is no significant difference in the MAE conditions between the two media used in this study, namely, the cell culture and needle of *Taxus baccata* L. The optimized 2D-HPLC conditions that was used in the analytical scale was applied in the semi preparative scale for the final purification of paclitaxol.

EXPERIMENTAL

Materials

The standard of paclitaxel (98%) was purchased from Sigma (St. Louis, Mo, USA). The paclitaxel standard was dissolved in methanol to obtain a suitable concentration. HPLC grade solvents (methanol, acetonitrile) were obtained from Caledon Company (Caledon, Canada). Ultra pure water was obtained



Scheme 2. Pathway for purification of paclitaxel from complex media.

from the Milli-Q water system. All of reagents used for cell line initiation were obtained from Sigma (St. Louis, Mo, USA).

Samples

Cell Culture

Suspension cells originated from *Taxus baccata*, were maintained under darkness at 24°C until 26 days, with shaking at 120 rpm. The temperature was increased from 24 to 29°C at 26–30th day. Suspension cells were cultured in Gamborǵs B5 medium supplemented with 30 g/L sucrose, 2 mg/L NAA, 0.2 mg/L 2, 4-D, and 0.2 mg/L Kin. In the prolonged culture for paclitaxel production 1% sucrose (w/v) was added to the culture medium at day 21 and $CoCl_2$ (20 μM), with $AgNO_3$ (30 μM) were added on the initiation of the culture as ethylene inhibitor elicitors. Methyl jasmonate (100 μM), salicylic acid (100 mg/L), and fungal elicitor from *Rhizopus estelonifera* (25 mg/L) were added to the culture medium at the 25th day. After the culture, biomass was recovered using a high speed centrifuge at 6000 rpm at the 30th day.

Plant

The investigation was performed on needles of *Taxus baccata* L., collected from the Botanical Garden of Tehran University in November 2005. The Taxus needles were air dried at room temperature. Dry needles were then removed from the stem and ground.

Apparatus

Microwave Extraction

MAE experiments were performed on a Milestone (Ethos, Italy) (1000 W, 2450 MHz) equipped with 10 closed PTFE vessels. Maximum values for pressure and temperature inside of the extraction vessels were 250 psi and 300°C.

An amount of 1 g of cell suspension material was frozen with liquid N_2 and subsequently freeze dried. Forty mg of dried material were weighed. According to working conditions imposed by the full factorial design (Table 1), samples were placed in vessels. Solvent volume was 5 mL. The control vessel was connected with the temperature control sensor after putting the vessel into the microwave cavity. Extractions were performed at fixed temperature at 25% microwave oven power with 2 min needed to reach the selected extraction temperature.

After extraction time was completed, the vessels were allowed to cool down to room temperature before they were opened. The vessels were then taken out and opened; then, the suspensions were centrifuged.

Solid Phase Clean Up

The solid phase apparatus includes a Empore (3M Corp., St. Paul, MN, USA) extraction disk, 47 mm size, which was used in conjugation with a Millipore all glass filtration apparatus (Millipore Bedford, MA, USA).

Sample clean up was performed by solid phase. After centrifuge and filtration of the sample, aliquots (2 mL) were subjected to solid phase clean up. Conditioning was accomplished with 15 mL ethyl acetate, 15 mL methanol, and 15 mL Milli-Q water under gentle vacuum. Under very gentle vacuum, 10 mL Milli-Q water was added to the reservoir followed immediately by 2 mL of crude extract. The disk was washed with 10 mL Milli-Q water, 10 mL 20% aqueous methanol, and the taxoid fraction was eluted with 20 mL methanol. The solvent was evaporated in a rotary evaporator (30–35°C) and it was again dissolved in 2 mL methanol. Aliquots (20 μL) were injected into the HPLC system. The pacliaxel content in the crude extract was 0.01%.

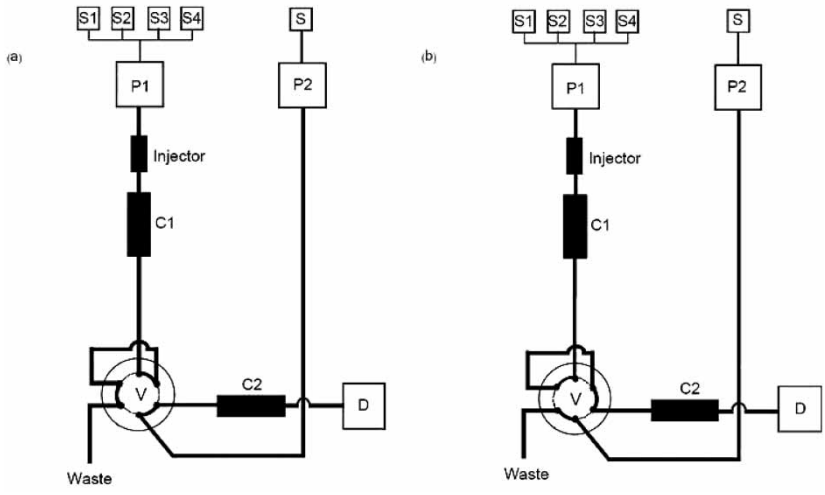
Table 1. Factors levels, design matrix, response values in the factorial design developed to investigate MAE conditions in the extraction of paclitaxel from cell culture

				Levels	
Variable		Key	Unit	Low (−)	High (+)
Temperature		A	°C	75	95
Ratio of methanol to water		B	% v/v	70	90
Time		C	min	7	11
Run	A	B	C	Yield (area)	Estimated effect
1	−	−	−	10905	26642
2	+	−	−	13139	22951 (A)
3	−	+	−	16804	19483 (B)
4	−	−	+	14708	−3640 (C)
5	+	+	−	73000	14718 (AB)
6	+	−	+	28940	−6264 (AC)
7	−	+	+	18249	−13442 (BC)
8	+	+	+	37392	−12263 (ABC)

HPLC Method

HPLC analyses of extracted paclitaxel from cell culture for the factorial design to find the optimization condition of MAE, were carried out using a Knauer pump (K-1001) and a Knauer diode array detector (DAD, K-2800) (Berlin, Germany). The column was 250 × 4 mm I.D. (with integrated guard column) Nucleusil ODS-100 A column (Knauer, Germany). The flow rate in all analyses was 1 mL/min, the injection volume was 20 μL. The experiments were carried out under isocratic elution mode at ambient temperature using water and acetonitrile in the ratio 40:60 (v/v). The total run time was 20 min. and was performed using 227 nm.

Chromatographic analysis, in 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 3) as heart-cutting 2D-HPLC. The first column, C_8 (250 × 4 mm, 5 μm), was eluted with water/acetonitrile (40:60 v/v) at a flow rate of 1 mL/min and detection was carried out at a wavelength of 227 nm throughout the analysis. Switching time was determined by injection of the paclitaxel standard onto this column before performing the 2D system. The mobile phase for the second column, C_{18} (250 × 4 mm, 5 μm) was water/acetonitrile (45:55 v/v), pumped at a flow rate of 1 mL/min and detection wavelength was 227 nm. There were two positions for the 2D system used in the present experiment, load, and injection position. In load position



Scheme 3. 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).

(Scheme 3a) eluant from the first column was transferred to the waste, while in injection position 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 the load position at the end of the standard peak.

Semi-preparative separation was carried out on a 2D system using the chromatographic condition similar to ones used for the analytical scale.

Extracted sample was injected onto the first column and the fraction containing paclitaxel was transferred onto the second column by switching the valve to injection position during the beginning and the end of the standard peak. A Eurospher-100 C_8 (120×16 mm, 5 μm) and a Eurospher-100 C_{18} (120×16 mm, 5 μm) both eluted with 40:60 water/acetonitrile at a flow rate of 11 mL/min were used for 2D semi-preparative liquid chromatography.

RESULTS AND DISCUSSION

MAE for Extraction of Paclitaxel from Cell Culture

The effect of three parameters on the extraction yield of paclitaxel from cell culture by MAE, including temperature (A), ratio of methanol to water (B),

and time (C), as well as their interactions were simultaneously studied by means of eight randomized experiments with the help of a full factorial design. The value of levels given to each factor, were selected based on the previously published report for MAE of paclitaxel from needles of yew trees *Taxus baccata* L.[21] Microwave extracts were submitted to the proposed cleanup procedure and finally, the extracts were analyzed by a reversed phase condition, which was used for monitoring of paclitaxel in a cell culture.[22] The data on the factorial design parameters are shown in Table 1.

In the present work, the Yates method was used to calculate the essential parameters which include the effects of temperature, ratio of methanol to water, and time of MAE. These parameters were plotted on a normal probability paper. In the normal plot method, the non-significant effects tend to fall on a straight line, while significant effects deviate from the line. Also, a positive sign of effect indicates extraction yield increased when the factor was changed from low to high level, whereas a negative sign indicates the opposite behavior. Figure 1 indicates that the two main parameters are that of A and B, which have significant positive effects that their value should be set at the high level to achieve maximum MAE efficiency. Also, one two-factor (AB) and three-factor (ABC) interaction effects were the most important ones with positive and negative signs, respectively. The main effect of time had an adverse effect on the extraction yield, which means that yield increases when the time is at the lower level. The paths of these variations are shown in Fig. 2 as an estimated response surface.

Higher temperature causes the increase of the extraction yield because in closed vessel system, temperature reaches well above the boiling point of the solvent. This high temperature results in improved extraction efficiencies since desorption of paclitaxel from active sites in the cell culture would increase. Also, high temperature causes the increase of paclitaxel solubility.

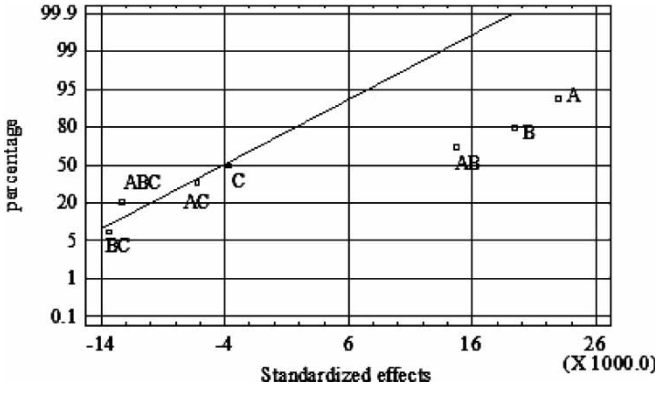


Figure 1. Normal probability plot of effects for the full factorial design.

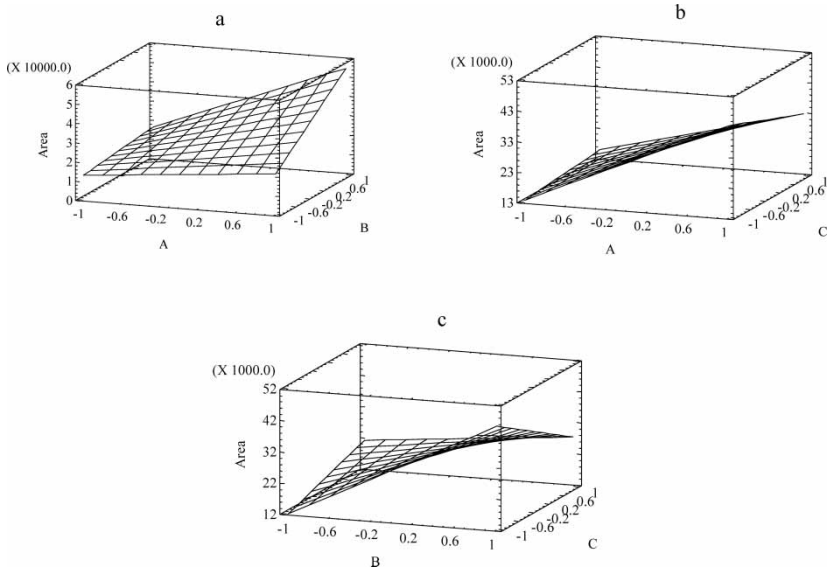


Figure 2. Estimated response surfaces for the central level of (a) time, (b) ratio of methanol to water and (c) temperature for the MAE of paclitaxel from cell culture.

Enhancing of methanol percentage leads to increasing of paclitaxel solubility and extraction recovery, but the boiling point of methanol is low and the presence of water permits the use of higher temperatures for MAE. The negative effect of time can be seen when comparing the 7th and 8th run. This effect is due to the degradation of paclitaxel with a longer extraction time.

The results of extraction of paclitaxel from the cell culture are very similar and in agreement with our previously published results for MAE from needle of yew *Taxus baccta* L. sample[21] and shows that the type of matrix does not significantly influence the extraction of paclitaxel by the MAE method. Although temperature is an essential parameter, when the temperature of MAE of paclitaxel increases it influences significantly the release of paclitaxol from the cell culture or needle media in solvent (B), causing the degradation of paclitaxol during this time, therefore, this phenomena requires limiting the use of high temperature and time of the microwave. Similar behavior was observed with zearalenon, a sensitive temperature compound, when it was extracted from different media; such as wheat and corn.[23] The influence of plant matrix on MAE processes was investigated and showed that for a non-sensitive temperature compound, such as diosgenin, the time of MAE permits variation at constant levels of other factors.[24]

Simultaneous solving of the equations of effects of temperature, solvent, and time and supplementary experiments show the optimum conditions for MAE are obtained at A (temperature) = 92°C, B (ratio of methanol to

water) = 94%, and C (time) = 7 min. Good yields and purity have been obtained at this condition. Therefore, MAE coupled with solid phase can be used for pre-purification of paclitaxel from complex media, such as cell culture and needle of the plant. For further purification, use of the chromatographic method has been required.

2D HPLC Conditions

Analytical HPLC

The injection of the sample after MAE and solid phase onto the first column in analytical HPLC causes separation of taxoids, but did not show a suitable resolution (Fig. 3). In spite of using a polar mobile phase to increase the interactions of taxoids with the stationary phase, overlapping is observed in the separated peaks. The study of UV-VIS spectra of these signals by photodiode array detector in HPLC indicated that a majority of these compounds belong to taxoids. The overlapping pattern of the profile can be attributed to the high structural similarity between the members of this class of natural compounds. To achieve final resolution, 2D HPLC was employed (Fig. 4) and it shows that it facilitated the isolation of paclitaxel from other compounds in the analytical column. There is a negative peak in the

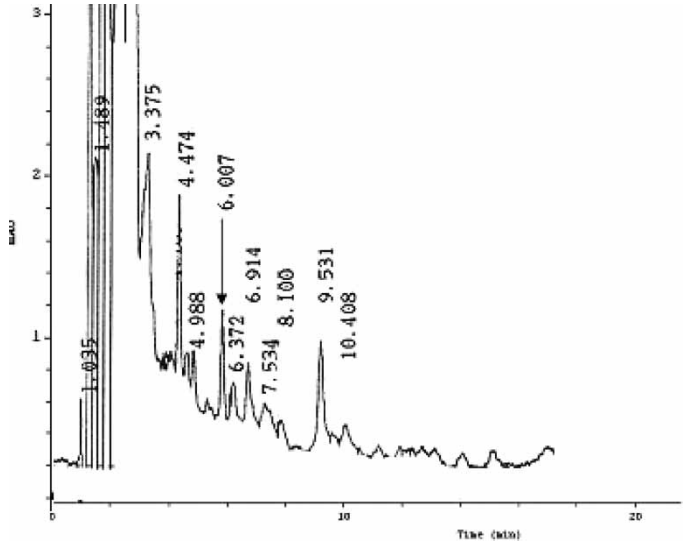


Figure 3. A analytical liquid chromatogram of paclitaxel ($t_R = 6.007$ min) that extracted from cell suspension culture for chemometric study of MAE (Nucleosil ODS-100 A, 250 × 4 mm, 5 μm, water/acetonitrile 40:60 v/v).

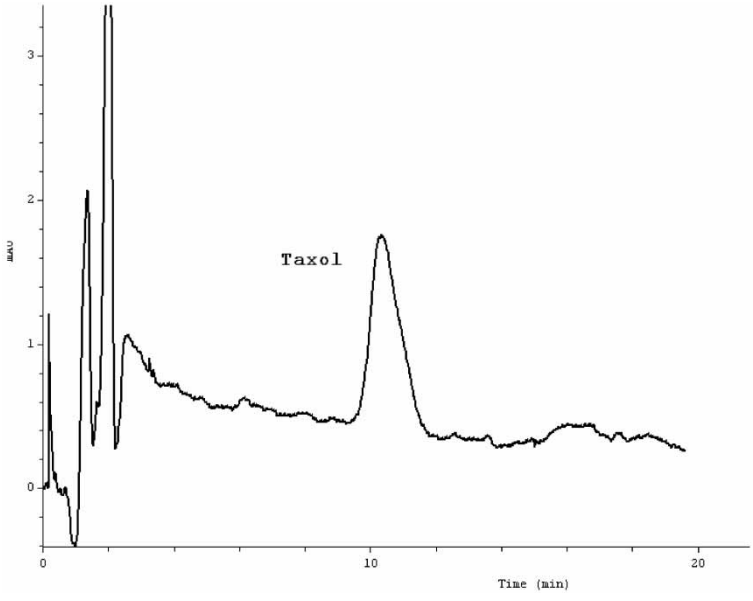


Figure 4. A typical chromatogram of paclitaxel after analytical 2D liquid chromatography (Eurospher C8, 250 × 4 mm, 5 μm, water/acetonitrile 40:60 nucleosil ODS-100 A, 250 × 4 mm, 5 μm, water/acetonitrile 45:55 v/v).

chromatogram, which belongs to the variations in the system pressure when switching between columns and a few small peaks of solvents, due to difference in composition of the mobile phase (water/acetonitrile) of the second LC column and sample solvent (methanol).

The column switching process should have a high repeatability, which can be achieved by switching the valve at the same rate interval during repetitions. To retain the first column to be highly efficient and repeatable, it can be eluted overnight and regenerated occasionally.

Semi-preparative HPLC

Although it is recommended to keep the flow rate higher to decrease the dead volume in preparative HPLC, it can be modified to satisfy resolution. Poor resolution in the first column could have several sources, such as high similarity of existing compounds, high flow rate, and maybe low efficiency of the employed column. This can be explained by the fact that the presence of contaminants in the preparative use of chromatography has strong effects on the column efficiency, as well as obedience of the separation process from a certain isotherm.

The fraction belonging to paclitaxel was collected and reanalyzed by analytical HPLC to measure its purity. For further purification of these

samples to obtain >97%, only one step of semi-preparative HPLC with the C_{18} column can be used. Gradient elution may be helpful in the case that several fractions are to be collected, but it will impose high costs, since recycling process will be impossible. As a result of overloaded injection, the chromatogram has a rectangular shape. To use the highest capacity of the column, volume overload and mass overload should coincide.

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