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FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
AND PHARMACEUTICAL ANALYSIS

DIPLOMA THESIS

Optimization of separation conditions for HPCCC of paclitaxel

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"I declare that this thesis is my original copyrighted work. All literature and other resources I used while processing are listed in the bibliography and properly cited. The thesis was not misused for obtaining the same or different academic degree."

In Hradec Králové

INDEX

ABSTRAKT 6

ABSTRACT 7

1. INTRODUCTION 8

2. THE AIM OF WORK 9

3. THEORETICAL PART 10
   3.1 Taxanes in general 10
   3.2 Taxanes in Teva (Opava) 13

4. LIQUID-LIQUID EXTRACTION 15
   4.1 History of liquid-liquid extraction 15
   4.2 Present of liquid-liquid extraction 20
   4.3 Theory of countercurrent extraction 21
   4.4 Taxanes separation by countercurrent chromatography 28

5. EXPERIMENTAL PART 29
   5.1 Targeted molecules 29
   5.2 HPCCC characterisation 32

6. RESULTS AND DISCUSSION 36
   6.1 Test tube experiments 36
      6.1.1 Distribution coefficients for various solvent systems 37
      6.1.2 Concentration dependence of distribution coefficient 39
   6.2 HPCCC bleeding monitoring 41
      6.2.1 Stationary phase loss {MIBK/methanol/water (3:3:4)} 41
      6.2.2 Stationary phase loss {MIBK/methanol/water + 1% $CaCl_2$ (3:3:4)} 43
   6.3 Preparation of experiment conditions 45
   6.4 Preliminary HPCCC tests 47
      6.4.1 MIBK/acetone/water (2:3:2) – dual mode 47
      6.4.2 MIBK/methanol/water (3:3:4) - reverse phase mode 49
      6.4.3 MIBK/methanol/water (3:3:4) - reverse phase – Rafinated Extract 52
      6.4.4 MIBK/acetone/water (2:3:2) - reverse phase – Rafinated Extract 55
   6.5 Monitoring of stationary phase loss; dependence on rotation and flow 57
   6.6 Preparative scale HPCCC conditions 59

6.6.1 MIBK/acetone/water 2:3:2, 50 mg RE..59
6.6.2 MIBK/acetone/water 2:3:2, 200 mg RE + DHB and PAC...............................63
6.6.3 MIBK/acetone/water 2:3:2, 800 mg RE + DHB and PAC...............................67
6.6.4 Toluene/acetone/water 4:2:4, 50 mg RE...72
6.6.5 Toluene/acetone/water 4:5:1, 50 mg RE...77

7. CONCLUSION...81

8. ABBREVIATIONS..83

9. TABLE INDEX...84

10. FIGURE INDEX...85

11. GRAPH INDEX..88

12. BIBLIOGRAPHY..89

ABSTRAKT

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Cílem této práce základního výzkumu je seznámit se s problematikou izolace přírodních látek, v našem případě paclitaxelem a jeho meziproduktů (především 10-deacetylbaccatinem) pomocí vysoce moderní techniky vysoce účinné protiproudé chromatografie (HPCCC). Pro účely testování separace je nutné prakticky vyzkoušet možné mobilní a stacionární fáze složené z různých rozpouštědel a jejich poměrů.

Protiproudá („countercurrent“) chromatografie patří mezi moderní separační techniky techniky využívající dvě vzájemně nemísitelné kapaliny.. Jedna z kapalin zastupuje fázi stacionární, druhá mobilní. Stacionární fáze je držena v koloně pomocí odstředivé síly zatímco mobilní fáze je pumpována skrz kolonu. Nastříknutý vzorek tak prochází spolu s mobilní fází přes kolonu a podle svého distribučního koeficientu jsou jednotlivé komponenty drženy na stacionární fázi nebo prochází dále přes detektor do sběrače frakcí.
Výhodou této nedestruktivní metody je například možnost analyzovat větší množství vzorku, práce při vyšších průtocích s čímž souvisí kratší doba analýzy nebo vhodnost při separaci přírodních materiálů. Nevýhodou je vyšší cena za potřebné vybavení.

Během této práce jsem otestovala spoustu systémů rozpouštědel a vypočítala distribuční koeficienty, díky kterým bylo možné určit vhodný systém pro další práci. Bylo také nutné optimalizovat daný přístroj a minimalizovat ztrátu stacionární fáze, ke které bohužel díky odstředivému pohybu a tlaku mobilní fáze může docházet a proto jsem jej testovala při různých otáčkách, průtocích a koncentraci vzorku v nástřiku.

Z vytvořené tabulky distribučních koeficientů byly vybrány tři vhodné systémy rozpouštědel a separační podmínky byly nastaveny na 1200 otáček/min a průtok 5 ml/min. Pro většinu experimentů byl také využit tzv. duální mód, který umožňuje separaci směsi látek s odlišnými distribučními koeficienty v mnohem kratším čase.

Klíčová slova: paclitaxel, 10-deacetylbaccatin, vysoce účinná protiproudá chromatografie, duální mód, systém rozpouštědel, distribuční koeficient

ABSTRACT

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Name of diploma thesis: Optimization of separation conditions for HPCCC of paclitaxel

The aim of this work is a feasibility study of natural substances isolation (in my case it is paclitaxel and its intermediate 10-deacetylbaccatin) using modern technique called High performance countercurrent chromatography (HPCCC) available in Teva in Opava. For this basic research it was necessary to study the literature and test various solvent systems. Chosen solvent systems were then tested together with natural substance in HPCCC, results were processed and discussed.

Countercurrent chromatography is considered a modern separation technique based on two immiscible liquids. One of them is called a stationary phase and is held inside the column by centrifugal force, the second liquid is called a mobile phase and is pumped through the column. Then the injected liquid sample is also pumped through the column and depending on distribution coefficients the sample components are either held in the stationary phase or pass through the column and detector into fraction collector.
Advantages of this method are e.g. total sample recovery, high mass and volume injection loadings, high flow rates connected with reduced analysis time or suitability for separation of crude natural materials. The disadvantage can be bigger cost of equipment.

This work consists of series of test tube experiments and calculation of distribution coefficients that was necessary to perform for selection of convenient solvent systems. Other target was to optimize the HPCCC instrument and reduce the stationary phase loss which is caused by the centrifugal force and the pressure of mobile phase being pushed through the column. For that reason I tested different rotation speed, flow rates and distribution coefficient concentration dependence.

The summary of distribution coefficients in tested solvent systems is a valuable groundwork for further experiments. Three solvent systems were selected and separation conditions were set to 1200 rpm and 5 ml/min. For most experiments dual mode was used allowing us to separate compounds presenting different polarity (different distribution coefficients) in shorter time.

Key words: paclitaxel, 10-deacetylbaccatin, high performance countercurrent chromatography, dual mode, solvent system, distribution coefficient

1. INTRODUCTION

Paclitaxel (PAC) belongs to the group of anti-cancer chemotherapy drugs. Its mechanism of action is basically blocking the growth of the cancer by stopping cancer cells separating into two new cells. It is used as a treatment for various types of cancer such as ovarian, metastatic breast, lung, oesophageal or prostate.

Nowadays there are a few methods for paclitaxel isolation. The most common process is a semi-synthesis from 10-deacetylbaccatin, 9-dihydro-13-acetylbaccatin III, baccatin III or 10-deacetylpaclitaxel (others methods are: total synthesis - economically unaffordable; isolation from roots and bark of yew tree – original method but the product is too dirty; fermentation – taxus tissue cultures, takes very long time; endophytic fungi – taxol-producing microorganisms). The target is to get the purist product in reasonable time and price.

One of the purification method is chromatography. Old chromatographic systems used gravity for the process of separation but new types of machine use centrifugal force generating very high g-force and some of them even consist of two axis around which the column rotates creating planetary motion. This instrument is called High performance countercurrent chromatography (HPCCC).

This diploma thesis is directing on behaviour of paclitaxel and its intermediates in HPCCC and finding the best possible conditions for the separation of these compounds from the natural product. It required seeking of the suitable solvent system and also monitoring various system adjustments.

2. THE AIM OF WORK

The aim of this thesis is a feasibility study for paclitaxel and intermediates 10-deacetylbaccatin (10-DAB), 9-dihydro-13-acetylbaccatin III (DHB) isolation and purification by high performance countercurrent chromatography. This study belongs to basic research.

The main target is to test an ability of HPCCC to isolate paclitaxel and other intermediates from crude natural product and in general the behaviour of these compounds during countercurrent chromatography.

Next target is to test various solvent systems (coming from the literature or internal Teva documentation) by test tube experiments and select the ones with convenient distribution coefficients (“D”) of paclitaxel and intermediates.

The other targets are optimization of HPCCC conditions such as rotation speed or flow rate to reduce stationary phase loss and also testing of selected solvent systems for real natural substance in preparative scale.

3. THEORETICAL PART

3.1 Taxanes in general

Taxanes (=taxoids) belong to a class of chemical compounds composed of two terpene units called diterpenes. They were originally derived from natural sources - plants of the genus *Taxus* (yews) but some of them have also been synthesized artificially.

There are many species and hybrids of the genus *Taxus* such as *T. baccata* (European yew), *T. brevifolia* (Pacific yew), *T. canadensis* (Canada yew), *T. cuspidata* (Japanese yew), *T. chinensis* (China yew) and many others.

There are two well known members of taxane class – paclitaxel (trade name Taxol) and docetaxel (Taxotere) and less known cabazitaxel (all of them are FDA approved). Paclitaxel (PAC) was the original compound in this group and was originally isolated from the bark of *T. brevifolia* (Pacific yew) and later syntetised whereas docetaxel is a semi-synthetic analogue of the latter; an esterified derivative of 10-Deacetylbaccatin - natural product extracted from *T. baccata* (European yew). [1]
These hydrophobic structures are poorly soluble in water, soluble in alcohols, have numerous chiral centres and their oral bioavailability is also very low which is a limitation in development of treatment by the oral route. [2]

The unique mechanism of action is in contrast to other antimitotic drugs, such as *vinca* alkaloids or *colchicine* which inhibit tubulin polymerization (prevent mitotic spindle formation). Taxanes block cell cycle progression through centrosomal impairment, induction of abnormal spindles and suppression of spindle microtubule dynamics (inhibition of microtubule depolymerization). Triggering of apoptosis by aberrant mitosis depends on cell type and drug schedule. [3] To sum it up, taxanes cause inhibition of cell division, chromatid separation, growth and ultimately cell death. Therefore they are commonly known as „mitotic inhibitors“ or „mitotic poisons“. [4]

The two mentioned antineoplastic agents are widely used in the therapy of ovarian, metastatic breast, [5] lung, oesophageal, prostate, [6] pancreas, gastric, bladder and head and neck cancers. [7]

As every antitumour drug taxanes may also cause a multitude side effects/toxicity, shown in a 3-week clinical study in metastatic breast cancer treatment in 222 patients, see Table 1. [8]

Table 1: Adverse effects of docetaxel and paclitaxel in 3-week clinical study.

Adverse effect	docetaxel (%)	paclitaxel (%)
Any adverse effect	98.6	94.1
Pain	23.4	27.0
Asthenia	74.3	55.0
Peripheral edema	45.9	13.1
Neurosensory	64.4	59.0
Nausea	49.1	32.0
Stomatitis	51.4	16.2
Diarrhea	38.3	16.7
Infection	33.3	9.9
Myalgia	23.4	33.3
Skin disorders	37.4	14.4
Vomiting	27.9	15.8

As evident from Table 1, almost all toxicities were more frequent with docetaxel except from pain and myalgia. [8]

There are also new studies on taxol derivatives and analogues trying to find new potential structures with reduced toxicity and at least analogous potency. One of them is cabazitaxel (FDA approved in 2010), used as the 2nd line treatment of metastatic prostate cancer. [9] For the rest of analogues see Table 2.

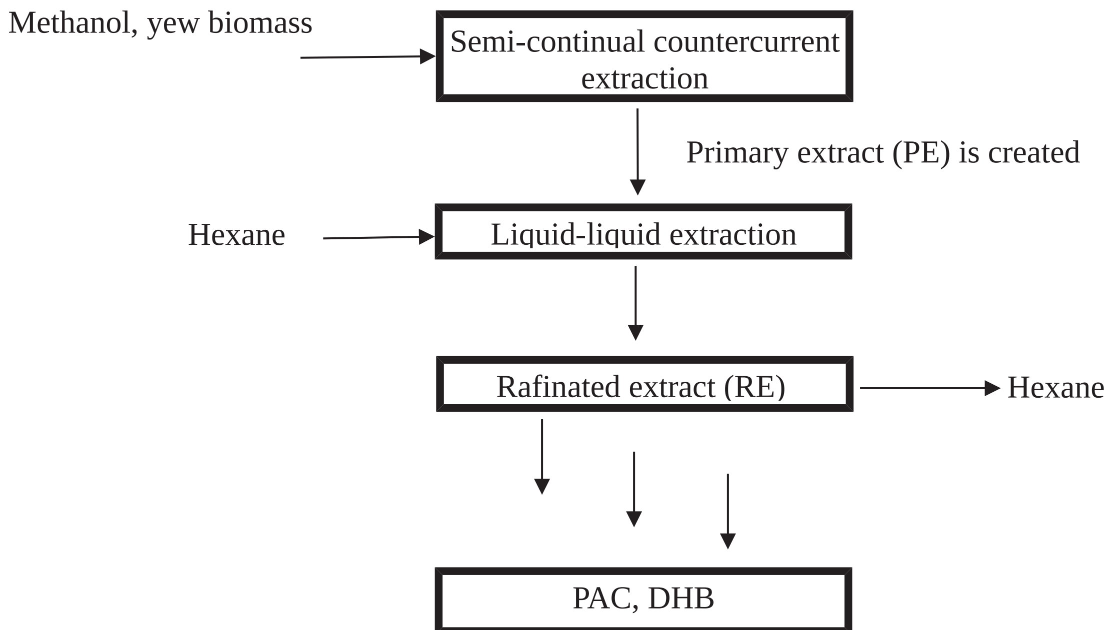
Table 2: Summary of developed taxane analogues including potential compounds for the future cancer treatment. [10]

Agent	Administration	Potential Indications	Toxicity	Development Stage
Cabazitaxel	Intravenous (IV)	Prostate	Neutropenia	FDA-approved
		Breast	Neurotoxicity	Phase II
			Diarrhea	
DJ-927	Oral	NSCLC	Neutropenia	Phase I/II
		Breast	Anemia	
		Melanoma	Nausea	
		Bladder	Fatigue	
BMS-184476	IV	NSCLC	Neutropenia	Phase II
BMS-275183	Oral	NSCLC	Neurotoxicity	Phase I
IDN-5109	Oral	NSCLC	Febrile Neutropenia	Phase II
	IV		Nausea	
			Vomiting	
XRP9881	IV	NSCLC	Neutropenia	Phase II
		Breast	Fatigue	
			Diarrhea	
Milataxel	IV	Colorectal cancer	Neutropenia	Phase II
	Oral	Advanced solid tumors	Leukopenia	
TPI-287	IV	Neuroblastoma	Neutropenia	Phase I
		Medulloblastoma	Anemia	

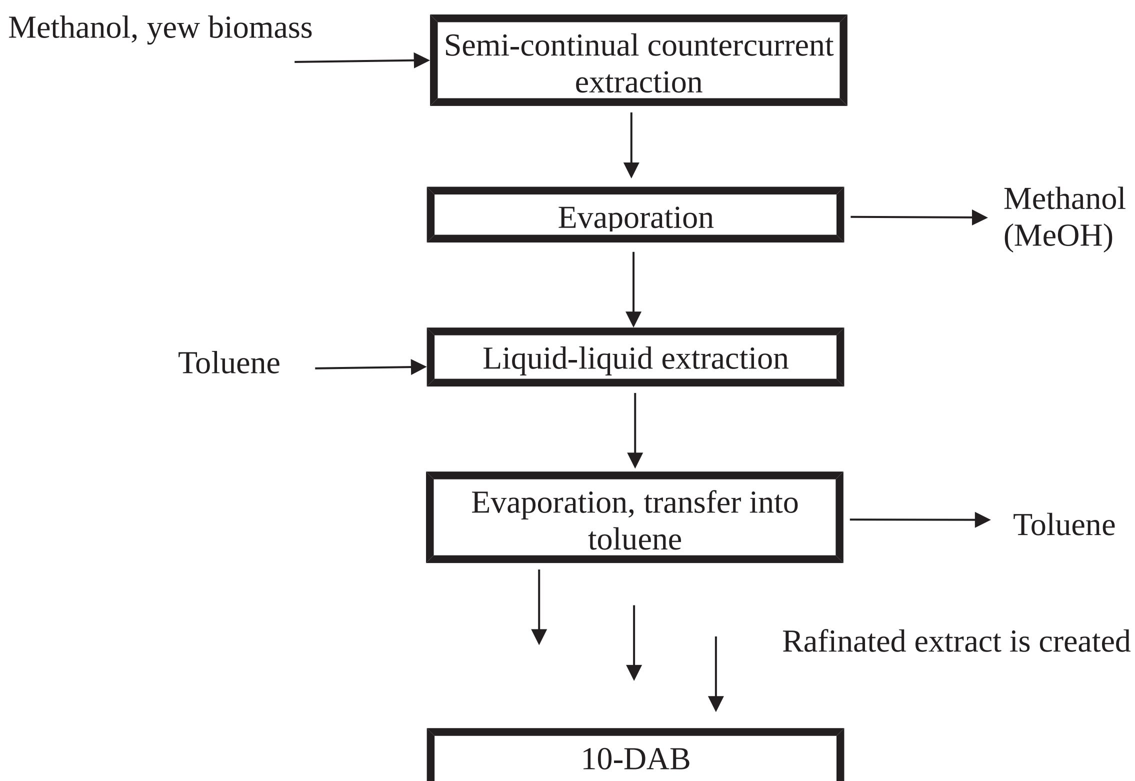
3.2 Taxanes in Teva (Opava)

There are a few techniques used for isolation of taxanes. During the process the yew biomass is extracted with methanol and creating “Primary extract”. After another extraction with hexane the “Rafinated extract” is obtained and processed, see the scheme below.

Flow chart of PAC & DHB



Flow chart of 10-DAB



During the separation process there are many in-process controls (IPC). Every IPC is executed in quality control (QC) laboratory, final results are recorded and approved by qualified person (QP).

“Teva in Opava now processes natural PAC (and DHB later transferred semi-synthetically into PAC) isolated from *T. canadensis* but this production is slowly terminating and another process is starting. Only *T. baccata* (delivered from Netherlands) will be manufactured and PAC will be then semi-synthesized from 10-DAB isolated from this kind of yew tree. The new method is considered more advantageous and less expensive.” (Ing. Ladislav Cvak, Ph.D., personal communication, February 7, 2017)

The year production of pure PAC in Teva Opava is 200 kg per year which requires about 1 200 tons of needles to be processed.

The year demand for PAC in the whole world is about 700 kg/year which makes Teva (Opava) one of the most significant world supplier.

The drug content

“The concentrations of taxoids in the drug are variable and depend on the season. [11] Approximately 30 % of the total alkaloid fraction from *Taxus baccata* consist of a mixture of compounds called “taxine” which contains the main alkaloids called taxine B and isotaxine B [12] and is responsible for the toxicity of the yew plant. These compounds have been isolated in yields of 1.2 % dry weight in needles. [13] In contrast, the content of PAC and 10-DAB is lower and varies from 0 to 0.05 % and 0 to 0.48 % dried needles, respectively. [14] The concentrations of 10-DAB, cephalomannine, and baccatin III (impurities) were found in lower concentrations, ranging from 0 to 0.05 % dry weight in needles. [14] On the surface of twigs of *Taxus baccata*, baccatin III, PAC, and 10-DAB were present in lowest concentrations (<0.003 % fresh weight). The concentrations of paclitaxel were usually the lowest.” [14, 15]

4. LIQUID-LIQUID EXTRACTION

4.1 History of liquid-liquid extraction

At the very beginning of High performance countercurrent chromatography there was a simple and well-known process of liquid-liquid (LL) extraction. [16] All that was needed to process was an impure sample specified for testing which was dissolved in a two-phase solvent system in a separatory funnel, see Figure 1.

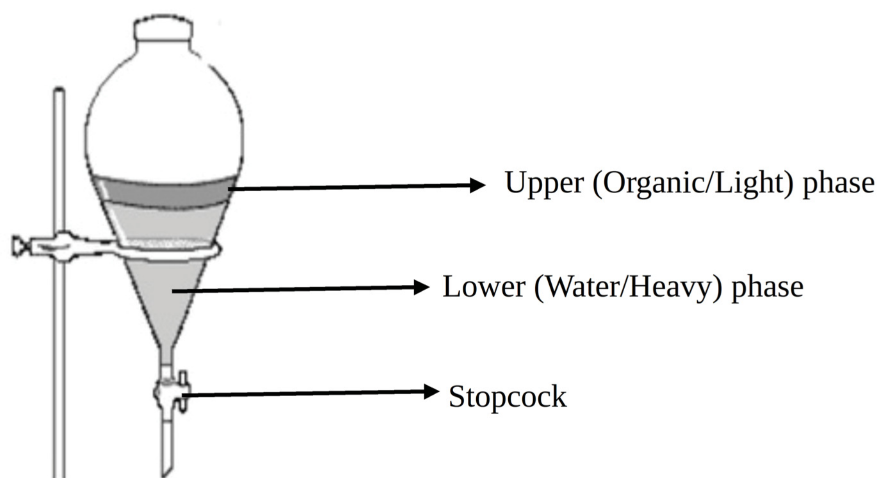


Figure 1: Separatory funnel.

Generally, liquid-liquid extraction involves two immiscible liquids which form an interface when placed in the same funnel. The typical example can be ether (as a light phase) + water (as a heavy/aqueous phase). The location of an extraction solvent is determined by its density, e.g. when water is placed in a funnel with ether – ether is on the top but when water is paired with oil – water is always on the bottom. [17]

After the sample is dissolved in solvent system the separatory funnel is vigorously shaken and left to settle for a while allowing the two phases to separate properly. The sample will then partition preferentially into one of the phases.

If necessary, the whole process of extraction can be repeated by removing the heavy phase (into a beaker and transporting to a new separatory funnel) and adding another amount of light phase (or analogously adding further quantity of heavy phase to the original separatory funnel).

This simple, quick and still very often used technique is effective in separating compounds with very different distribution constant=partition ratios=K_d (if the ratios are similar, the process must be repeated hundreds of times for a complete separation).

The apparatus designed and produced by Craig and Post (see Figure 2) in late 1940s [20] improves an old technique of LL extraction.

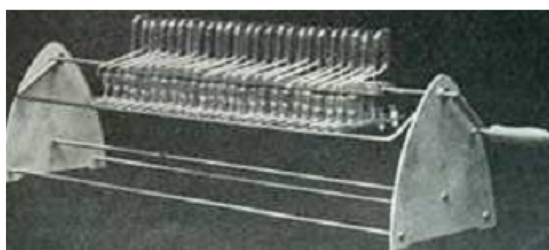


Figure 2: Craig's countercurrent distribution apparatus consisting of 25 tubes (manually operated). [18]

They created a series of tubes arranged to transfer a liquid phase from one tube to the next one. "The liquid-liquid extractions take place simultaneously in all tubes of the apparatus which is usually driven electro-mechanically creating a cycle." [18] The cycle is shown in Figure 3.

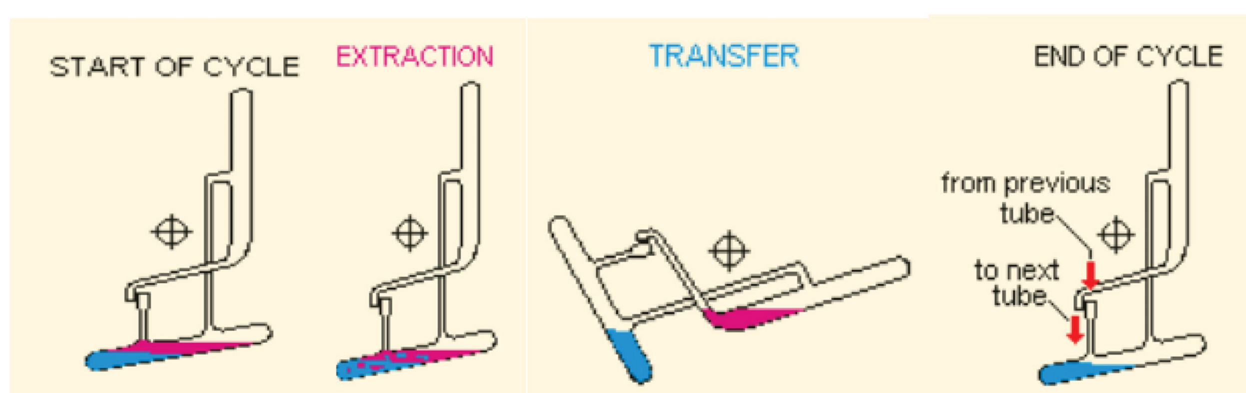


Figure 3: Mechanical cycle of Craig apparatus. [18]

The next progress in countercurrent chromatography was the invention of Droplet countercurrent chromatography (DCCC) in 1970 by Tanimura, Pisano, Ito, and Bowman. [19] The system is composed of series of vertical glass tubes connected top-to-bottom by capillaries (see Figure 4). It is all-liquid separation technique, where liquid stationary phase (SP) is retained in tubes while mobile phase (MP, containing sample) in form of steady stream of droplets is passing through surrounding SP. This shows big disadvantage of DCCC – it is limited only to those biphasic solvents systems that form stable droplets.

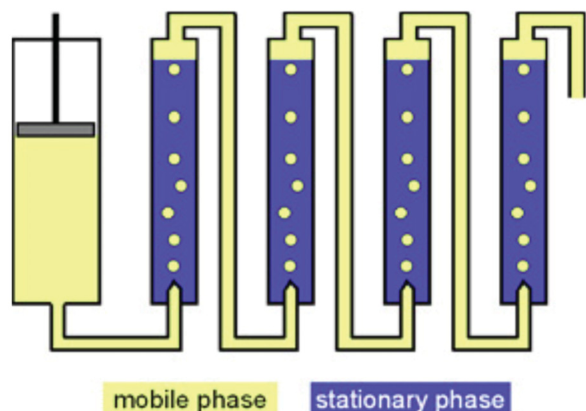


Figure 4: DCCC machine composed of series of tubes filled with SP and droplets of MP being pushed through them. [20]

“MP is either pumped to the top of each tube (descending mode) if the phase is denser than SP, or to the bottom (ascending mode), if lighter.
Compounds which distribute preferentially into the MP will pass through the apparatus and will be eluted more quickly than those which distribute less preferentially into the MP and a separation will take place due to this distribution (partitioning) process.” [16]

But there was still one disadvantage. The SP was held in columns only by gravity and so DCCC was not effective enough. [19]

The next progress in this field and the first modern countercurrent chromatography (CCC) instrument was introduced to the world in 1982 by Japanese company Sanki Engineering. This system uses a similar arrangement of extraction cells linked in cascade by ducts (see Figure 5) and connected in disks in a circle around a rotor – shown in Figure 6. [21] but instead of gravity the stationary phase is immobilized by strong centrifugal force (circa 200 g). [22]

The instrument was named **Centrifugal partition chromatography** (CPC). At that time the instrument was unique because any biphasic LL system could have been used as MP and SP. [23]

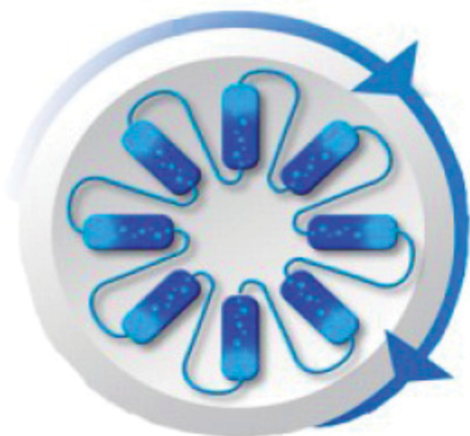


Figure 5: Theoretical detail of CPC instrument. [34]

Figure 6: CPC instrument containing series of extration cells. [21]

CPC machines are known as <u>hydrostatic</u> instruments because they rotate around only one axis. Later the new goal became actual - to develop instruments rotating the column on two axes – <u>hydrodynamic</u> instruments.

Hydrodynamic instruments (modern technology)

The inventor and brain of modern countercurrent chromatography is without doubts Japanese investigator Dr. Yoichiro Ito (Figure 7) who was working in late 1960s in his laboratory at the National Institutes of Health in Bethseda, Maryland (USA). He has successfully published over 600 articles and holds 50 patents for his multiple chromatographic advances. He also dedicated his life to scientific research in chromatography. [24]

Figure 7: Yoichiro Ito. [24]

One of current and widely used chromatographic method is called **High speed countercurrent chromatography** (HSCCC). [25] This up-to-date system has become known as the "J" type configuration because the flow tubes form a letter J.

These machines have helically coiled tubing wound on a bobbin that rotates on its own axis and which itself rotates around a central axis (Figure 8) to achieve a planetary motion – Figure 9.

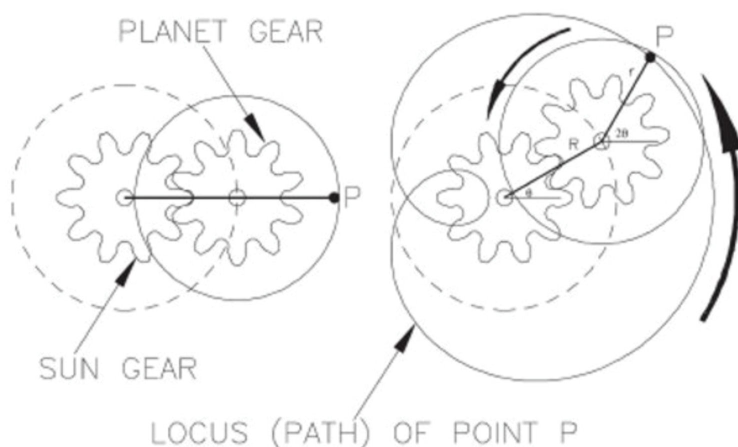


Figure 8: Two axis creating planetary motion. [26]

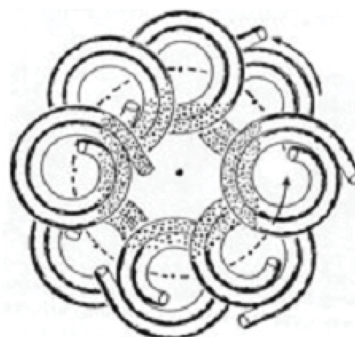


Figure 9: Planetary motion. [26]

“This motion sets up an oscillating hydrodynamic force field, which causes a mixing and settling step. This hydrodynamic force field also causes phases of differing density to travel to opposite ends of the coil; it is this phenomenon alone that retains the stationary phase.” [16]

One of big advantages of this system is that it operates at low pressure allowing higher mobile phase flow rates and hence shorter separation times. [27]

However, the stationary phase retention of HSCCC machines was still poor in comparison with CPC machines. Moreover, HSCCC machines are only able to generate a g-level between 55-80 g which causes relatively low mobile phase flow rates if high SP retention (explained in 4.3) is to be maintained. These low flow rates mean that cycle times are still measured in hundreds of minutes (separations take usually between 3 to 6 hours, a time scale generally unacceptable to industry).

In the early 2000s, Dynamic Extractions designed and built the first true high speed and high g-level machines (240 g) called **High performance countercurrent chromatography** (HPCCC). See the picture of real machine in Figure 10.

Figure 10: HPCCC machine developed by Dynamic Extractions. [28]

Some important differences between HPCCC and HSCCC are mentioned in Table 3.

Table 3: Differences between HPCCC and HSCCC presented on example of separation of two isomers isolated from Chinese medicines. [26]

	HPCCC	HSCCC
Sample capacity per run (g)	43	2
Run time (min)	45	450
Productivity (mg/min)	970	4.44
Purity of isolated compounds (%)	>99.9	>98.5

More about HPCCC will be described in 5.2.

4.2 Present of liquid-liquid extraction

In the market there are only few companies dealing with liquid-liquid chromatography using centrifugal force to stabilize the stationary phase.

1. Important CCC machines providers are:
   a) Dynamic Extractions Ltd. *(Uxbridge, UK)* – offering HPCCC for laboratory (gram) and production (kilo) scale separation (see more information about two currently offered types of HPCCC in Table 4) and other ancillary equipment; the advantage of Dynamic Extractions is the combination of analytical and semi-preparative column in one instrument; Teva (Opava) purchased one of their Spectrum HPCCC machines in June 2013 [29]

   Table 4: HPCCC types offered by Dynamic Extractions Ltd. and their specification.

HPCCC instruments				
	SPECTRUM		**MIDI**	
Column volume (ml)	18	136	19	940
Sample injection range (g)	0.01-0.3	1-2	0.01-0.3	15-25
Flow rate MAX (ml/min)	2	10	2	100

   b) AECS *(Bridgend, UK)* – offering CCC and CPC machines, developing new instruments [30]
   c) Tauto Biotech Shanghai *(China)* – manufacturing of HSCCC (semi-preparative and preparative scale) working with 16-4800 ml column volumes, 400-2000 revolution speed range and 0.5-50 ml/min flow rate range [31]
   d) Pharma-Tech Research Corporation *(Baltimore, MD, USA)* – HSCCC manufacturing

2. Important CPC machines providers are:
   a) Sanki Engineering Co., Ltd. *(Kyoto, Japan)* – first CPC machines
   b) Armen Instrument *(France)* – semi-preparative and preparative systems; 50-1000 ml column capacity, 0.1-30 g injection range, 0.5-50 ml/min flow rate range [32]
   c) Kromaton & Rousselet Robatel *(France)* – Fast CPC; 25-18000 ml rotor volume, 0.01-30 g injection range, 1-50 ml/min flow rate range, 2000-3000 rpm rotation speed range [33]
   d) Rotachrom *(Dabas, Hungary)* – Industrial scale CPC; 300-50000 ml column capacity, 200-10000 ml/min flow rate range [34]

4.3 Theory of countercurrent extraction

In LL extraction in general there is one fluid called **mobile phase** in which the sample (mixture) is dissolved in. This phase carries the mixture through another fluid called **stationary phase**. Depending on differential partitioning of the compounds between MP and SP the separation is provided (see more in 6.1)

Modes of operation

Normal phase – when this mode is performed the column is filled up with the SP and retained there. The MP is then pumped through the column. In this case the SP is the lower phase (heavy phase, more polar) collected from the separatory funnel while the MP is the upper phase (light/organic/non-aqueous).

Reverse phase – the column is filled up with less polar/upper phase as the SP while the aqueous/lower phase is used as the MP. This mode enables more polar components to elute first because of their affinity to the MP.

Stationary phase retention

= a volume of the SP held inside the column during the whole process of separation
In CCC the column is filled with the SP and the coil is then rotated at a proper speed. After that MP is pumped in at a certain flow rate. As the MP progressively flows through the column, it slowly sets up an equilibrium between the two phases (see "hydrodynamic equilibrium" in paragraph below). Even after an equilibrium is set some stationary phase is pushed by the MP out of the coil (this is called „**bleeding**")

The SP retention in equilibrium (= initial SP_{ret}) can be calculated:

$$SP_{eq} = \frac{V_{column} + V_{dead} + V_{loop} - V_{pushed}}{V_{column}} * 100 \quad [\%]$$

SP_{eq} is basically the volume of SP that is inside the column when the equilibrium is set.
V_{column}...total column volume, see in 6.3
V_{dead}...all the volume in a chromatographic system except from column volume, see more in paragraph "Dead volume" - page 22, calculated as 1.6 ml (6.3)
V_{loop}...loop volume for sample injection - 4 ml, see in 6.3
V_{pushed}...volume of SP pushed out of the column before equilibrium

After an experiment is over the volume of remaining SP is calculated.

$$SP_{end} = \frac{V_{residual}}{V_{column}} * 100 \quad [\%]$$

$V_{residual}$ ... the rest of SP collected after the experiment is ended
V_{column}...total column volume, see in 6.3

The volume of SP retained in the column can vary with MP flow, rotation speed and physical properties of the solvent system.

In CCC a minimum of 10 % SP retention is required for the separation but the retention of over 50 % is satisfactory (the more the better).

Hydrodynamic equilibrium

"Hydrodynamic equilibrium" is a situation when the column is partially filled with SP and partially with MP flowing through and no SP is collected. The system is then ready for a sample injection.
Before an equilibrium is set, some SP is pushed out of the column due to low centrifugal force or the MP trying to force through.

Distribution constant (=partition ratio) or distribution coefficient

=„K_d" in CCC expressed as „D" is the equilibrium constant for the distribution of an analyte between two immiscible solvents

$$D=\frac{C_S}{C_M}$$

D....distribution coefficient
C_S...concentration of the sample component in the SP
C_M...concentration of the sample component in the MP

This formula tells us that a component of the sample with <u>high D</u> will have a higher concentration in the SP than in the MP and vice versa (<u>low D</u> means higher concentration in the MP).

If D=0 the component stays in the MP.
If D=1 the concentration in the SP and MP is equal.
If D=∞ the component is present only in the SP.

For my work the ideal D=1-5 so that the separation of components from the mixture is satisfactory (PharmDr. Tomáš Holas, Ph.D., personal communication September 15, 2015).
Higher D would mean long time of separation and low D would cause very fast and inaccurate separation (poor peak resolution).

Theory of CCC is based on countercurrent distribution (CCD). It is usually carried out using large number of test tubes - each partly filled with lower phase (LP) and upper phase (UP). Then the sample mixture is put into the first test tube, shaken properly and left to settle - the components in the sample mixture will distribute over the phases by their partition coefficient. The UP of every test tube is moved to the next one. The process continues until the desired components are eluted from the test tubes. [35, 36] Components with strong affinity to the LP stay in first few tubes whereas components being held in the UP move with this phase to next tubes. See Figure 11 where e.g. "D=32" corresponds with compounds with strong affinity to the LP.

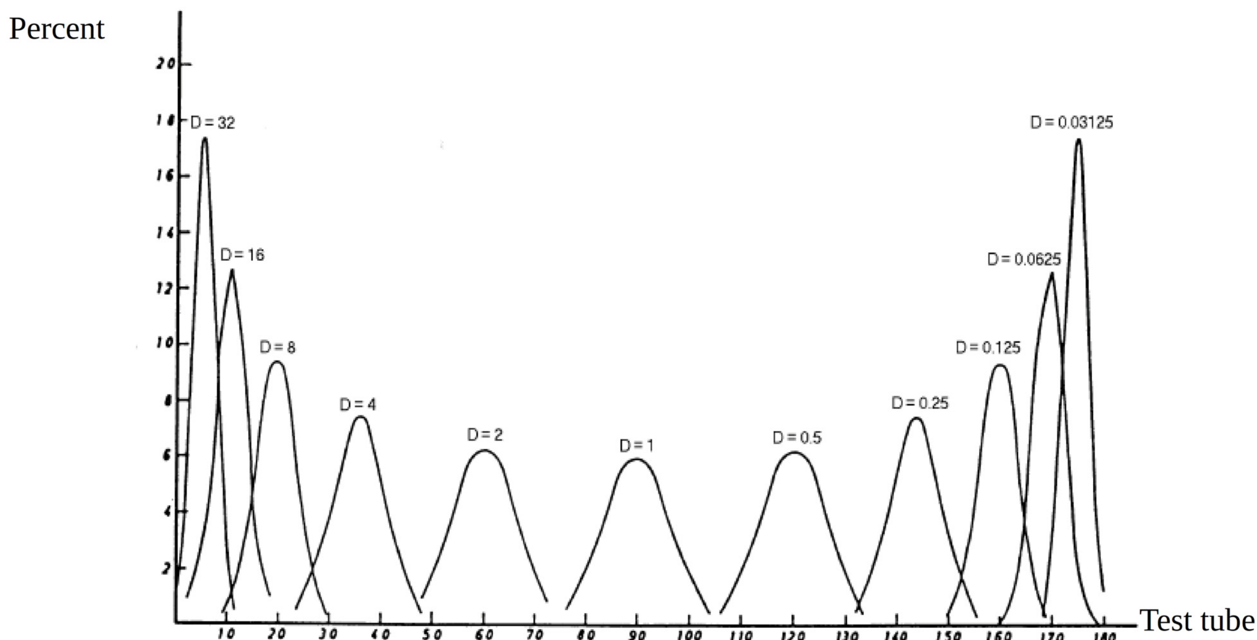


Figure 11: Theoretical countercurrent distribution of components with different distribution constants. [37]

Dead volume

Dead volume refers to all the volume in a chromatographic system from the injector to the detector other than the column such as tubing used to connect components, volume within the detector cell, etc. This volume only has the ability to broaden the peak but has no influence on separation. The goal is to reduce the dead volume as much as possible. In countercurrent chromatography this requires short tubing, small-volume detector cells, etc. The volume was calculated in 6.3.

Selectivity factor „α“

This factor describes the separation of two solutes (A and B) on the column. It is defined as the ratio of the retention factor of two sequent peaks:

$$\alpha = \frac{k_2}{k_1} \qquad k = \frac{K_d \times V_S}{V_m}$$

V_S ....SP volume, V_M ...MP volume

The selectivity ratio is always equal 1 or higher than 1.
If α=1 no separation is possible.

Solvent system (SS)

As CCC is a system with both SP and MP being immiscible liquids, the column is filled with two equilibrated phases forming a biphasic liquid system.
There is also an impact of the temperature, therefore we have to check it so that it does not change rapidly during the separation. [38]

Biphasic SS is created by:

- mixing 2 immiscible solvents – after the equilibrium is reached, each phase is saturated with the other
- mixing 3 solvents – when the wider choice of properties is required, the graphic description is called the „**ternary diagram**“ (see in Figure 12), 2 solvents are immiscible, the third is miscible with only one or both
- mixing 4 or 5 solvents (e.g. HEMWat system, see 4.3c)

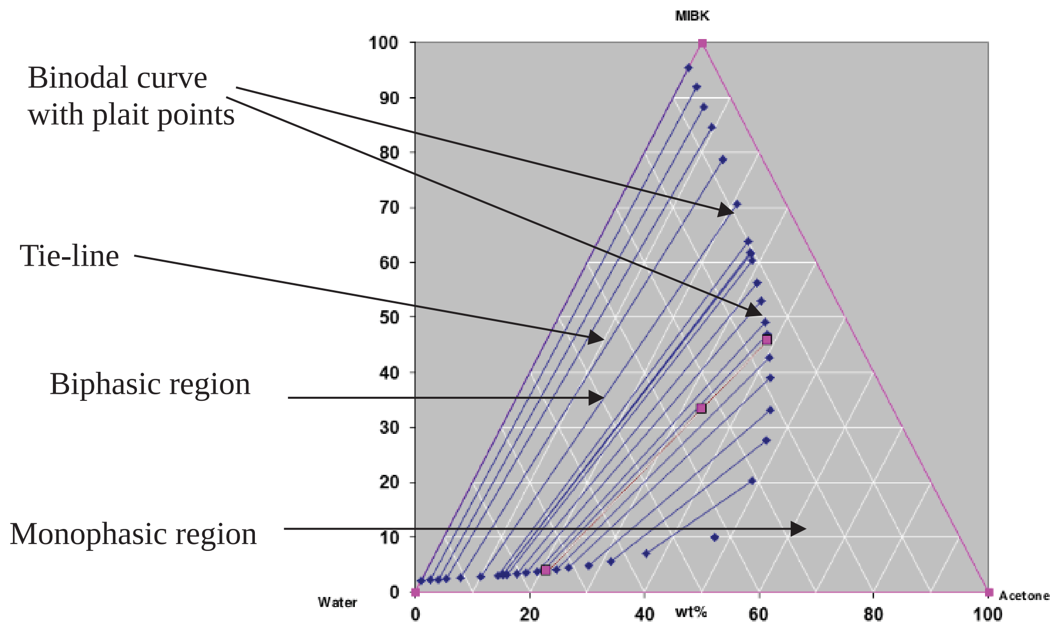


Figure 12: Model ternary diagram of MIBK, acetone and water creating biphasic system.

Plait points – in these spots the system is almost monophasic (in CCC the goal is to have the SS chosen from the biphasic region as far from the plait point as possible)
Tie-line – the line touching the binodal curve

Ternary diagram adjustments

1. It is possible and sometimes useful to change the solvent system ratios for minimising solvent consumption (see Figure 13 as an example). First we test the system for ratio “2“. If tested sample partitions preferentially into organic phase (lower) but there is not enough LP volume for the experiment - the possible improvement is moving to ratios “3-5” (adding more chloroform, reducing water and MeOH). The ratio is changed in benefit for organic phase.

The only difference between “1-5” is the phase volume ratio but their compositions are identical and CCC separation will give exactly the same results.

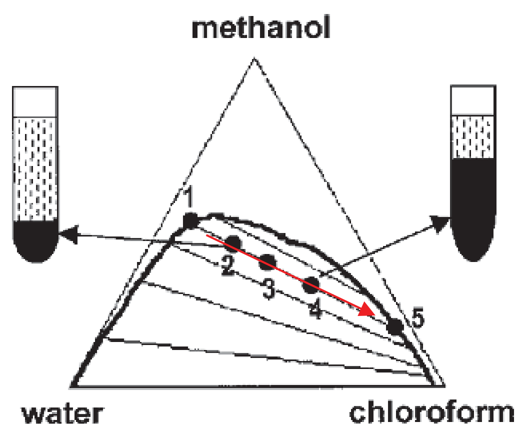


Figure 13: Changing the SS ratio for more preferential phase volume. [38]

2. Moving perpendicularly to the tie-line it is possible to change the distribution coefficient of the sample (=presence of the sample in either upper or lower phase) by changing the SS ratios.

Example:
Water and chloroform create a biphasic system (water is the upper phase). MeOH is the co-solvent, miscible with both phases.
If our sample is first mostly present in more polar phase (mostly water) but we need it to move to the less polar phase (mostly chloroform), we add more methanol (and remove water, chloroform volume stays the same) – see the red arrow in Figure 14. This strategy was also used for this work.

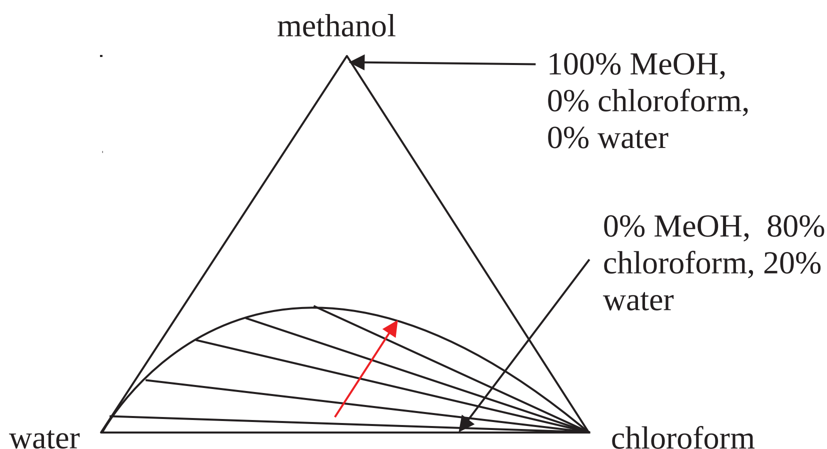


Figure 14: Change of the distribution coefficient using ternary diagram.

Strategies for solvent system selection: [39]

- The „best solvent“ approach (first select the best solvent where the sample will dissolve and then choose two immiscible solvents having strongly different polarities – creating different partitioning of compounds and separating them due to different polarities of these compounds)
  - e.g. if the „best solvent“ is MeOH the more polar solvent is water and the less polar is toluene → together they create a biphasic system used in CCC

- Multisolvent system aproach:
  a. *The **Oka** approach*
     =n-hexane/ethyl acetate/n-butanol/methanol/water
     - 18 binary or ternary mixtures with different polarities
     - best for polar samples (SS always contains water)
  b. The **HBAW** approach
     =n-heptane/acetonitrile (ACN) (1:1) and n-butanol/water (1:1)
     - binary, ternary or quaternary systems
     - useful for samples soluble in acetonitrile or n-butanol
  c. The **ARIZONA** approach, also called **HEMWat**
     =n-heptane/methanol system (1:1, less polar phase) and ethyl acetate/water system (1:1, polar phase), see Table 5 for HEMWat ratios.

Table 5: HEMWat solvent system ratios.

HEMWat system #	relative proportions of solvents			
	hexane	ethyl acetate	methanol	water
-8	10	0	10	0
-7	9	1	9	1
-6	8	2	8	2
-5	7	3	7	3
-4	7	3	6	4
-3	6	4	6	4
-2	7	3	5	5
-1	6	4	5	5
0	5	5	5	5
+1	4	6	5	5
+2	3	7	5	5
+3	4	6	4	6
+4	3	7	4	6
+5	3	7	3	7
+6	2	8	2	8
+7	1	9	1	9
+8	0	10	0	10

Less polar

More polar

  d. The **expanded ARIZONA** approach HEMWat solvent system ratios
     =n-heptane/methanol/acetonitrile system (1:1) and
     ethyl acetate/MTBE-water system (1:1)
     - MTBE=methyl *tert*.-butyl ether
     - the solubilization is upgraded
  e. **Acetone-based solvent scale**
     =heptane/toluene/acetone/water
     - acetone is a good solvent for many crude mixtures of secondary plant metabolites
     - toluene can be replaced by methyl isobutyl ketone (MIBK) or ethyl acetate (EA) for another ternary diagram

4.4 Taxanes separation by countercurrent chromatography

In the literature there are only few methods of taxanes separation using various types of genus, instruments and most of all solvent systems (see Table 6). The most cited method is from 1994 using MIBK/acetone/water as SS but was performed with CPC.

Table 6: The list of taxanes separations created after literature and Internet search.

SAMPLE	GENUS	INSTRUMENT	SOLVENT SYSTEM	D	YEAR
10-DAB	*T. chinensis*	HSCCC	n-hexane/ethyl acetate/ethanol/water (2:5:2:5) + hexane/chloroform/methanol/water (5:25:34:20)	n.s.	1994 [40]
PAC	*T. baccata*	not specified (n.s.)	hexane/ethyl acetate/methanol/water (6:4:5:5)	n.s.	1998 [41]
PAC	*T. baccata*	n.s.	hexane/ethyl acetate/methanol/water (6:3:5:5)	n.s.	1998 [41]
10-DAB	*T. baccata*	CPC	MIBK/acetone/water (2:3:2)	n.s.	1994 [42]
10-DAB	*T. baccata*	CPC	MIBK/acetone/water (2:3:2)	1.72	2015 [43]
10-DAB	*T. baccata*	CPC	MIBK/acetone/water (2:3:2)	1.35	2015 [43]

5. EXPERIMENTAL PART

5.1 Targeted molecules

PACLITAXEL (= PAC, Taxol)

Paclitaxel (see figure 15) was first isolated in 1971 from the bark of the Pacific yew (*T. brevifolia*) and approved for medical use in 1993.

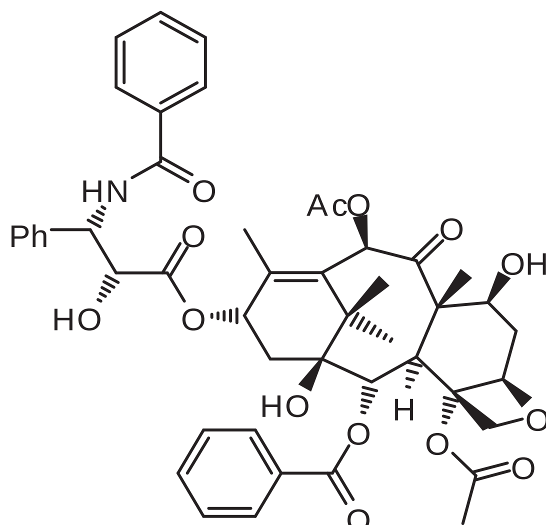


Figure 15: Paclitaxel.

Molecular Weight: 853.906 g/mol

CAS No.: 33069-62-4

IUPAC name: (2α,4α,5β,7β,10β,13α)-4,10-Bis(acetyloxy)-13-{[(2*R*,3*S*)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate

Empirical Formula: $C_{47}H_{51}O_{14}$

Appearance: White solid

Solubility: Soluble in dimethyl sulfoxide (DMSO), MeOH, ethanol (EtOH), ACN (insoluble in water)

10-DEACETYLBACCATIN (= 10-DAB)

10-DAB (see Figure 16) is natural organic compound, the fifth intermediate in the semi-synthetic manufacturing of paclitaxel and starting raw material for docetaxel (and other taxanes), isolated from needles and roots of the yew tree – *Taxus brevifolia, Taxus canadensis, Taxus baccata* and related species.

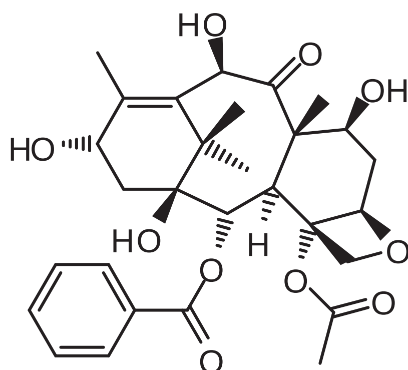


Figure 16: 10-DAB.

Molecular Weight: 544.6 g/mol

Empirical Formula: $C_{29}H_{36}O_{10}$

CAS No.: 32981-86-5

IUPAC name: (2a*R*,4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*,12b*S*)-12b-(Acetyloxy)-12-(benzoyloxy)-1,2a,3,4,4a,6,9,10,11,12,12a,12b-dodecahydro-4,6,9,11-tetrahydroxy-4a,8,13,13-tetramethyl-7,11-methano-5*H*-cyclodeca(3,4)benz(1,2-b)oxet-5-one

Appearance: White solid

Solubility: Soluble in DMSO, MeOH, EtOH, ACN (insoluble in water)

9-DIHYDRO-13-ACETYLBACCATIN III (= DHB, 9-DHAB III, 13-Acetyl-9-dihydrobaccatin III)

DHB (see Figure 17) is the most abundant taxane found in needles of the Canadian yew tree (*Taxus canadensis*). [44]

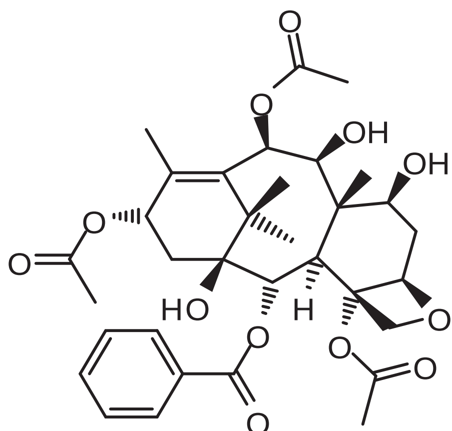


Figure 17: DHB.

Molecular Weight: 630.68 g/mol

Empirical Formula: $C_{33}H_{42}O_{12}$

CAS No.: 142203-65-4

IUPAC name: (2α,5β,7β,9α,10β,13α)-4,10,13-tris(acetyloxy)-1,7,9-trihydroxy-5,20-epoxytax-11-en-2-yl benzoate

Appearance: White solid

Solubility: Soluble in DMSO

5.2 HPCCC characterisation

HPCCC instrument is based on LL partitioning and is considered modern and beneficial purification method. There should not be a competition seen between HPLC and CCC machines because they both have different use and advantages. Moreover the combination of HPLC and CCC is possible and can reduce the number of purification steps.
The difference between HPLC and CCC is that in CCC the solute can access the whole volume of the SP (liquid) x in HPLC it only accesses the interface between MP and SP (solid) – this creates much faster overloading in HPLC.
The CCC rotation speed, injection volume and flow rate ranges are mentioned in 4.2.

The HPCCC apparatus consists of a Teflon tubing coiled around a bobbin on a drum which is centrifugally rotated in a double-axis planetary motion. The separation principle was mentioned in 4.3 and the whole separation process is described below. The LL partition based on different affinity of the compound for the SP is the only phenomenon responsible for the retention of solute.

The HPCCC separation procedure in short

The column is initially filled with the SP of the suitable solvent system and the apparatus is rotated at a proper speed while the MP is pumped into the column. After the hydrodynamic equilibrium is established (see page 21) the prepared sample solution is injected into the column through the injection valve (it is important not to make any bubbles for accurate separation). The temperature is maintained, compounds are monitored by a detector and fraction are collected. Then the rotation is stopped and rest of the SP is collected as well.
The scheme of HPCCC by Dynamic Extractions is shown in Figure 18 (scanned from instruction manual).

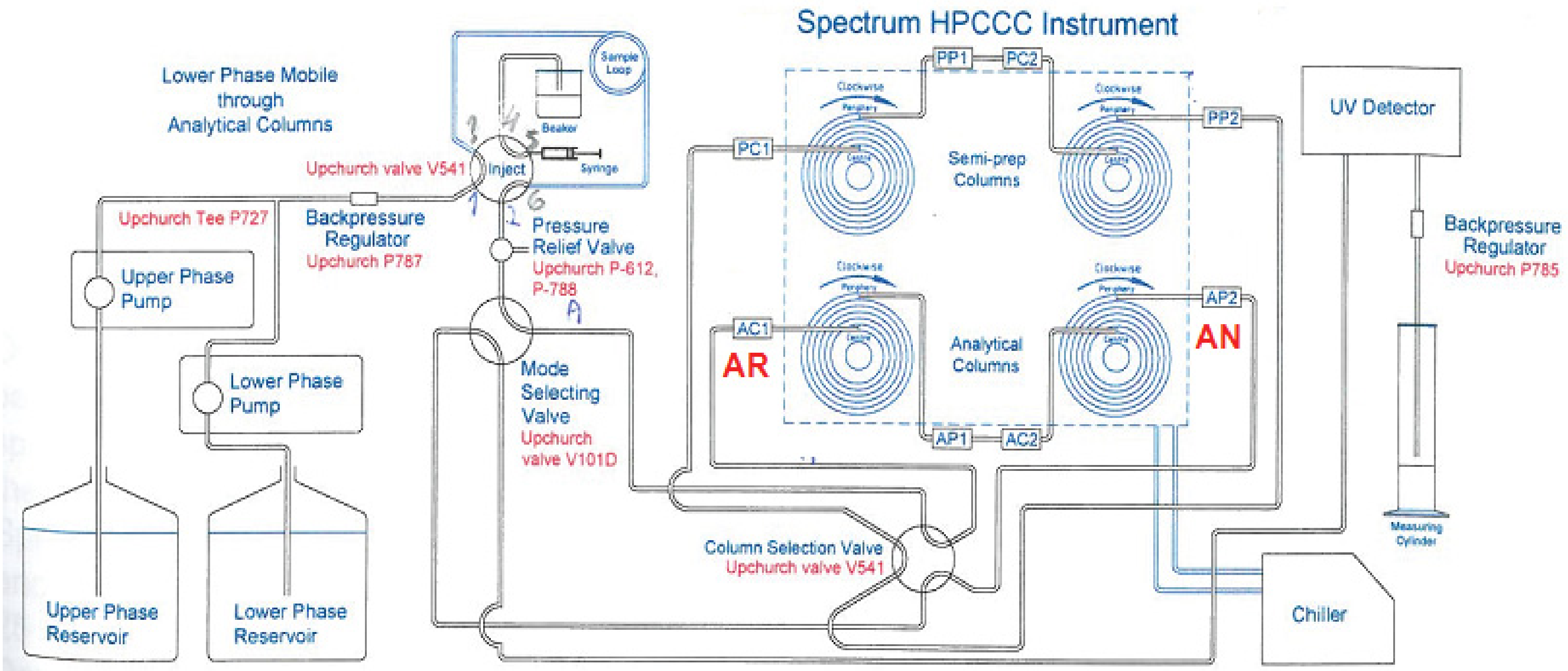


Figure 18: Spectrum HPCCC instrument (by Dynamic Extractions) in detail.

Description:
Liquid phases are pumped through backpressure regulator and injection valve. Depending on current valve setting it is either possible to switch the valve to pump liquids directly into column (used before an equilibrium is set, e.g. numbers 3→1 leaving the loop disconnected) and at the same time inject sample into loop (6→2) or connect the loop containing sample with column (as it is seen in Figure 18, 1→3→6→2). Mode switching valve allows to change modes (even during the separation process, see "Dual mode" in this chapter). Figure 18 shows reverse mode (pumping AR→AN) using analytical columns.

AR = analytical column used in reverse mode, the liquid is pumped through the column from the centre ("**head-to-tail mode**")
AN = analytical column used in normal mode, the liquid is pumped from the end of the column ("**tail-to-head mode**")
Analytical column contains of two bobbins, both can be entered centrally (AC1 and AC2) or peripherally (AP1 and AP2)

Standard HPCCC system set-up

Since CCC uses only liquid SP special column adjusted for centrifugal motion is required. Other equipment used is basically the same as for HPLC (see Figure 19):

- two phase reservoirs containing solvent system already prepared – one reservoir for UP and one for LP
- suitable pump to deliver the solvent
- HPCCC instrument with valve box – allowing one to easily change between analytical/preparative column, normal /reverse phase and easily inject and load samples
- detection system (e.g. UV lamp)
- fraction collector
- computer with chromatographic software
- chiller – cooling the rotating parts of centrifuge

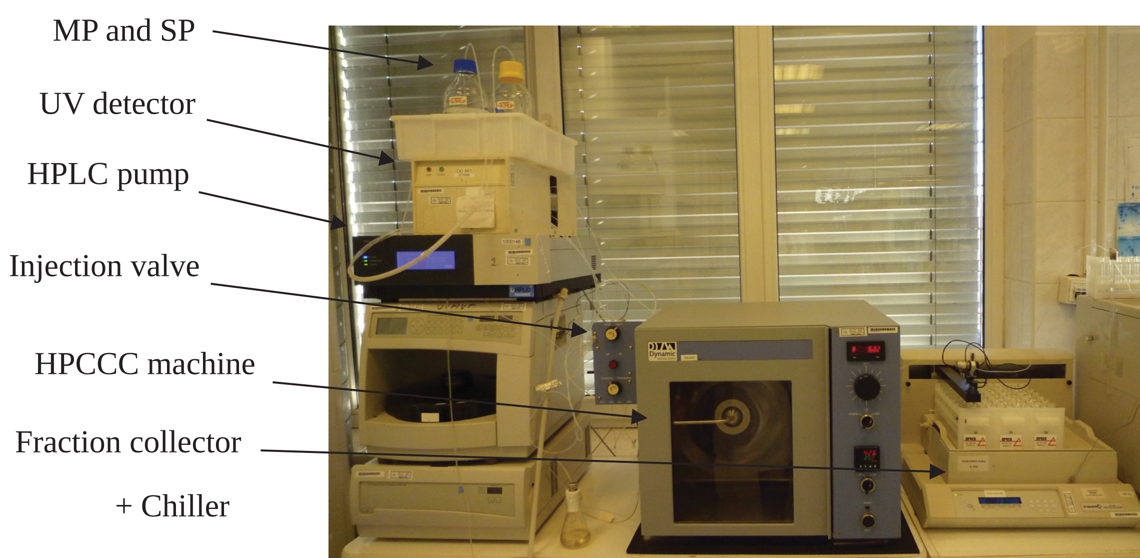


Figure 19: Example of HPCCC system set-up.

Advantages of HPCCC

- the mildest form of chromatography (no loss of substrate by binding to the column)
- high mass and volume injection loadings (vs. preparative HPLC, see 4.2)
- wide choice of biphasic solvent
- can be used when the solubility of the sample is problematic
- reduced sample preparation
- total sample recovery (=non-destructive method)
- scalable - CCC is able to range from milligrams to tens of grams on the same instrument.
- no expensive chromatographic solid phase to purchase
- low pressure operation (4-7 bar)

Elution strategies

'Standard' or 'Classical' elution mode
In CCC both phases (UP or LP) can be mobile or stationary and therefore it is possible to perform a separation in:

- **normal phase** (NP) mode where the less polar (always organic) liquid is used as the MP
  - less polar (lipophilic) components of the sample are eluted first because they do not have a strong affinity for the polar SP
- **reverse phase** (RP) where the more polar (aqueous) liquid is the MP (Figure 20)
  - more polar components are eluted first because of their affinity to polar MP

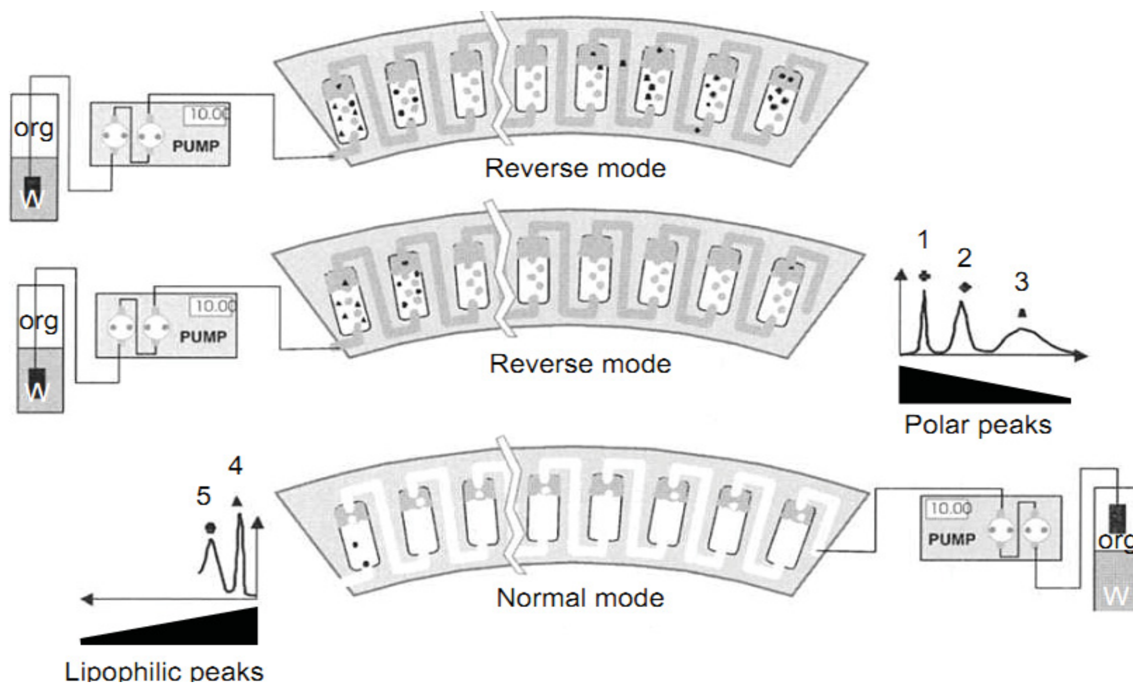


Figure 20: Reverse and normal phase mode. [45]

Dual mode
When operating CCC there is a possibility of switching the phases during a run (NP → RP or RP → NP). This is called a dual mode elution strategy. Basically you first pump one of the phases as the mobile phase and after a set period of time you switch the mode selecting valve and start pumping the other phase as mobile. The direction of flow is also changed, [46] see the "mode selecting valve" in Figure 18. You can repeat this switching procedure until your desired resolution is achieved. [47] Big advantage of this method is that components with strong affinity for the original stationary phase (that would be otherwise strongly retained with a long classical mode elution time) can be separated and eluted quickly = beneficial for fractionation of molecules presenting very different polarities.
It can also be inexactly comprehended as a kind of backward running. Figure 20 - looking at first two cavities in reverse mode there are some lipophilic compounds held inside (black dots in the middle picture) after the normal mode switch they are immediately eluted.

6. RESULTS AND DISCUSSION

6.1 Test tube experiments

Test tube experiment is a process of measuring the distribution constant (known as "D").

The separatory funnel (or test tube or any other mixer-settling tank) is filled with tested solvents in defined ratio. Two phase system is shaken properly (for at least 1-2 minutes) for proper phases saturation. When the mixing stops the immiscible phases settle and create an interface. Exact volume of both phases (e.g. 10 ml and 10 ml) is transferred to another extraction funnel and tested molecule is added. The sample distributes between phases whilst the mixture is shaken intensively again. Samples from UP and LP are analyzed by HPLC or other method.
Samples for HPLC are prepared as follows: 1.0 ml of UP is put in vial No.I and 1.0 ml of LP is put into vial No.II → dried with N_2 → diluted in MeOH.

From HPLC area "D" is calculated. Other technique is performing Thin-layer chromatography=TLC but results are only approximate (proper amount of the UP and the LP is put on TLC plate, developed and visually compared). [48]

6.1.1 Distribution coefficients for various solvent systems

For data input it was necessary to test various SS and their ratios using test tube experiments (see 6.1). Some of them were inspired by literature search, other had already been prepared in the laboratory and together they created initial data – see Table 7.

Solvent systems containing hexane are too lipophilic and give very low D, for that reason they are unsuitable for our experiments.

Red highlighting stands for solvent systems with convenient distribution coefficients for our experiments in reverse mode and therefore can be considered usable for further experiments. Only three of them were eventually selected:

- MIBK/acetone/water 2:3:2
- toluene/acetone/water 4:2:4
- toluene/acetone/water 4:5:1

MIBK/methanol/water 3:3:4 was tested preliminary and showed significant bleeding, see experiments 6.2.1+6.2.2 and 6.4.2+6.4.3.

Blue highlighting stands for SS convenient for being tested in normal mode.

SS volume ratio is included in the table because it is easier to measure liquids by volume.
Mass ratio was also included because this type of measurement was used in ternary phase diagrams generated by DynoChemResources in Teva files. Density of individual solvent had to be bear in mind.
Solvent systems containing MIBK were processed using density. The only SS that occurred in the literature was MIBK/acetone/water 2:3:2 (volume ratio). To make a series of at least 3 ratios it was convenient to use ternary diagram created in DynoChemResources. These diagrams use mass volumes and therefore ratios had to be recalculated into volume ratios.

Information about separation is also important and can be beneficial for total time of experiment.

Table 7: Summary of test tube experiments containing distribution coefficients.

	SS	SS ratio (vol)	SS ratio (mass)	Notes	RP (UP/LP)			NP (LP/UP)			selectivity	
					DAB	*DHB*	*PAC*	DAB	DHB	PAC	α (DAB/DHB)	α (PAC/DHB)
1	H:M:W	4:2:4	32:19:49	slow separation	0.0014	0.0015	0.0081	691.44	647.41	123.39	1.1	5.2
2	H:M:W	4:3:3	33:30:38	reasonable	0.0011	0.0006	0.0010	841.71	1527.7	919.42	1.8	1.7
3	H:M:W	4:4:2	34:41:26	fast separation	0.0004	0.0004	0.0002	2473.3	2346.4	3829.8	1.1	1.5
4	H:M:W	4:5:1	35:52:13	fast separation	0.0003	0.0002	0.0001	2785.9	3423.3	9585.7	1.2	2.8
5	H:A:W	4:2:4	32:19:49	fast separation	0.0011	0.0082	0.0179	903.32	121.48	55.649	7.4	2.2
6	H:A:W	4:3:3	33:30:38	fast separation	0.0018	0.0109	0.0134	546.29	91.333	74.532	6.0	1.2
7	H:A:W	4:4:2	34:41:26	fast separation	0.0084	0.0271	0.0225	117.96	36.844	44.295	3.2	1.2
8	H:A:W	4:5:1	35:52:13	very fast	0.0327	0.1612	0.1853	30.530	6.2015	5.3943	4.9	1.1
9	T:M:W	4:2:4	38:17:44	opaque phases	4.8603	28.923	238.66	0.2057	0.0345	0.0041	6.0	8.3
10	T:M:W	4:3:3	39:27:34	opaque phases	24.438	50.884	304.12	0.0409	0.0196	0.0032	2.1	6.0
11	T:M:W	4:4:2	40:37:23	opaque phases	0.0127	0.2168	0.4980	78.722	4.6114	2.0080	17.1	2.3
12	T:M:W	4:5:1	41:47:12	opaque phases	39.622	7.2728	5.5495	0.0252	0.1374	0.1801	5.4	1.3
13	T:A:W	4:2:4	38:17:44	fast separation	0.8236	28.992	23810	1.2140	0.0344	0.0000	35.2	8213
14	T:A:W	4:3:3	39:27:34	fast separation	1.4113	25.545	477.56	0.7085	0.0391	0.0020	18.1	18.7
15	T:A:W	4:4:2	40:37:23	fast separation	2.0198	17.810	246.67	0.4950	0.0561	0.0040	8.8	13.8
16	T:A:W	4:5:1	41:47:12	fast separation	1.9173	11.778	81.962	0.5215	0.0849	0.0122	6.1	7.0
17	Mi/A/W	32:33:35	3:3:4	fast separation	5.2703	69.311	260.21	0.1897	0.0144	0.0038	13.2	3.8
18	Mi/A/W	33:22:44	3:2:5	fast separation	6.7409	178.86	595.05	0.1483	0.0055	0.0016	26.5	3.3
19	Mi/A/W	2:3:2	27:40:33	fast separation	3.4054	22.799	47.651	0.2936	0.0438	0.0209	6.7	2.1
20	Mi/M/W	33:22:44	3:2:5	fast separation	7.7731	104.36	1093.4	0.1286	0.0095	0.0009	13.4	10.5
21	Mi/M/W	34:11:55	3:1:6	fast separation	10.287	282.78	1953.8	0.0972	0.0035	0.0005	27.5	6.9
22	Mi/M/W	32:33:35	3:3:4	very slow	4.0178	12.030	94.919	0.2488	0.0831	0.0105	3.0	7.9

SS (H=hexane, M=methanol, T=toluene, W=water, A=acetone, Mi=MIBK)

6.1.2 Concentration dependence of distribution coefficient

Sample content: Natural material from genus *Taxus* contains many impurities. Some of them are known and described. Few of them are important to monitor because they are difficult to separate and therefore can appear in final product. Teva monitored impurities are: TB1, TB5, TB6, TB7, TB8, DACM, DAPAC, TB20 and flavonoids 1-6 (for this work it is not important to describe them further).

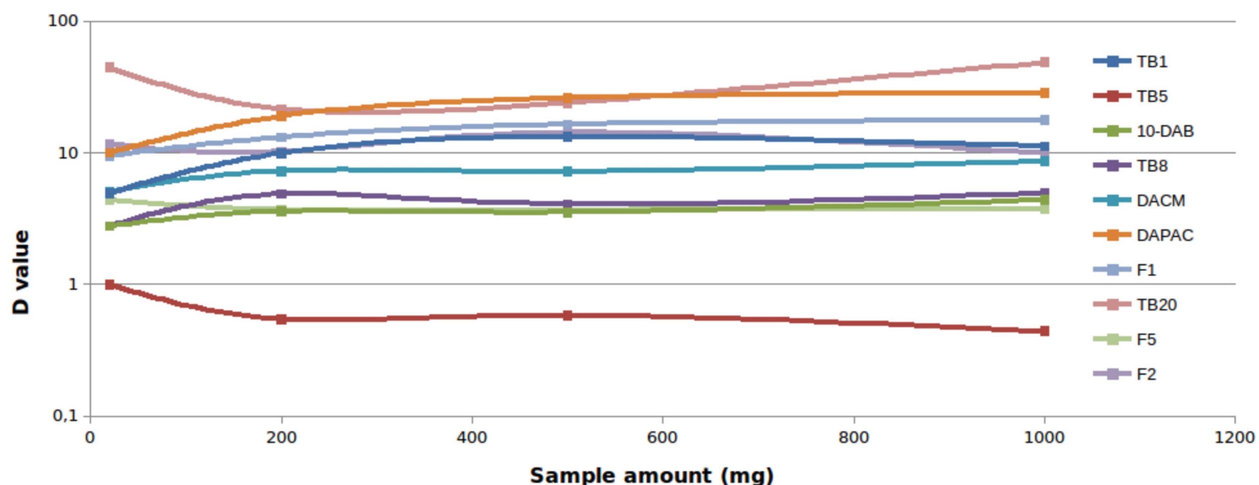
Target: Distribution coefficient dependence in different sample concentrations.

Solvent system: MIBK/acetone/water 2:3:2 chosen for most often cited SS.

Procedure: Various amounts of evaporated Rafinated extract (20, 200, 500 and 1000 mg) were dissolved in 10 ml UP and 10 ml LP (prepared according procedure mentioned in chapter 6.1) and shaken for 30 s in separatory funnel. Then the lower and upper phases containing sample were separated. Exact volume of every phase (see Table 8) was put into vial (e.g. 1000 µl from UP containing 20 mg and 1000 µl from LP containing 20 mg etc.) and diluted with 1.0 ml methanol. All 8 samples were HPLC analyzed, results were processed, plotted in Graph 1 and Table 9 and also D was calculated.

Table 8: Phase sampling.

Input (mg)	Phase volume (µl)
20	1000
200	100
500	40
1000	20



Graph 1: Concentration dependence of distribution coefficient, HPLC evaluation.

Table 9: Concentration dependence of distribution coefficient.

	Sample amount (mg)			
D	**20**	**200**	**500**	**1000**
TB1	4.96	9.99	13.28	11.25
TB5	1.00	0.55	0.58	0.44
10-DAB	2.78	3.59	3.54	4.40
TB8	2.78	4.87	4.08	4.99
DACM	5.08	7.29	7.21	8.67
DAPAC	10.06	19.05	26.18	28.60
F1	9.54	13.16	16.46	17.72
TB20	44.73	21.60	24.05	48.80
F5	4.36	3.69	3.67	3.77
F2	11.71	10.27	14.26	9.96

Discussion: All impurities distribution coefficients were calculated according to chapter 4.3.

From Graph 1 and Table 9 it is evident that there is only slight distribution coefficient change. Distributions coefficients coming from the analytical concentration test tube experiments thus can be used for preparative purposes (preparative column in bigger mass volumes) without significant problems.

6.2 HPCCC bleeding monitoring

6.2.1 Stationary phase loss {MIBK/methanol/water (3:3:4)}

Target: Stationary phase bleeding monitoring.

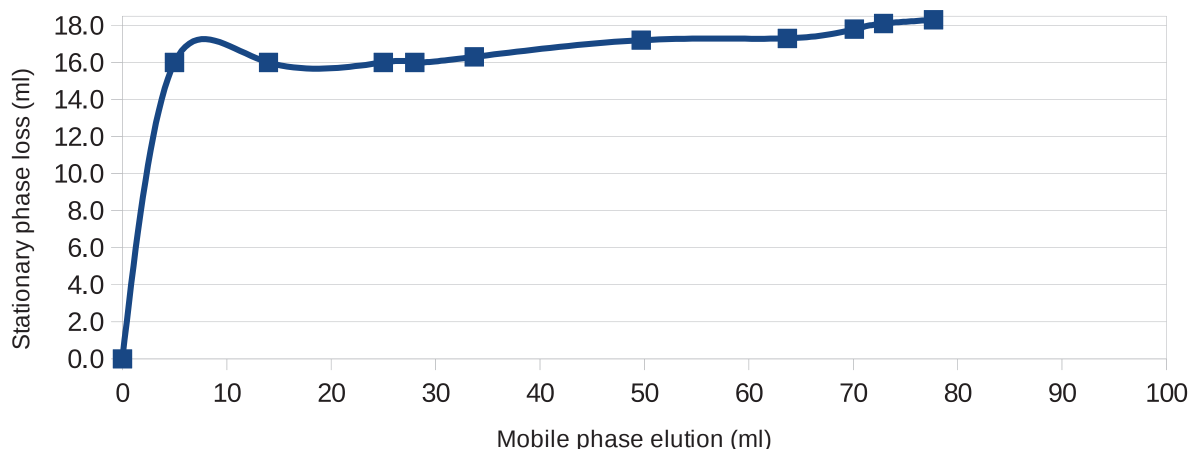
Procedure: SS containing MIBK/methanol/water (3:3:4) was chosen for its suitable D in reverse phase mode (see Table 7) and forms well separated two phases. They are opaque and they have trend to clarify over-night. One-day clear SS was subjected to this experiment.

HPCCC analytical column in reverse phase was filled with upper phase and rotation set up to 1600 rpm. Mobile phase (LP) was injected to the column and stationary phase (UP) volumes were recorded. The flow was 1 ml/min. Table 10 and Graph 2 describe stationary phase bleeding kinetic, Table 11 shows final results.

At the same time front dead volume was calculated and was determined as 1.6 ml (distance between pump and HPCCC AC1 input). Back dead volume was determined as 0.5 ml (from HPCCC AP1 to fraction collector waste tube).

Table 10: Monitoring of MIBK/methanol/water 3:3:4 SP loss without SP retarder.

Time	Stationary phase (UP)	Mobile phase (LP)
06:50	start	
07:11	16	5
07:20	16	14
07:31	16	25
07:34	16	28
07:41	16.3	33.7
07:57	17.2	49.7
08:11	17.3	63.7
08:15	17.8	70.1
08:21	18.1	72.9
08:26	18.3	77.7



Graph 2: Monitoring of MIBK/methanol/water 3:3:4 SP loss without SP retader.

Table 11: Difference in SP loss (start to end) expressed as a percentage.

	Volume (ml)	SP_{ret} at the beginning	SP_{ret} after 100 min
$V_{dead\text{-}front}$	1.6		
V_{column}	25.5	45 %	38 %
$V_{dead\text{-}back}$	0.5		
Total V	27.6		

Discussion: This SS {MIBK/methanol/water (3:3:4)} applied in reverse phase with flow 1 ml/min and 1600 rpm gave stationary phase retention 45 % at the beginning. During 100 minutes SP_{ret} decreased to 38 %. The initial SP_{ret} is too low and therefore has to be improved by $CaCl_2$ addition (working as SP retarder which controls the diffusion of a mobile phase in the stationary phase).

The initial noticeable SP volume increase seen in Graph 2 is the process of equilibrium establishment. The goal is to minimize this volume loss and also minimize substantial bleeding.

6.2.2 Stationary phase loss {MIBK/methanol/water + 1% $CaCl_2$ (3:3:4)}

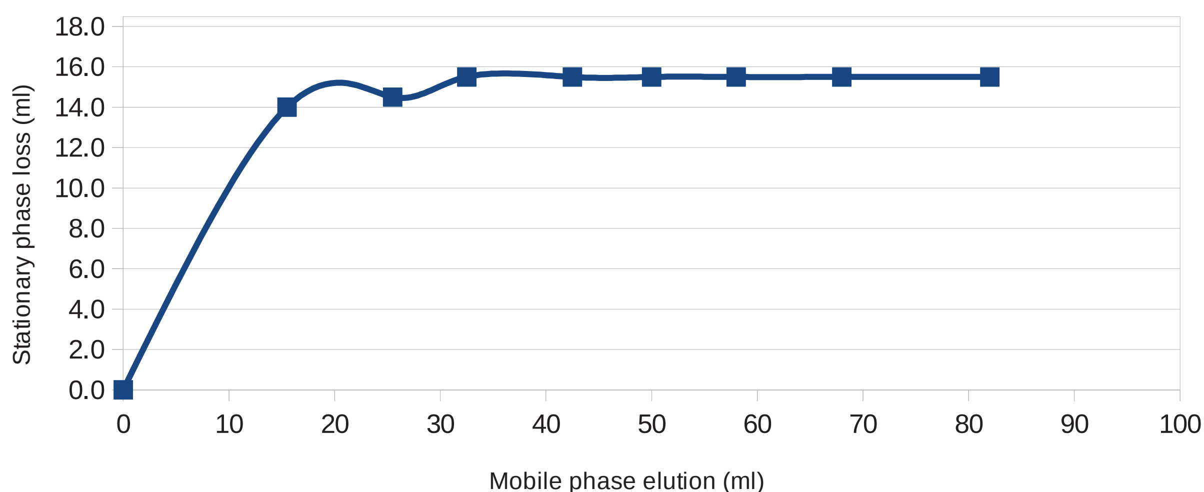
Target: Stationary phase bleeding monitoring, $CaCl_2$ as SP retarder

Procedure: SS containing MIBK/methanol/water (3:3:4; 1% $CaCl_2$ dissolved in water) forms well separated two phases, but they are opaque and they have trend to clarify over-night. New opaque SS was subjected to this experiment.

HPCCC analytical column in reverse phase was filled with upper phase and rotation set up to 1600 rpm. Mobile phase (LP) was injected to the column and stationary phase (UP) volumes were recorded. The flow was 1 ml/min. The Table 12 and Graph 3 describe stationary phase bleeding kinetic. Table 13 shows final results.

Table 12: Monitoring of MIBK/methanol/water 3:3:4 SP loss with SP retarder.

Time	Stationary phase (UP)	Mobile phase (LP)
11:25	start	
11:40	14	15.5
11:50	14.5	25.5
11:57	15.5	32.5
12:07	15.5	42.5
12:14	15.5	50
12:23	15.5	58
12:33	15.5	68
12:47	15.5	82



Graph 3: MIBK/methanol/water 3:3:4 + 1% CaCl2 stationary phase loss.

Table 13: Difference in SP loss (start to end) expressed as a percentage.

	Volume (ml)	SP_{ret} at the beginning	SP_{ret} after 100 min
$V_{dead-front}$	1.6		
V_{column}	25.5	47 %	43 %
$V_{dead-back}$	0.5		
Total V	27.6		

Discussion: 1% $CaCl_2$ addition to SS containing MIBK/methanol/water (3:3:4) tested in RP with flow 1 ml/min and 1600 rpm gave stationary phase retention in equilibrium about 47 %. This SP_{ret} has no tendency to worsen during prolonged time in comparison without $CaCl_2$ addition. The real residual SP retention formed 43 % which is in good relation with loss calculation. From the results we can conclude that 1% $CaCl_2$ can lower bleeding quantitatively. The overall problem of low SP_{ret} for SS MIBK/methanol/water describes probably its physical-chemical behaviour and there is most likely no chance of improvement. However this SS will be tested with real sample just to compare results and definitely exclude it from further experiments.

6.3 Preparation of experiment conditions

Column

- analytical – 25.5 ml
- semi-preparative – 128.5 ml

Both volumes were exactly measured in the laboratory during the process of machine qualification.

Dead volume

- *front* = distance between pump and HPCCC AC1 output → 1.6 ml (calculated during experiment 6.2.1.)
- *back* = distance between HPCCC AP2 and fraction collector waste tube → 0.5 ml

Loop

Calculated from its diameter and length as 4 ml.

In almost all experiments the sample volume was lower than 4 ml. Before the sample was injected loop was rinsed and filled up with lower phase. When the sample was finally injected, it partially pushed some of mobile phase from loop into waste. This process guarantees that loop does not contain any bubble (but while injecting the sample it is necessary not to make any bubble too).

Fraction collection

- preliminary experiments – 5 ml fractions
- preparative experiments – 20 ml fractions for MIBK/acetone/water
  – 10 ml fractions for toluene/acetone/water

HPLC preliminary analysis

HPLC preliminary analysis of Rafinated extract and mixture of 10-DAB+DHB+PAC were used as comparative chromatograms in experiments (Figure 21). In the second blue chromatogram first eluted peak represents 10-DAB, second is DHB and third is PAC.

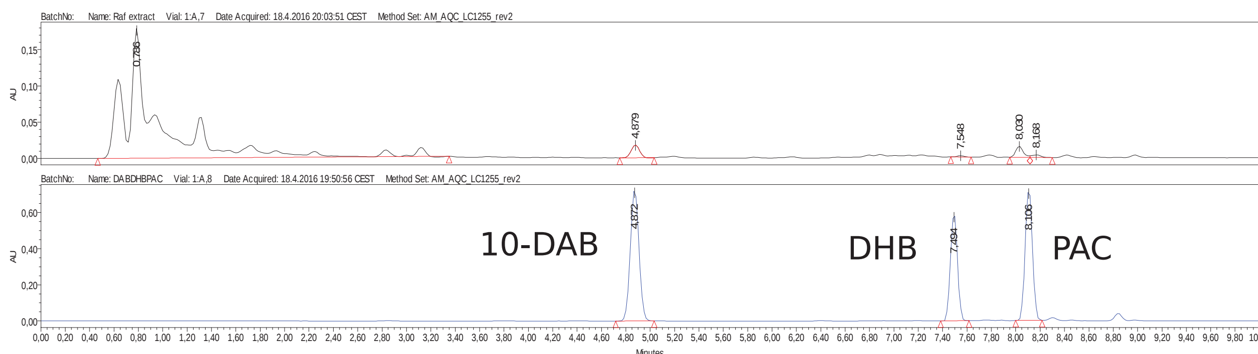


Figure 21: HPLC analysis of Rafinated extract and mixture of 10-DAB, DHB and PAC.

Tested Rafinated extract *(from T. baccata)* only consists of 10-DAB and impurities. Polar impurities are eluted first and make 94 % of the product (see Table 14)

Table 14: HPLC analysis of Rafinated extract.

	Name	Retention time	Area %
1		0.79	94.67
2	10-DAB	4.88	2.56
3		7.55	0.20
4		8.03	2.02
5		8.17	0.55

Figure 22 shows Rafinated extract analyzed using different method focused only on flavonoids (mostly lipophilic impurities occuring in genus *Taxus*). This method enables to see only flavonoids that would otherwise be difficult to see in method used in Figure 21.

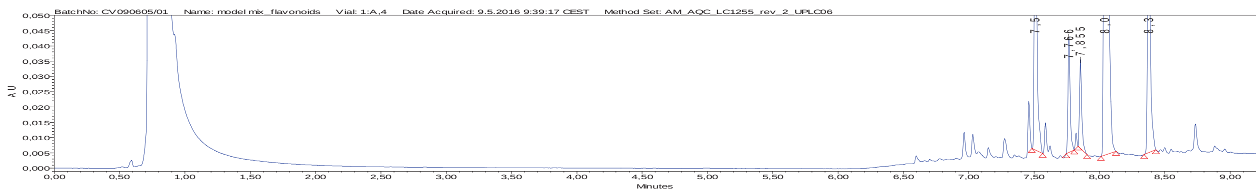


Figure 22: HPLC analysis of Model mix (black) and flavonoids (blue).

Model HPCCC chromatogram is presented in Figure 23.

Voltage

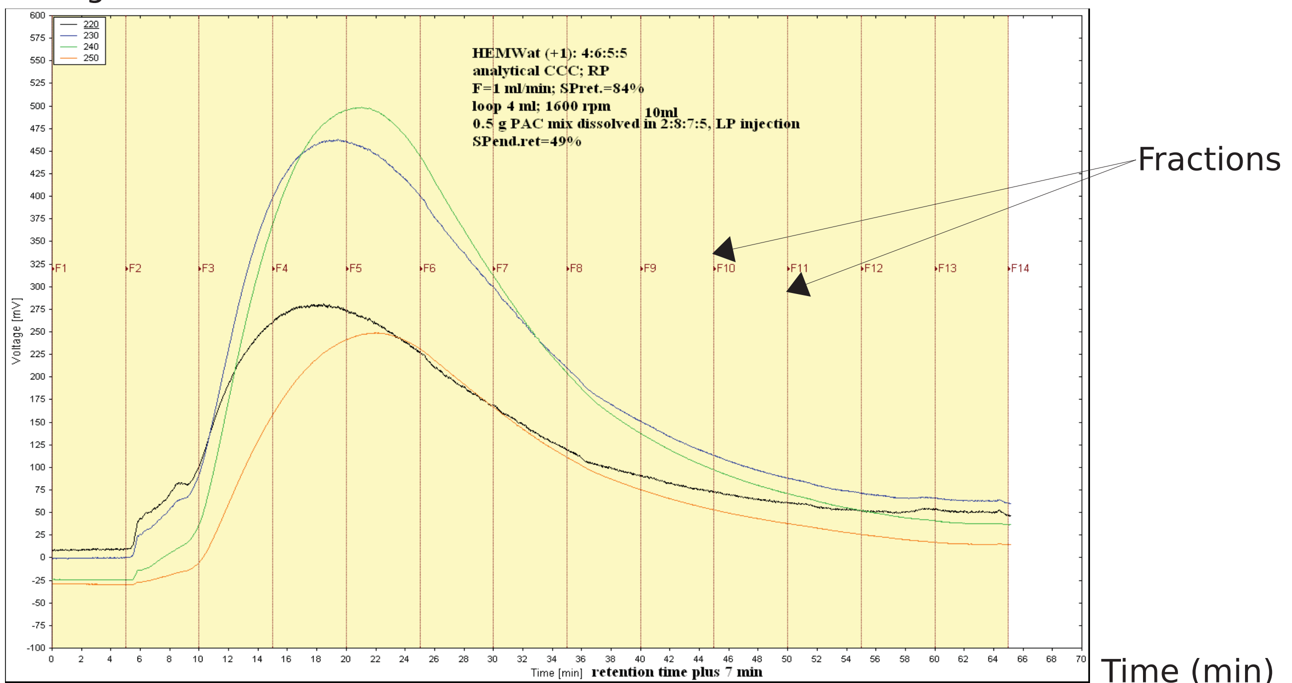


Figure 23: Model HPCCC chromatogram; measured in 4 different length waves (220 nm, 230 nm, 240 nm and 250 nm), in this case 14 fractions were collected.

6.4 Preliminary HPCCC tests

6.4.1 MIBK/acetone/water (2:3:2) – dual mode

Target: The origin of this SS comes from the literature. [42] TLC one spot analysis was used for judgement of 10-DAB, DHB and PAC distribution, see Figure 24.

HPCCC model mixture preparation: 140 mg 10-DAB, 90 mg DHB and 20 mg PAC were dissolved in 10 ml UP of SS.

Procedure: Various SS ratios were tested and results were stated in Table 7 (Figure 25 - see the ternary diagram of all three ratios from Table 7). SS composed of MIBK/acetone/water 2:3:2 showed reasonable distribution coefficient (see Figure 24).

All HPCCC parameters for this experiment are stated in Table 15.

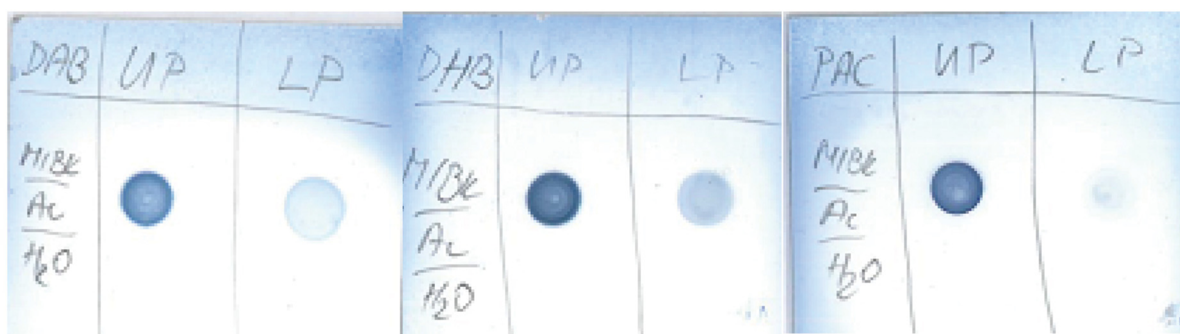


Figure 24: TLC one spot analysis used for MIBK/acetone/water 2:3:2.

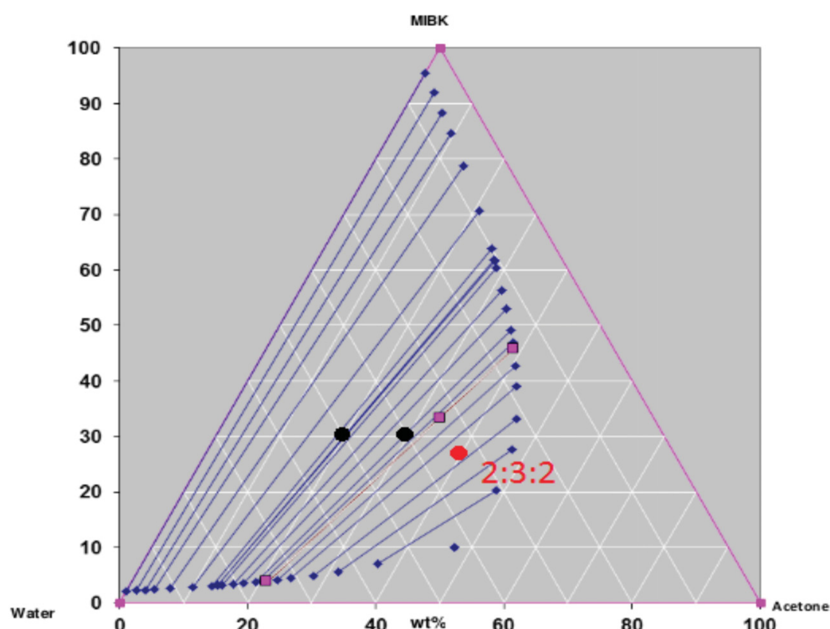


Figure 25: Ternary diagram for MIBK/acetone/water 2:3:2.

Table 15: HPCCC parameters of MIBK/acetone/water 2:3:2 tested on model mixture.

CCC – technical parameters	volume
CCC column volume (ml)	25.5
Total volume (pump-fraction waste; ml), no loop	30.0
Loop volume (ml)	4.0
Dead volume (pump - inj. valve; ml)	1.6
Flow MP (ml/min)	1.0
Fraction volume (ml)	5.0
Eluted SP (first cylinder; ml)	13.5
Injected SP (loop; ml)	4.0
Bleeded only in reverse phase (ml)	11.2
Residual SP (last cylinder; ml)	13
SP_{ret} initial	**65%**
SP_{ret} residual	**51%**

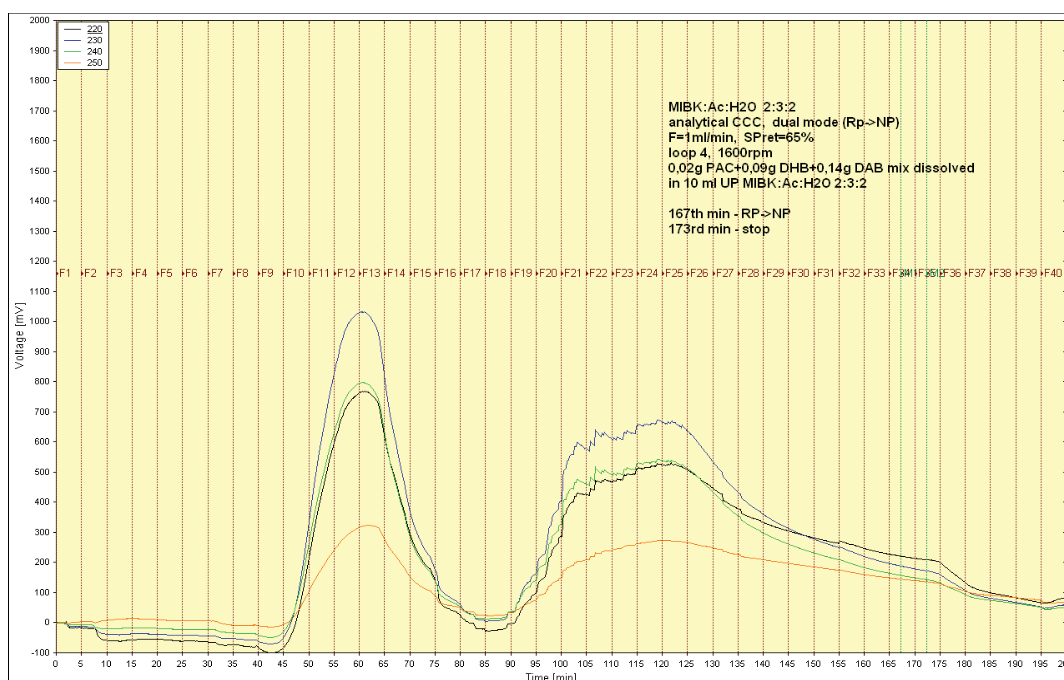


Figure 26: HPCCC UV profile of model mixture in MIBK/acetone/water 2:3:2.

Discussion: The SS suitability was checked by TLC - see Figure 24. According to TLC the expected distribution coefficients are 10-DAB=90:10, DHB=95:5 and PAC=99.9:0.01. Paclitaxel is difficult to dissolve in SS overall, therefore PAC concentration was lowest in this case. The first eluted UV dependent peak is 10-DAB with Rt=60 min. Later eluted broad peak should be DHB (Rt=120 min). Because of paclitaxel high lipophility it is probable that it would not be eluted in the reverse mode and therefore dual mode was used. The reverse phase was changed to normal phase (= dual mode) at 167th minute (first green marker in Figure 26) and the rotation was stopped at 173rd minute (second green marker). Because of PAC low concentration and broadening we did not see any sharp peak. In this experiment fractions were not analyzed by TLC.

6.4.2 MIBK/methanol/water (3:3:4) - reverse phase mode

Target: This suggested SS come from consideration of better sample solubility and acceptable D.

HPCCC model mixture preparation: 200 mg 10-DAB, 80 mg DHB and 20 mg PAC were dissolved in 10 ml UP of SS.

Procedure: Various SS ratios were tested and stated in Table 7 (see the dots in ternary diagram in Figure 27). Only SS composed of MIBK/methanol/water 3:3:4 showed reasonable distribution coefficient (see Figure 28 and red dot in Figure 27). Only TLC with 10-DAB is demonstrated because it is the most important compound.

All HPCCC parameters for this experiment are stated in Table 16, bleeding is shown in Graph 4 and TLP fraction analysis is shown in Figure 30.

Figure 28: TLC one spot analysis.

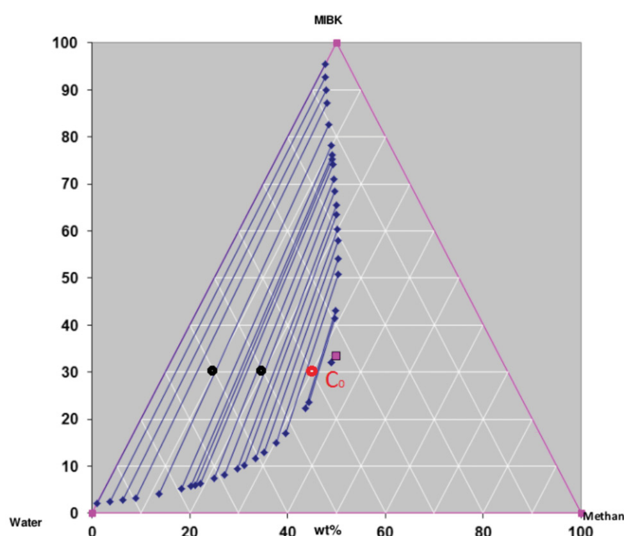


Figure 27: Ternary diagram for MIBK/methanol/water 3:3:4.

Table 16: HPCCC parameters of MIBK/methanol/water 3:3:4 tested on model mixture.

CCC – technical parameters	volume
CCC column volume (ml)	25.5
Total volume (pump-fraction waste; ml), no loop	30.0
Loop volume (ml)	4.0
Dead volume (pump - inj. valve; ml)	1.6
Flow MP (ml/min)	1.0
Fraction volume (ml)	5.0
Eluted SP (first cylinder; ml)	15
Injected SP (loop; ml)	4.0
Bleeded only in reverse phase (ml)	19.5
Residual SP (last cylinder; ml)	1
SP_{ret} initial	**59%**
SP_{ret} residual	**4%**

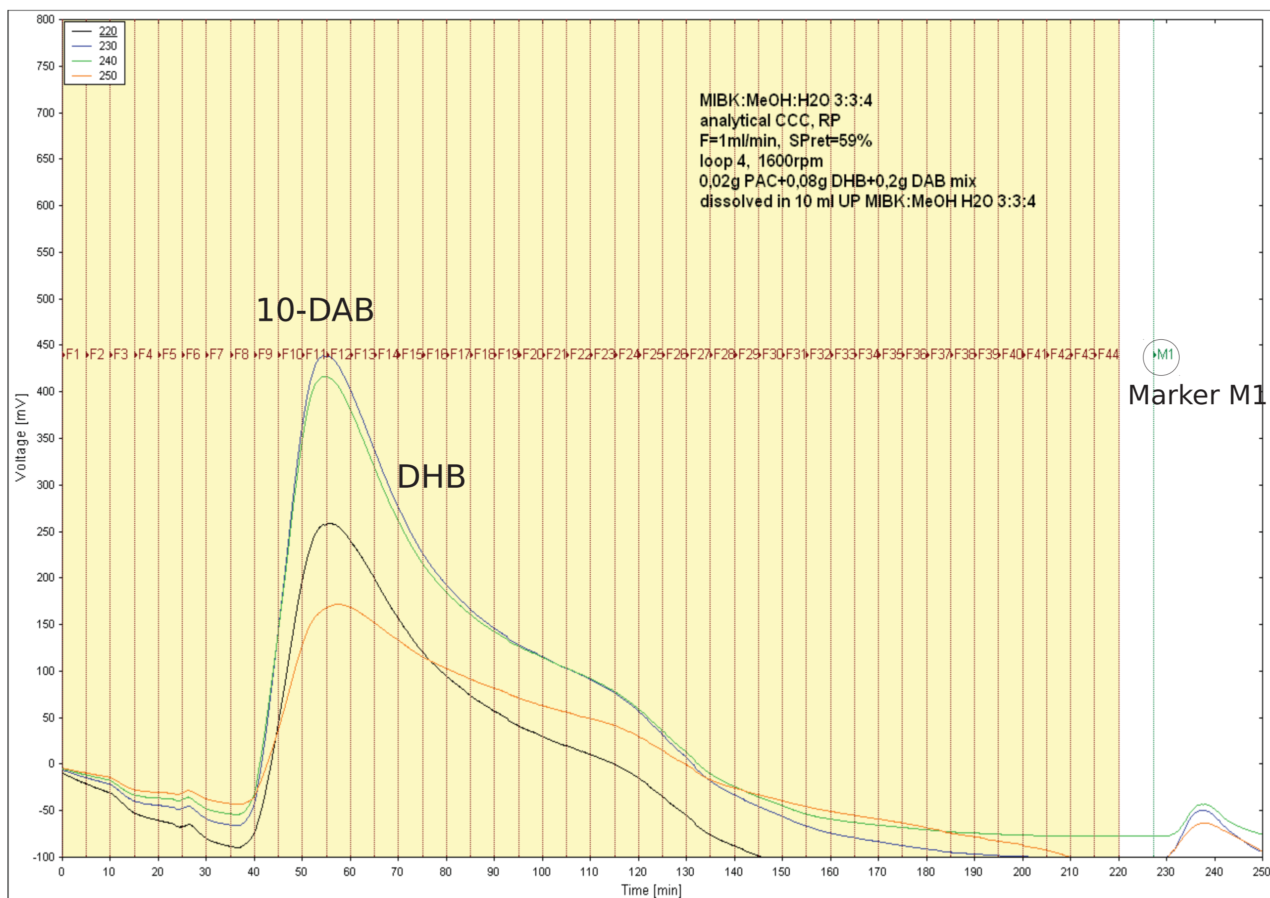
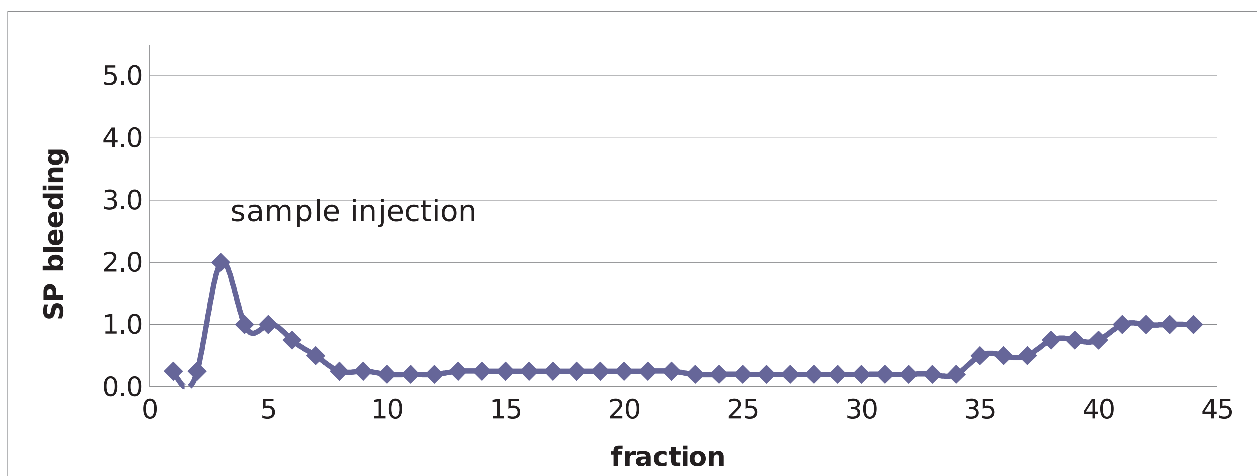


Figure 29: HPCCC UV profile of model mixture in MIBK/methanol/water 3:3:4.



Graph 4: Stationary phase bleeding of MIBK/methanol/water 3:3:4 tested on model mixture.

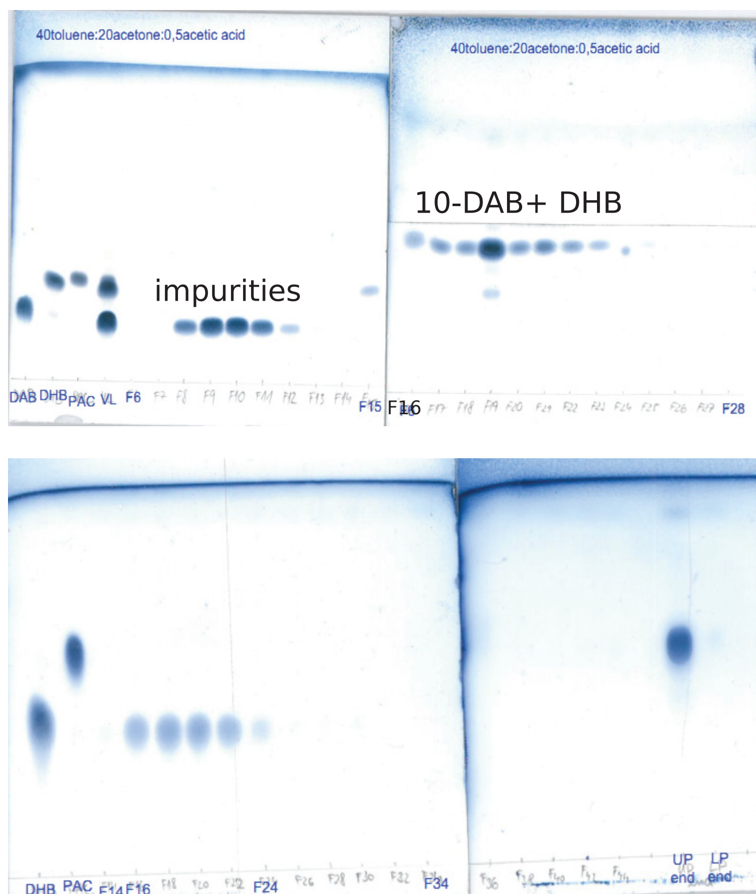


Figure 30: TLC support done for model mixture in MIBK/methanol/water 3:3:4.

Discussion: The first eluted peak is 10-DAB with Rt=55 min. Later eluted broader peak is DHB (Rt from 75th to 115th min). Peaks are wide and because of that compounds co-eluate. PAC is not eluted in this mode.

TLC description (Figure 30): first spots showed in fractions 8-12 – these were only polar impurities, easily separated from the rest of compounds. Next set of spots appeared in fractions 16-24. These were identified as 10-DAB co-eluted with DHB. For assurance there was another TLC done containing only DHB and PAC standards. It is apparent that DHB is really co-eluting in fractions 16-24. The colour of DHB spot is not rich because the concentration injected was lower than 10-DAB.

The analysis was stopped after 44 fractions collected (green marker M1 in HPCCC UV profile), the rest of UP ($SP_{ret\ end}$) and LP were also collected and TLC analyzed. In this final fraction PAC peak was found. It tells us that PAC is attached to stationary phase during this HPCCC analysis. The conclusion was proven by TLC support with toluene:acetone:acetic (40:20:0.5, v/v/v) acid mobile phase (see Figure 30). From SS bleeding it is evident that this condition is not possible to do in larger scale because of low SP_{ret} (see Table 16) and intensive bleeding (observed as UP) visible in all fractions (see Graph 4) which is connected to decrease of separation activity. Low SP_{ret} confirmed the theory from 6.2.2 but there will be one more experiment done with real Rafinated extract only to complete the survey.

6.4.3 MIBK/methanol/water (3:3:4) - reverse phase – Rafinated Extract

Target: This SS is chosen for completing the survey.

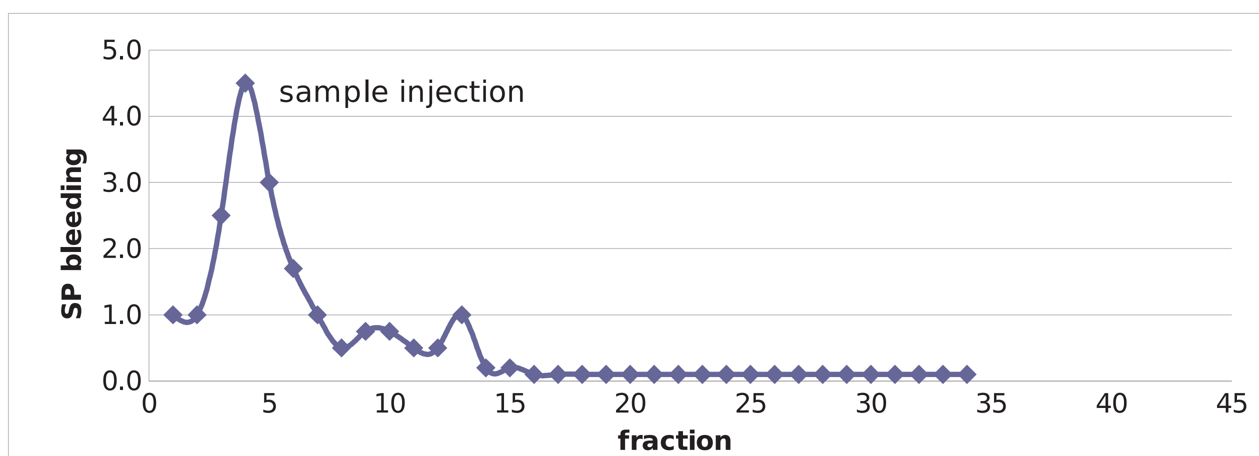
Sample preparation: Crude primary extract is evaporated to one third of the original weight (3 kg → 1 kg). 0.5 l of hexane is added, the mixture is shaken for 30 minutes and then let to separate the phases properly. After 12 hours the lower phase is collected and used as final Rafinated extract.

10 ml of this already prepared Rafinated extract is evaporated to dryness (3.2 g is weighed) and dissolved in 10 ml UP of SS (lots of material was not dissolved, for injection we choose the solution)

Procedure: The sample was injected to HPCCC (see HPCCC parameters in Table 17), bleeding was measured (Graph 5) and fractions were analyzed by TLC (Figure 32).

Table 17: HPCCC parameters of MIBK/methanol/water 3:3:4 tested on Rafinated extract.

CCC – technical parameters	volume
CCC column volume (ml)	25.5
Total volume (pump-fraction waste; ml), no loop	30.0
Loop volume (ml)	4.0
Dead volume (pump - inj. valve; ml)	1.6
Flow MP (ml/min)	1.0
Fraction volume (ml)	5.0
Eluted SP (first cylinder; ml)	16,5
Injected SP (loop; ml)	4.0
Bleeded only in reverse phase (ml)	21
Residual SP (last cylinder; ml)	1
SP_{ret} initial	**53%**
SP_{ret} residual	**4%**



Graph 5: Stationary phase bleeding of MIBK/methanol/water 3:3:4 tested on Rafinated extract.

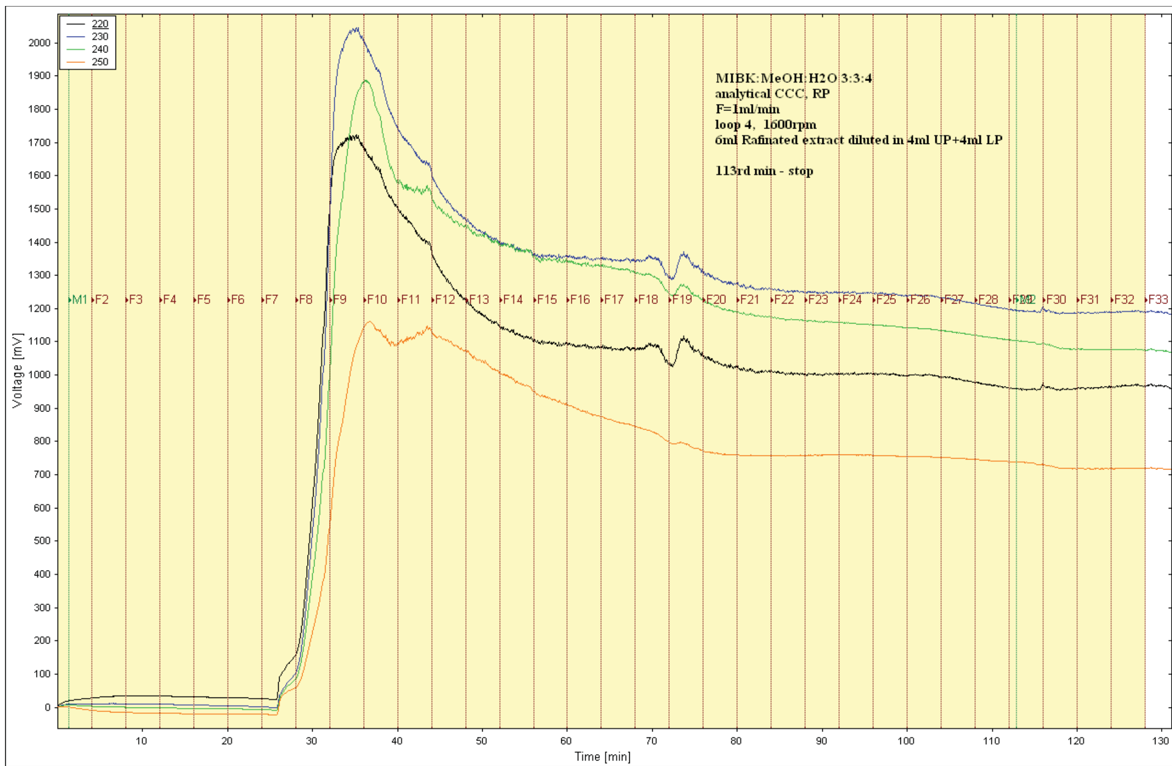


Figure 31: HPCCC UV profile of Rafinated extract in MIBK/methanol/water 3:3:4.

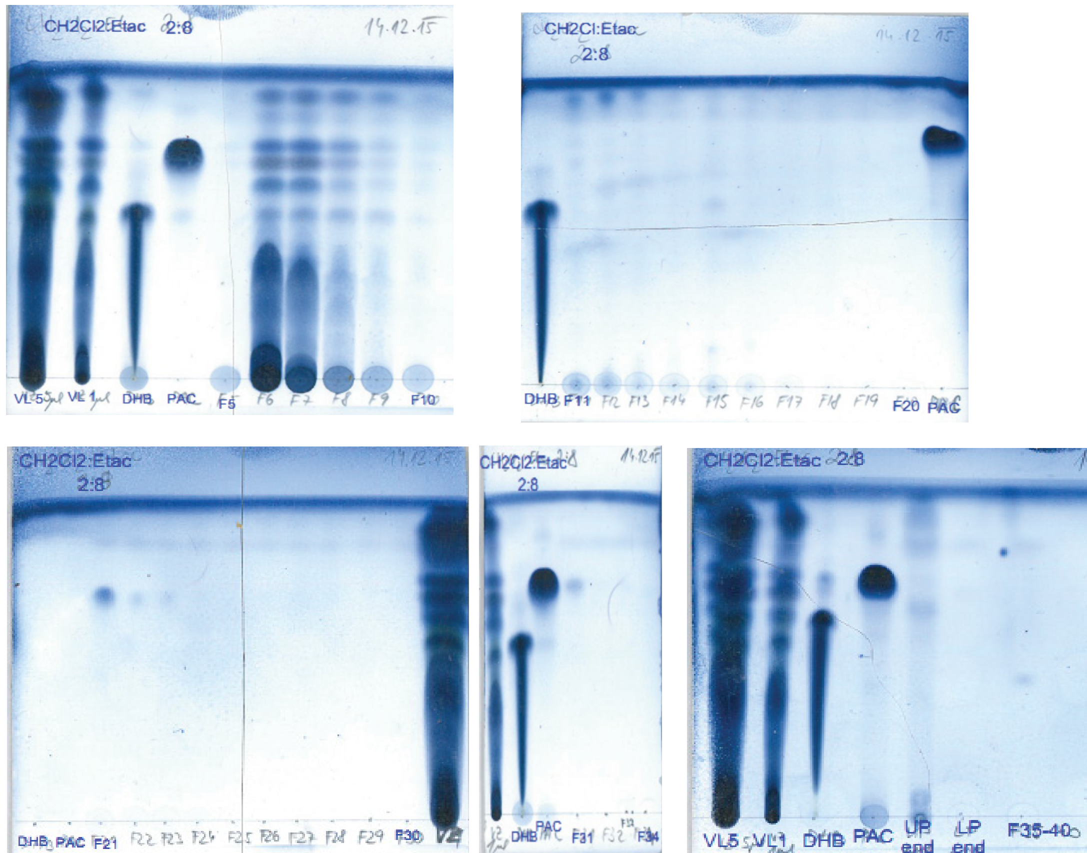


Figure 32:TLC support done for Rafinated extract in MIBK/methanol/water 3:3:4.

Discussion: Evaporated Rafinated extract was not possible to completely dissolve in UP of this SS. The solution was injected into HPCCC. The first eluted material was observed in 6th fraction. This fraction was dark green/brown rich colour. From TLC it looks like all compounds are co-eluted from 6th to 8th fraction. In this case there was probably no separation. SS MIBK/methanol/water 3:3:4 was therefore definitely eliminated from experimenting.

6.4.4 MIBK/acetone/water (2:3:2) - reverse phase – Rafinated Extract

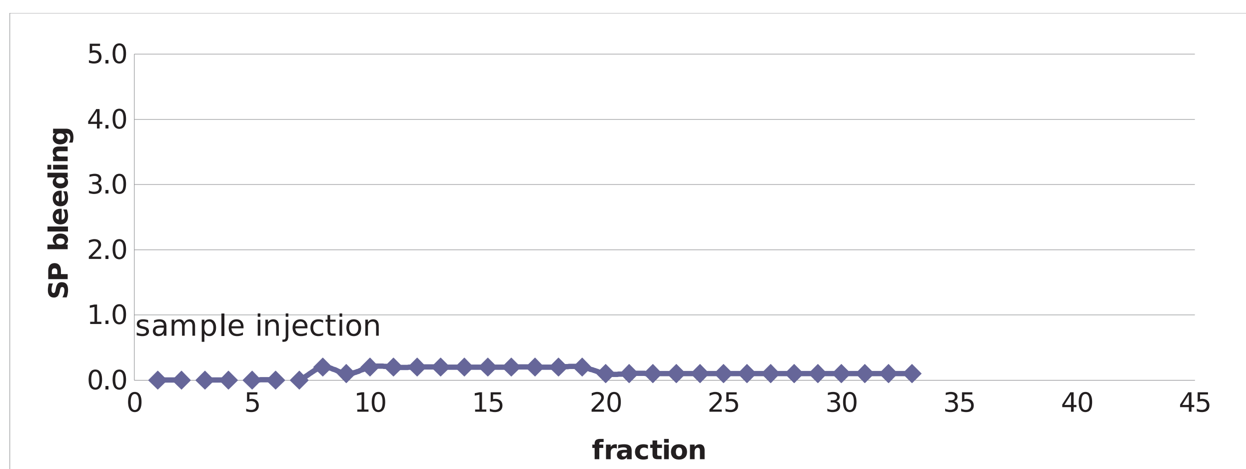
Target: Rafinated extract CCC chromatography without hydrodynamic equilibrium.

Sample preparation: Similar to chapter 6.4.3. and filtrated before use.

Procedure: The sample was injected to HPCCC without previous stationary phase equilibrium (see HPCCC parameters in Table 18), bleeding was monitored (Graph 6) and fractions were TLC analyzed (Figure 34).

Table 18: HPCCC parameters of MIBK/acetone/water 2:3:2 tested on Rafinated extract without phases equilibrium.

CCC column volume (ml)	25.5
Total volume (pump-fraction waste; ml), no loop	30.0
Loop volume (ml)	4.0
Dead volume (pump - inj. valve; ml)	1.6
Flow MP (ml/min)	1.0
Fraction volume (ml)	5.0
Injected SP (loop; ml)	4.0
Bleeded only in reverse phase (ml)	3.7
Residual SP (last cylinder; ml)	1.8
SP_{ret} residual	**7%**



Graph 6: Stationary phase bleeding of MIBK/acetone/water 2:3:2 tested on Rafinated extract without phases equilibrium.

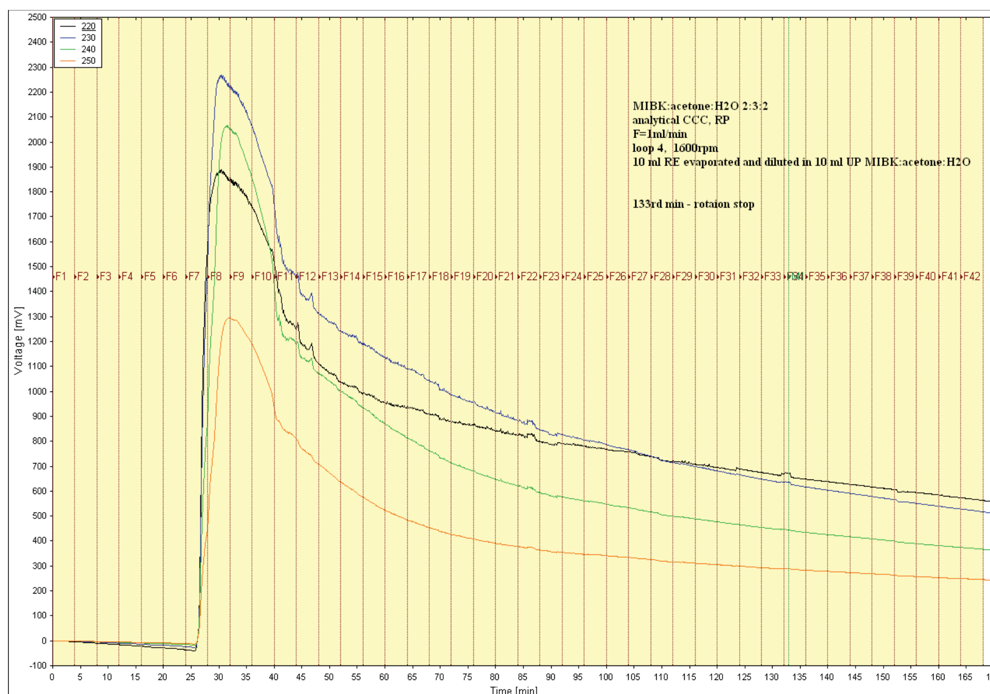


Figure 33: HPCCC UV profile of Rafinated extract in MIBK/acetone/water 2:3:2 without phases equilibration.

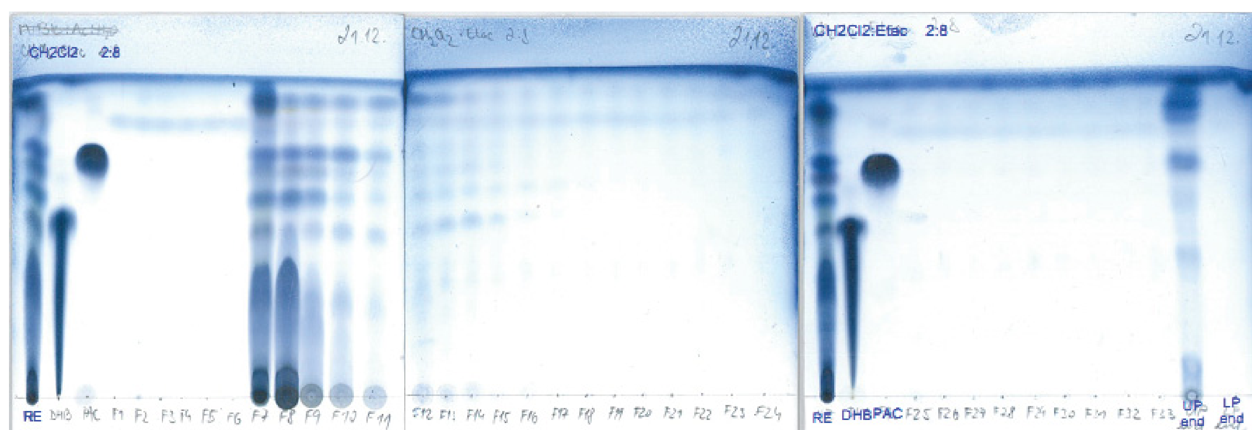


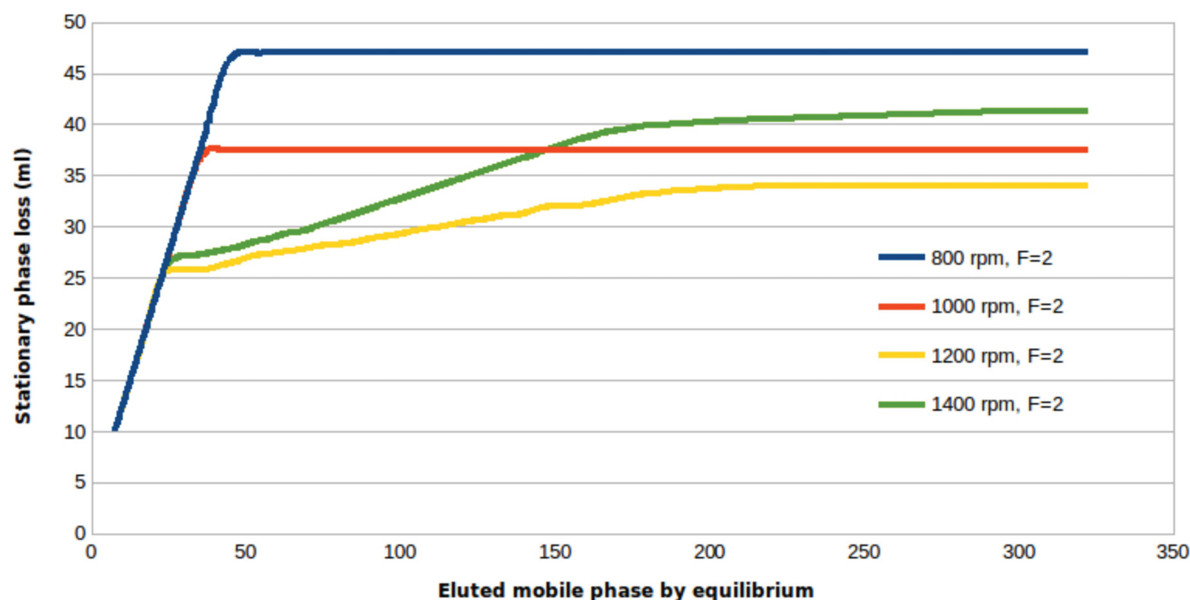
Figure 34: TLC support done for Rafinated extract in MIBK/acetone/water 2:3:2 without phases equilibrium.

Partial conclusion: The first eluted material was observed in the 7th fraction. This fraction was dark red/brown colour. From TLC it looks like that most of the material is eluted at the front. At 133rd minute the rotation was stopped (see the green marker in the chromatogram), UP and lower phase were collected and TLC analyzed. In the last washing fraction PAC eluted (it is not in UV profile). The stationary phase bleeding was incomprehensibly too low, possibly caused by skipping the phase equilibrium.
The bad solubility of Rafinated extract was improved by suspension warming for 30 °C and 5 min.
Skipping equilibration did not appear to be beneficial and for further experiments it was rejected.

6.5 Monitoring of stationary phase loss; dependence on rotation and flow

Target: For possible improvement of stationary phase retention we decided to test various rotation speeds and flow rates of HPCCC subsequently.

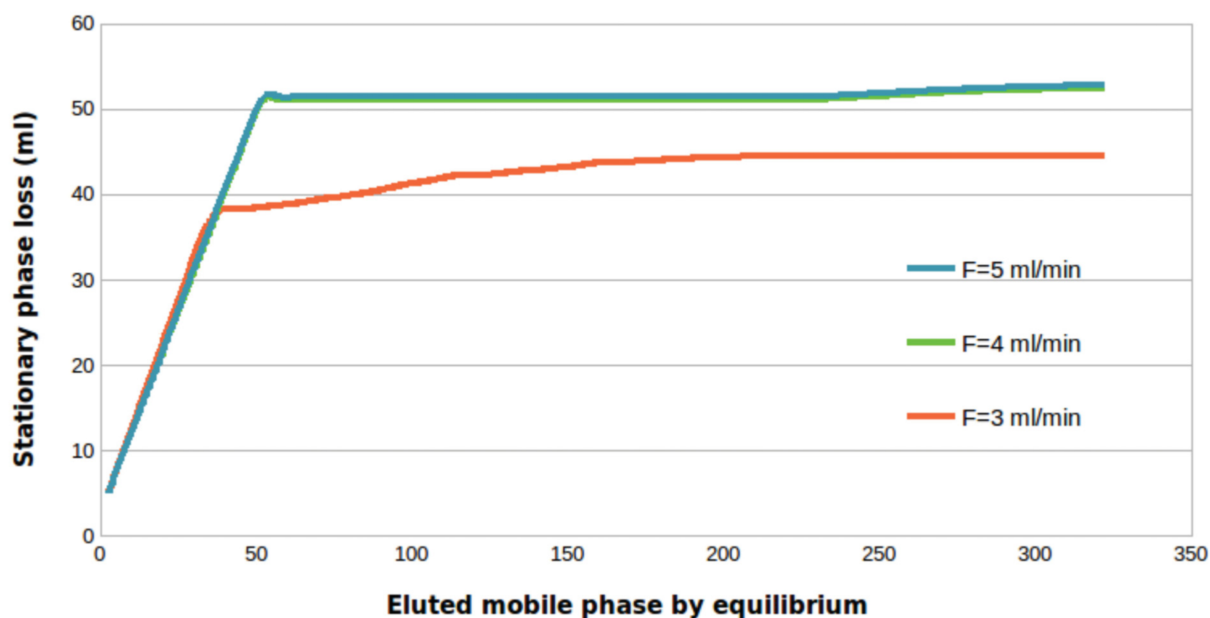
Design space: First of all the rotation interval was set to 800-1400 rpm (1600 rpm was tested in preliminary experiments – e.g. 6.1.1 and showed significant bleeding).
HPCCC bleeding was tested with solvent system MIBK/acetone/water 2:3:2 in reverse phase mode with semi-preparative column. The column with SP was spun at 800 rpm and MP flow was set to 2 ml/min. 5 ml fractions were collected and bleeding was calculated. The same experiment was performed with 1000, 1200 and 1400 rpm while the flow was still 2 ml/min (see Graph 7)



Graph 7: Monitoring of stationary phase bleeding of MIBK/acetone/water 2:3:2, rotation speed optimization.

Rotation speed tests discussion: From the results stated in Graph 7 it is clear that the best rotation speed with the lowest bleeding tendency is 1200 rpm. Even though the bleeding started early the total bleeding of stationary phase was the lowest. Therefore this rotation speed was proven to be optimal and the most convenient.

Flow rate tests: This experiment was done to test different flow rates. On the basis of optimal rotation speed the flow rate bleeding dependence was tested at 3 ml/min and subsequently at 4 ml/min and 5 ml/min.



Graph 8: Monitoring of stationary phase bleeding of MIBK/acetone/water 2:3:2, flow rate optimization.

Flow rate tests discussion: There is no substantial difference between the flow rate 3 ml/min and 5 ml/min, therefore for much faster separation process the 5 ml/min flow rate was chosen as more advantageous. Even though 2 ml/min seems to has the lowest SP loss this flow is inconvenient because of long very separation time.

From both Graph 7 and Graph 8 final optimized conditions were selected – 1200 rpm and 5 ml/min. These parameters were respected in final experiments in preparative scale.

6.6 *Preparative scale HPCCC conditions*

6.6.1 MIBK/acetone/water 2:3:2, 50 mg RE

Dual mode purification

HPCCC preparative CCC coil (128.5 ml)

Solvent system: MIBK/acetone/water 2:3:2 (UP about 60 volume %)

Rotation: 1200 rpm

Flow: 5 ml/min

UV detection: 210 nm, 220 nm, 230 nm, 250 nm

Initial SP_{ret}: [128.5+1.6+4–57.5/128.5]x100=**59.6 %**

Sample: *Taxus baccata* Rafinated extract (evaporated dry rest) **– 50 mg** dissolved in 1.5 ml upper and 1.5 ml lower phase.

Injection: loop volume 4 ml

Fraction volume: 20 ml; waste from 120th min (LP=60 ml)

Residual SP_{ret}: 60/128.5=**46.7 %**

Procedure: Dual mode (RP→NP). HPLC pump channels purging: A=UP, B=LP, CCC column stationary phase (A) filling; F=5 ml/min; rotation setting; mobile phase equilibrium (B); initial SP_{ret} calculation; 3 ml sample was injected to 4 ml loop, analysis started in reverse mode (B channel pumping); fraction collection; 60th min (16th fraction) switched to normal mode (A channel pumping, now as mobile phase), fraction collection, 120th min rotation stopped, end of analysis → the rest of SP (now after dual mode the SP is B=LP) collected → residual SP_{ret} calculation; all fraction bleeding calculation; fractions 1-15 (only RP phase) analyzed by HPLC (Figure 36), (bleeding observed in almost all fractions, see in Graph 9 for 50 mg). In Table 19 purity of target molecules in fractions is described.

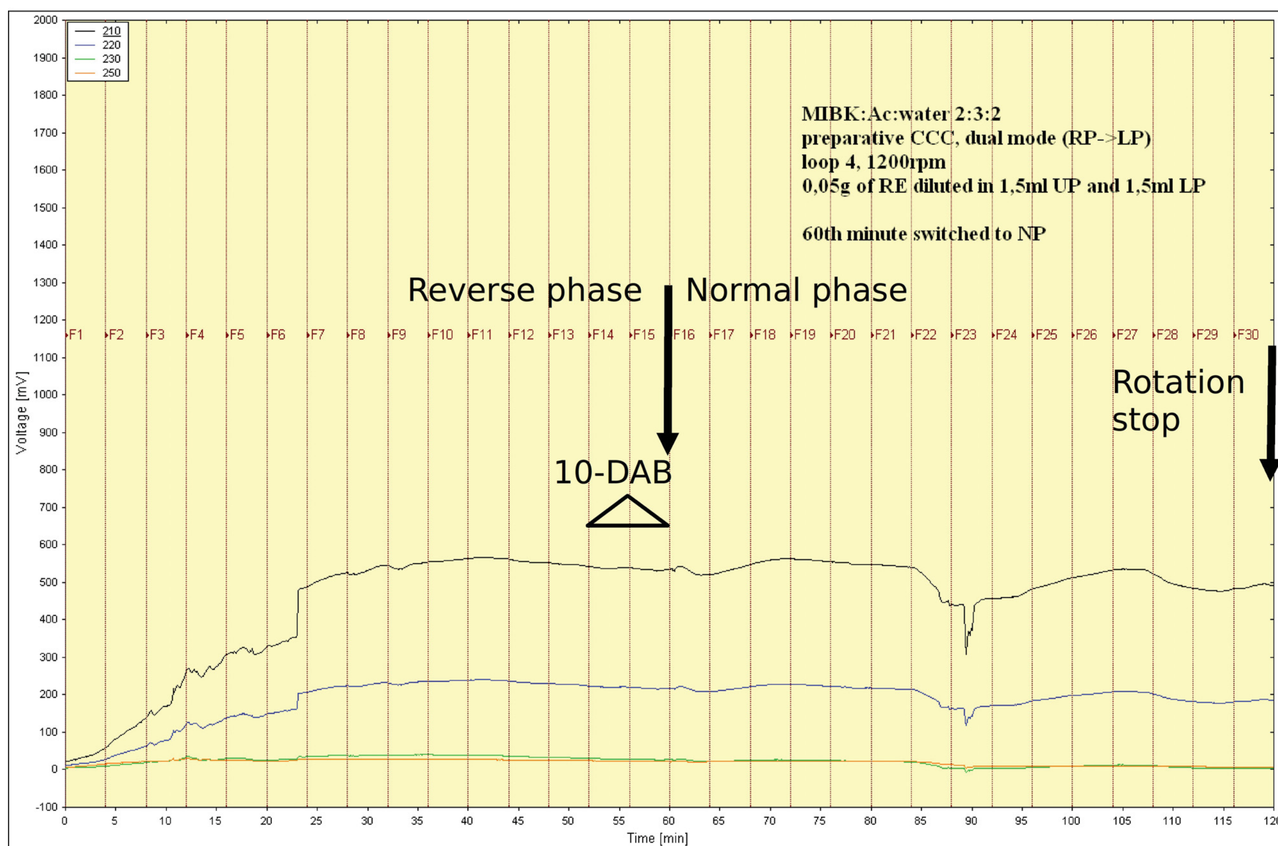
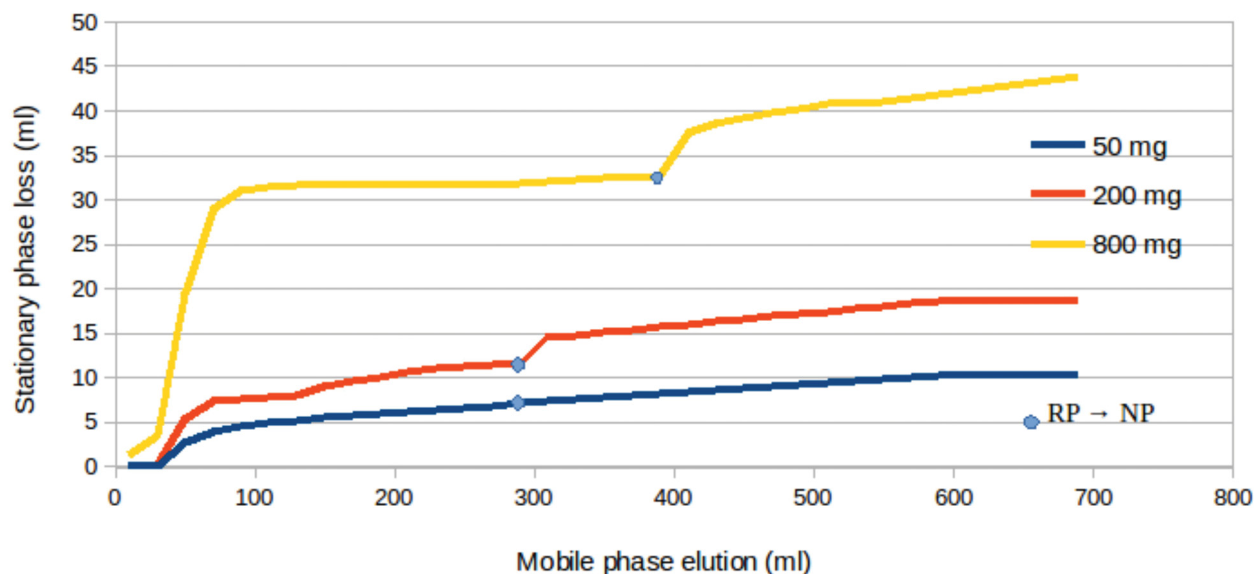
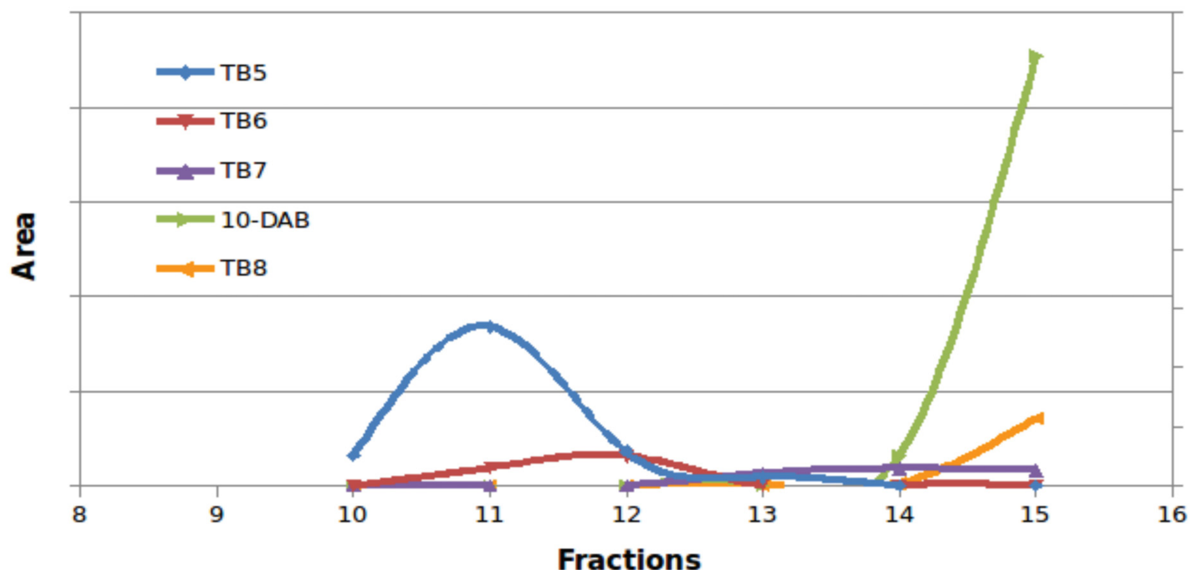


Figure 35: HPCCC UV detector profile for MIBK/acetone/water 2:3:3, 50 mg, dual mode.



Graph 9: Bleeding monitoring at 1200 rpm for 6.6.1-3, dual mode.

Three DUAL MODE separations were done (6.6.1-6.6.3) with injection of 50 mg, 200 mg and 800 mg with similar conditions. During experiments with 50 mg and 200 mg the phases were switched after 15th fraction which corresponds with 300 ml of MF (see Graph 9). With 800 mg injection the change was after 20th fraction (400 ml).



Graph 10: CCC chromatography profile – 50 mg (only revese mode).

Graph 10 - for comparison with 200 mg and 800 mg only 15 fractions were evaluated (mostly polar impurities) until 10-DAB as the most important compound was eluting. After the dual mode switch mostly lipophilic impurities are eluted.

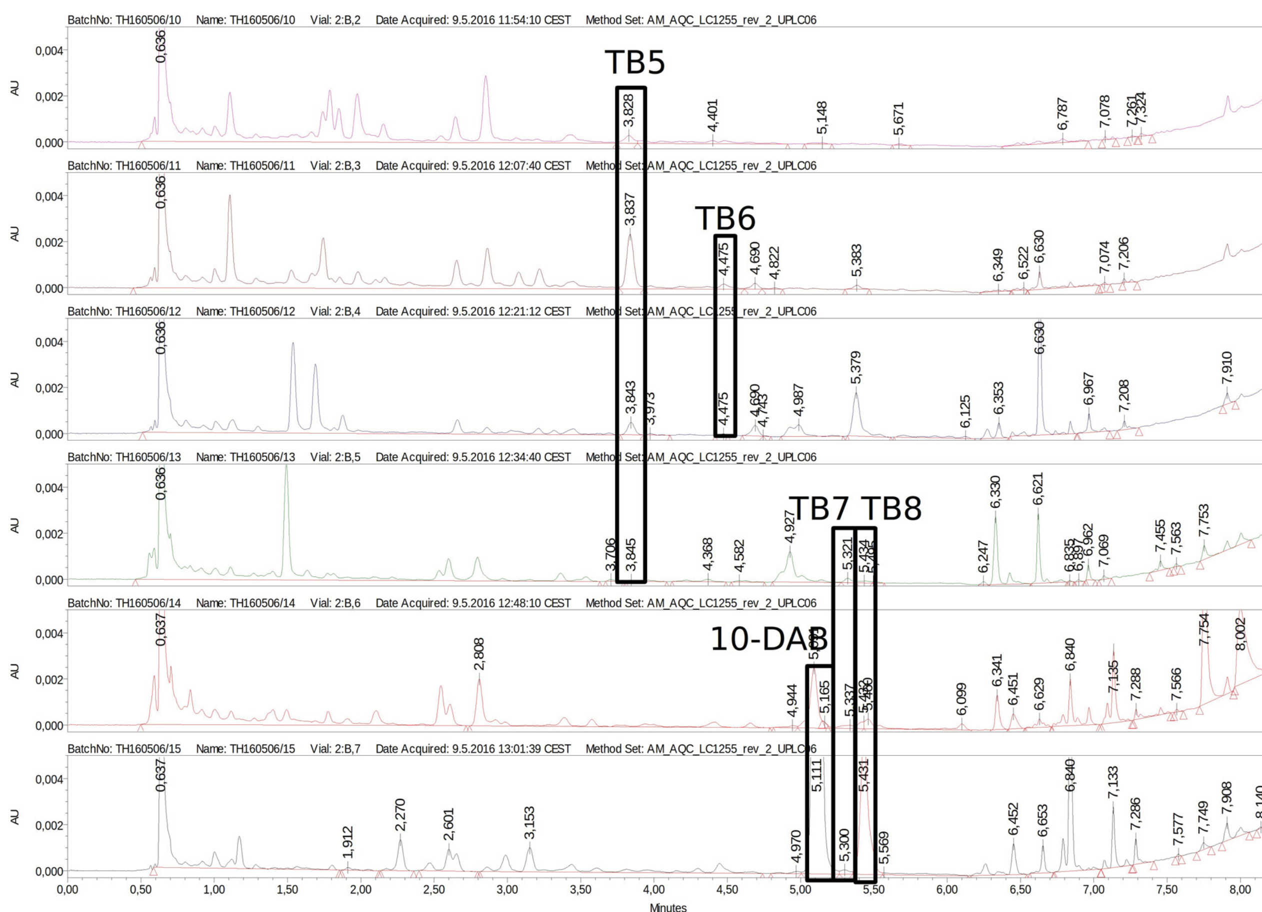


Figure 36: HPLC analysis of fractions 10-15 with important impurities, MIBK/acetone/water 2:3:2, 50 mg.

Table 19: Main fractions target molecule purity, MIBK/acetone/water 2:3:2, 50 mg.

Analysis	10-DAB purity (%)
RE	2.56
Fr. 14	6.49
Fr. 15	55.79

Discussion: Rafinated PE is a complex mixture with many impurities which have absorbance at 230 nm. Many TEVA known impurities are monitored also at 340 nm (flavonoids – lipophilic impurities). From previous analysis it is also known that this Rafinated extract does not contain any DHB and PAC, see Table 14 in 6.3.

Initial stationary phase retention by using MIBK/acetone/water 2:3:2 was 59.6 %. Injection of 50 mg Rafinated extract in mixture of UP/LP caused quite low bleeding of stationary phase (much bigger bleeding was observed with 800 mg injection – about 25 %). The most polar 10-DAB started to elute between 52-60th minute (260-300ml) in reverse phase mode. Subsequent mode changed to normal phase mode, the first fractions contained the most lipophilic compounds.

The mode was switched after 15th fraction. From Graph 10 it is obvious that 10-DAB was not eluted completely and therefore the rest of this compound was held in the column until the end of separation and collected probably after the rotation was stopped. For further experiments the dual mode switch should be postponed of at least 5 fractions.

10-DAB fraction HPLC purity was about 55 % (see Table 19). The similar CCC was done with 200 mg and 800 mg injection, see the following chapters.

6.6.2 MIBK/acetone/water 2:3:2, 200 mg RE + DHB and PAC

Dual mode purification

HPCCC semi-preparative CCC coil (128.5 ml)

Solvent system: MIBK/acetone/water 2:3:2 (UP about 60 volume %)

Rotation: 1200 rpm

Flow: 5 ml/min

UV detection: 210 nm, 220 nm, 230 nm, 250 nm

Initial SP_{ret}: [128.5+1.6+4–40/128.5]x100=**73.2 %**

Sample: *Taxus baccata* Rafinated extract (evaporated dry rest) – **200 mg** (spiked with 2 mg DHB and 2 mg PAC) dissolved in 1.5 ml upper and 1.5 ml lower phase.

Injection: loop volume 4 ml

Fraction volume: 20 ml; waste from 92.5 min (LP=58 ml)

Residual SP_{ret}: 58/128.5=**45.1 %**

Procedure: Dual mode (RP→NP), HPLC pump channels purging: A=UP, B=LP, CCC column stationary phase (A) filling; F=5 ml/min; rotation setting; mobile phase equilibrium (B); initial SP_{ret} calculation; 3 ml sample was injected to 4 ml loop, analysis started in reverse mode (B channel pumping); fraction collection; 60th min switched to normal mode (A channel pumping, now as mobile phase), fraction collection, 120th min rotation stopped, end of analysis → the rest of SP (now after dual mode the SP is B=LP) collected → residual SP_{ret} calculation; all fraction bleeding calculation (bleeding observed in all fractions, see in Graph 9 for 200 mg); fractions 4-18 analyzed by HPLC (Figure 38 and 39). Graph 11 shows elution of impurities and target molecules in fractions. In Table 20 purity of target molecules is described.

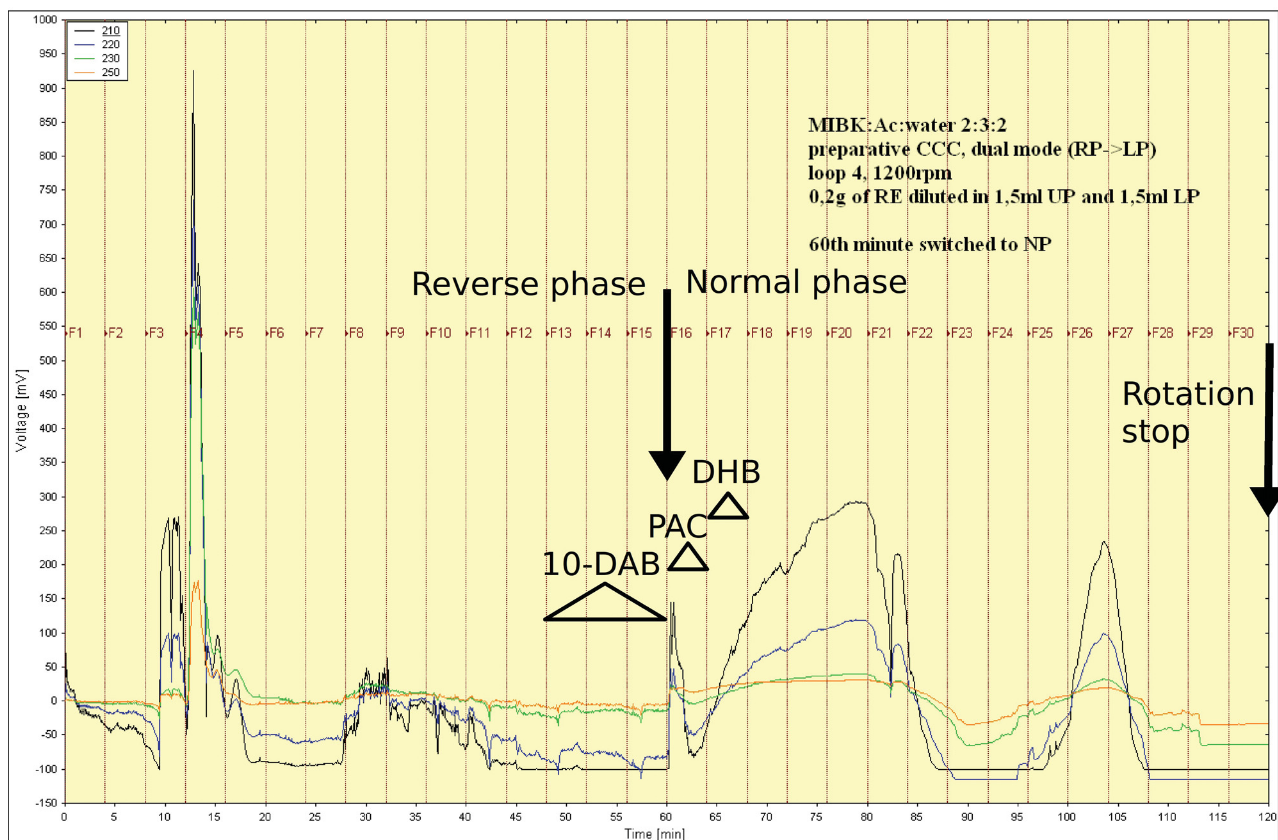
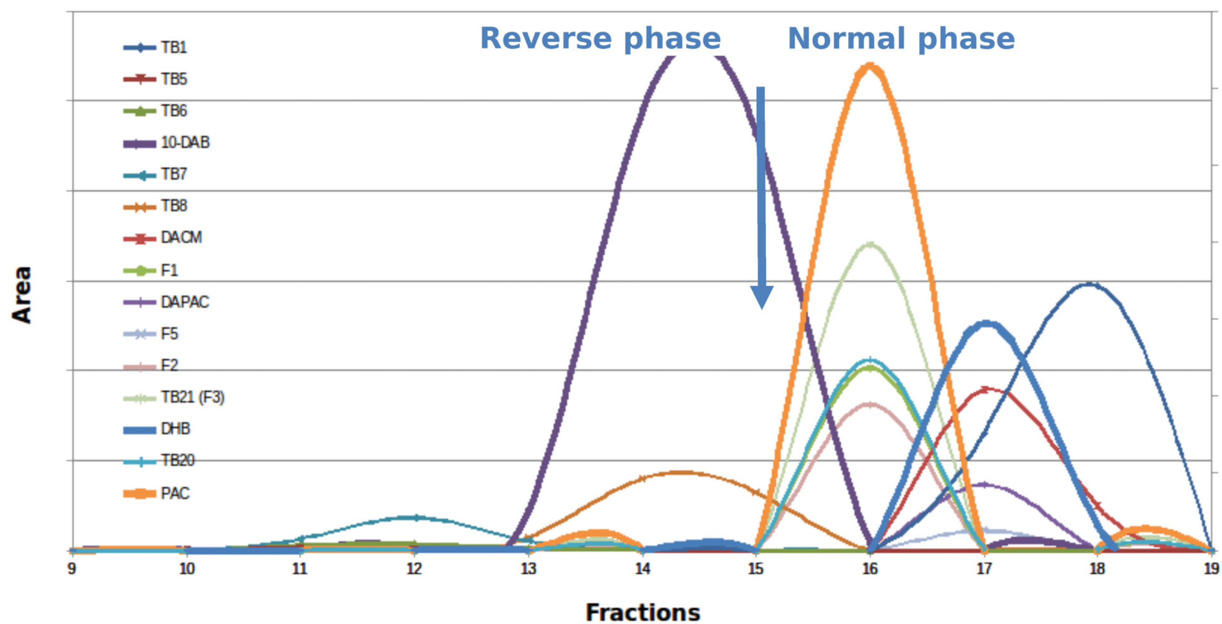


Figure 37: HPCCC UV detector profile for MIBK/acetone/water 2:3:3, 200 mg, dual mode.



Graph 11: CCC chromatography impurities and target molecules profile – 200 mg, dual mode.

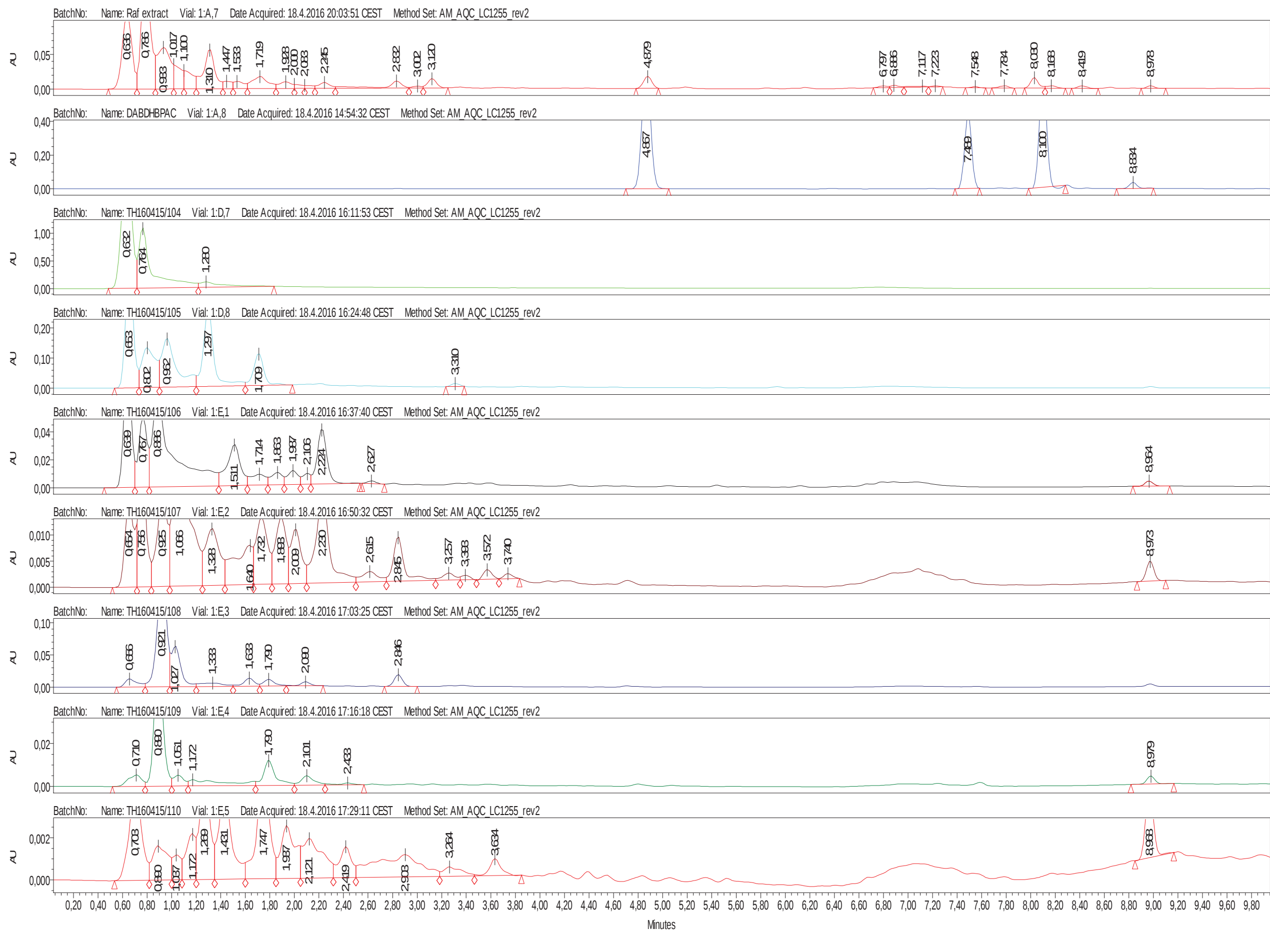


Figure 38: HPLC analysis of RE, mixture of 10-DAB+DHB+PAC, fractions 4-10, MIBK/acetone/water 2:3:2, 200 mg.

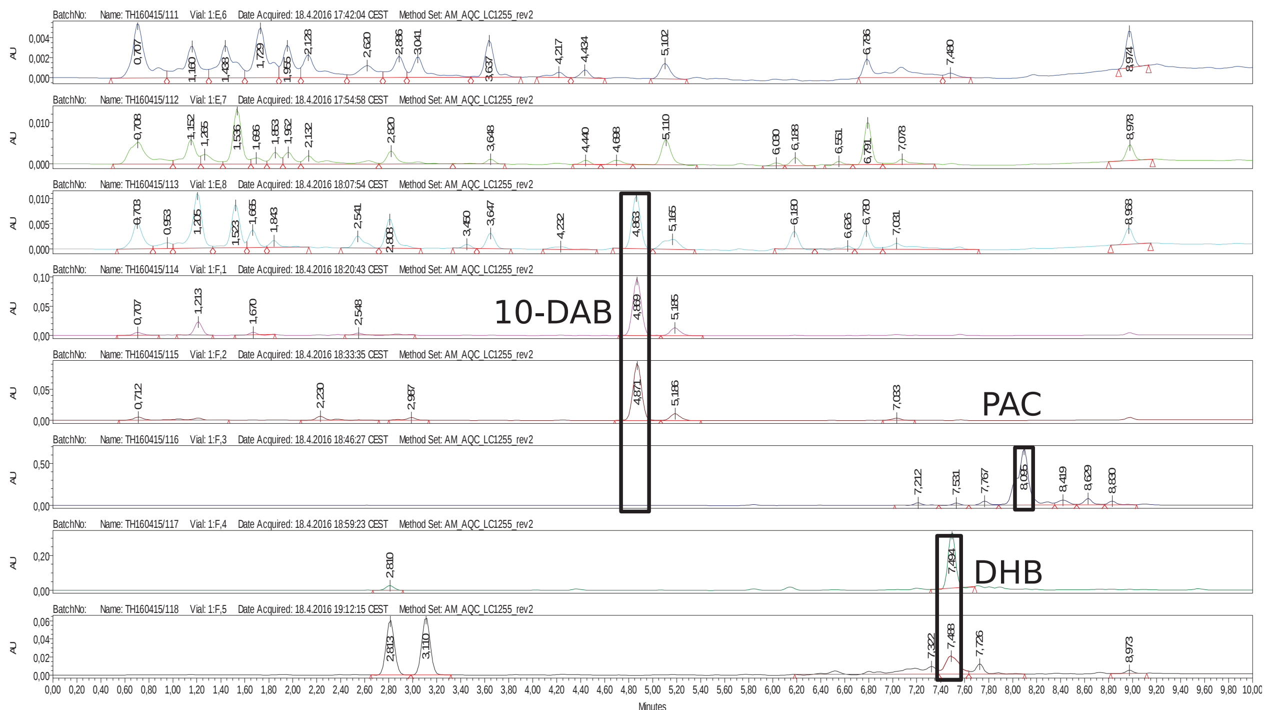


Figure 39: HPLC analysis of fractions 11-18, MIBK/acetone/water 2:3:2, 200 mg.

Table 20: Main fractions target molecule purity, MIBK/acetone/water 2:3:2, 200 mg.

Analysis	10-DAB purity (%)	DHB purity (%)	PAC purity (%)
RE	2.56		
Fr.13	11.92		
Fr.14	62.49		
Fr.15	67.23		
Fr.16	0.12		49.10
Fr.17		60.30	
Fr.18		15.33	

Discussion: From previous analysis we also know that our Rafinated PE does not contain any DHB and PAC, see Table 14, therefore PAC and DHB were reasonable added.

Initial stationary phase retention by using MIBK/acetone/water 2:3:2 was 73.2 %. Injection of 200 mg spiked Rafinated extract in mixture of UP/LP caused about 10 % bleeding of stationary phase (much more bigger bleeding was observed with 800 mg injection – about 20 %). Most polar or low molecular weight impurities are removed with front CCC. The polar 10-DAB started to elute between 50-60th minute = 13-15th fraction (250-300 ml) in reverse phase mode. Subsequent mode change to normal phase mode, the first fraction contained PAC and second DHB. 10-DAB fractions HPLC purity were about 65 % (see Table 20). Also fractions with PAC and DHB had purity about 50-60 %.

From the 10-DAB (%) in fractions 15 and 16 it is obvious that during the dual mode switch (after 15th fraction) there was some 10-DAB still inside the column and therefore the switch should have been postponed so that 10-DAB could eluate completely. In our case the rest of 10-DAB was collected after the rotation was stopped.

6.6.3 MIBK/acetone/water 2:3:2, 800 mg RE + DHB and PAC

Dual mode purification

HPCCC semi-preparative CCC coil (128.5 ml)

Solvent system: MIBK/acetone/water 2:3:2 (UP about 60 volume %)

Rotation: 1200 rpm

Flow: 5 ml/min

UV detection: 210 nm, 220 nm, 230 nm, 250 nm

Initial SP_{ret}: [128.5+1.6+4–48/128.5]x100=**67.0 %**

Sample: *Taxus baccata* Rafinated extract (evaporated dry rest) **– 800 mg** (spiked with 2 mg DHB and 2 mg PAC) dissolved in 1.5 ml upper and 1.5 ml lower phase.

Injection: loop volume 4 ml

Fraction volume: 20 ml; waste from 140th min (LP=70 ml)

Residual SP_{ret}: 70/128.5=**54.5 %**

Procedure: Dual mode (RP→NP)!. HPLC pump channels purging: A=UP, B=LP, CCC column stationary phase (A) filling; F=5 ml/min; rotation setting; mobile phase equilibrium (B); initial SP_{ret} calculation; 3 ml sample was injected to 4 ml loop, analysis started in reverse mode (B channel pumping); fraction collection; 80th min switched to normal mode (A channel pumping, now as mobile phase), fraction collection, 120th min rotation stopped, end of analysis → the rest of SP (now after dual mode the SP is B=LP) collected → residual SP_{ret} calculation; all fraction bleeding calculation (bleeding observed in all fractions, see in Graph 9 for 800 mg); fractions 4-25 analyzed by HPLC (see Figure 41-Figure 44). Graph 12 shows elution of impurities and target molecules in fractions. In Table 21 purity of target molecules is described.

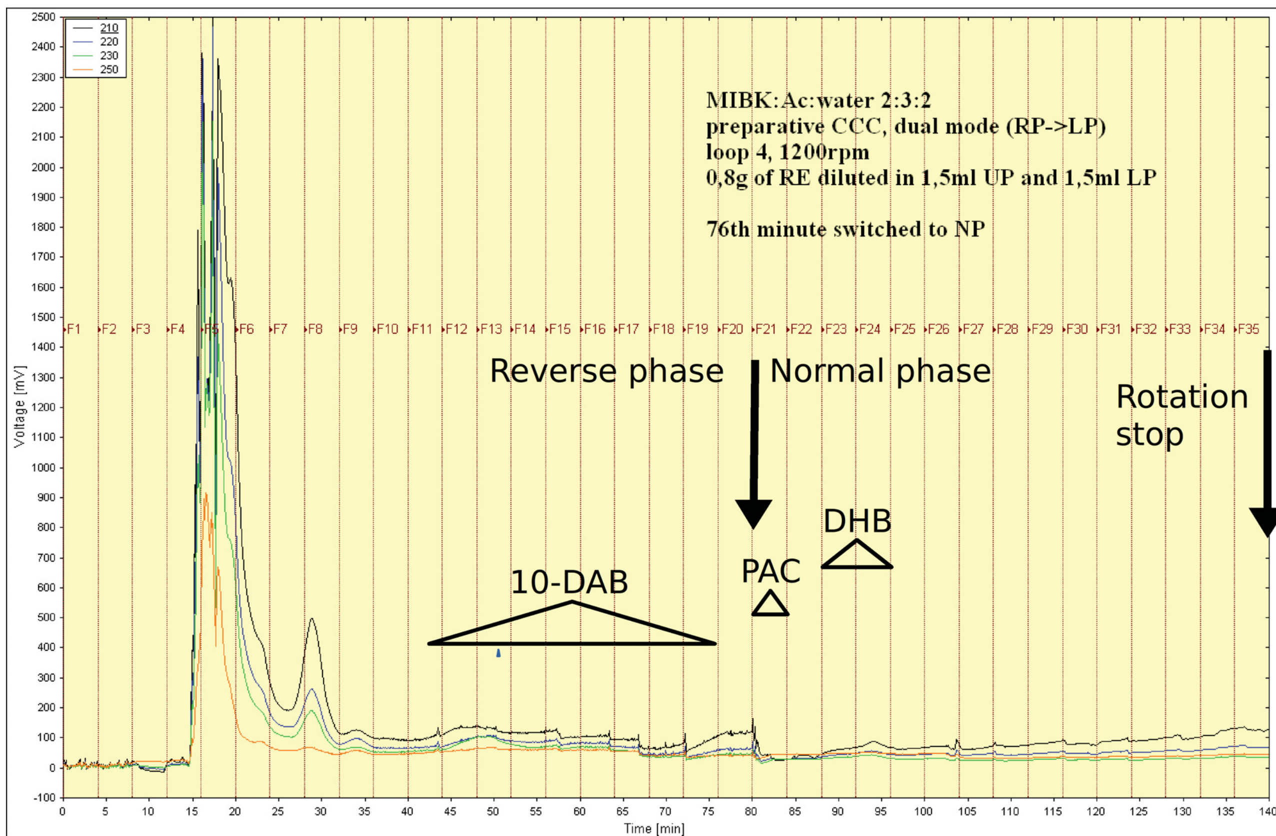
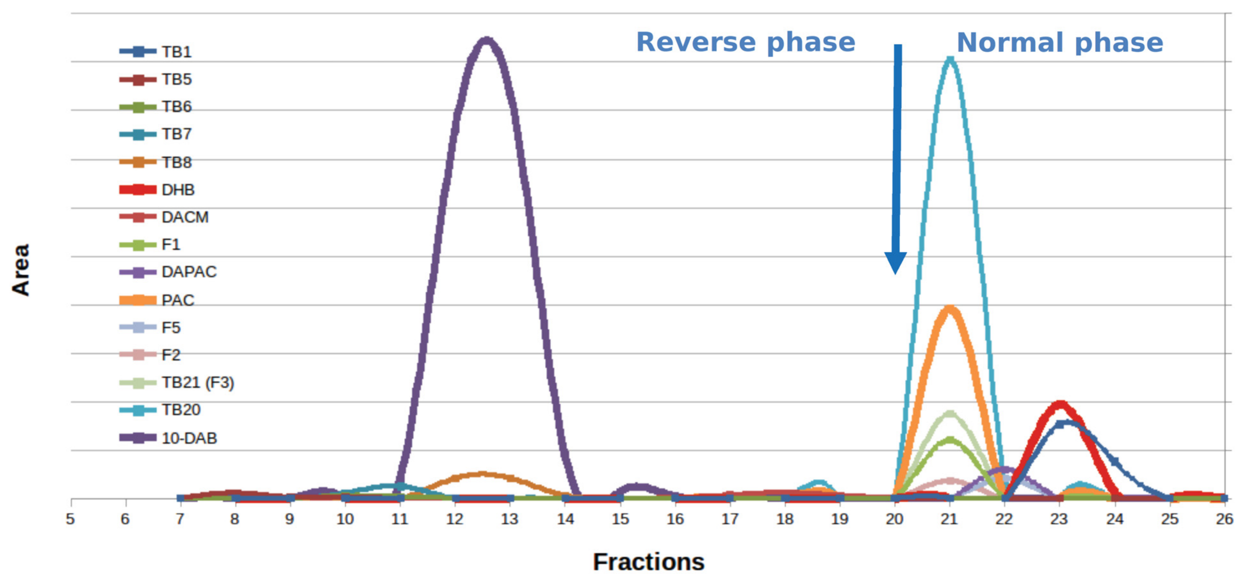


Figure 40: HPCCC UV detector profile for MIBK/acetone/water 2:3:3, 800 mg, dual mode.



Graph 12: CCC chromatography impurities and target mulecules profile, 800 mg, dual mode.

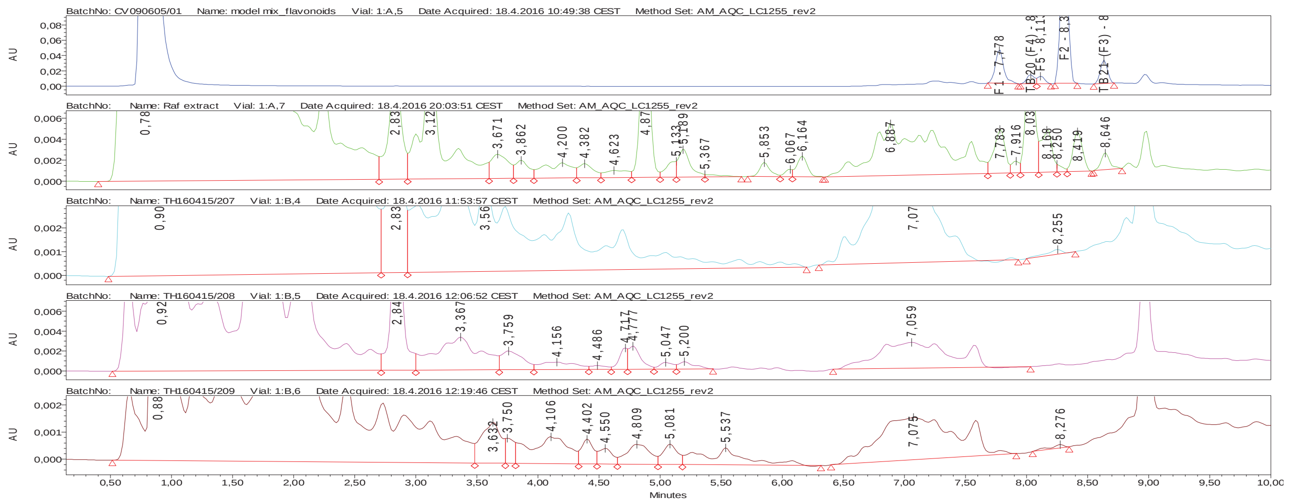


Figure 41: HPLC analysis of flavonoids, RE, fractions 7-9, MIBK/acetone/water 2:3:2, 800 mg.

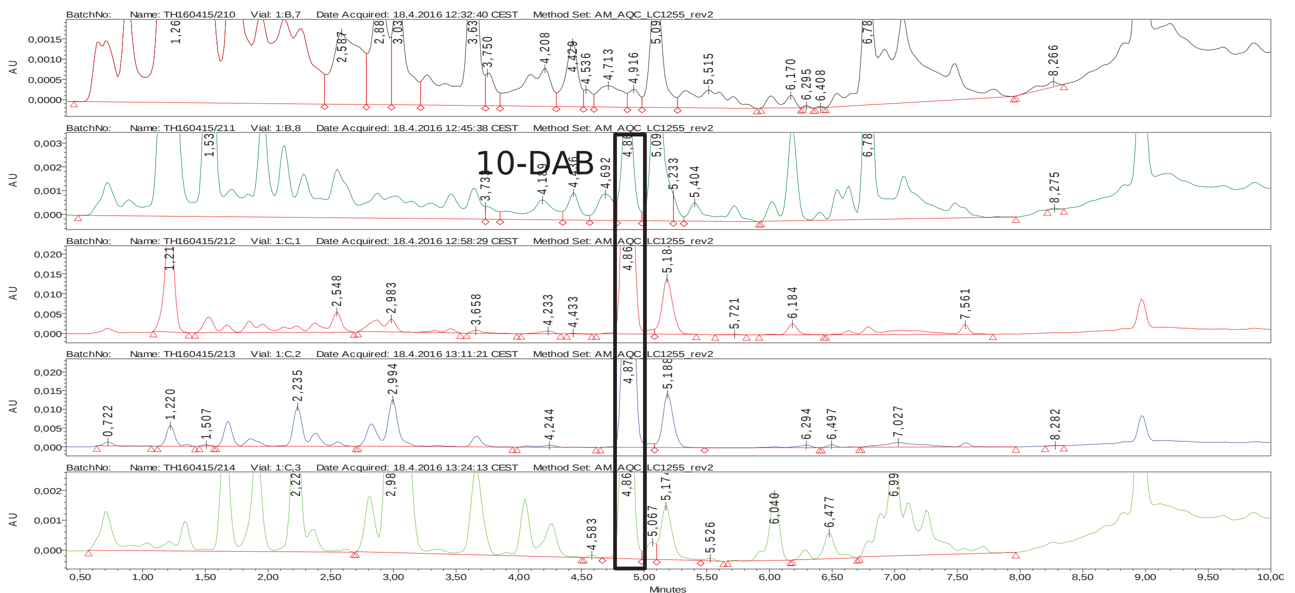


Figure 42: HPLC analysis of fractions 10-14, MIBK/acetone/water 2:3:2, 800 mg.

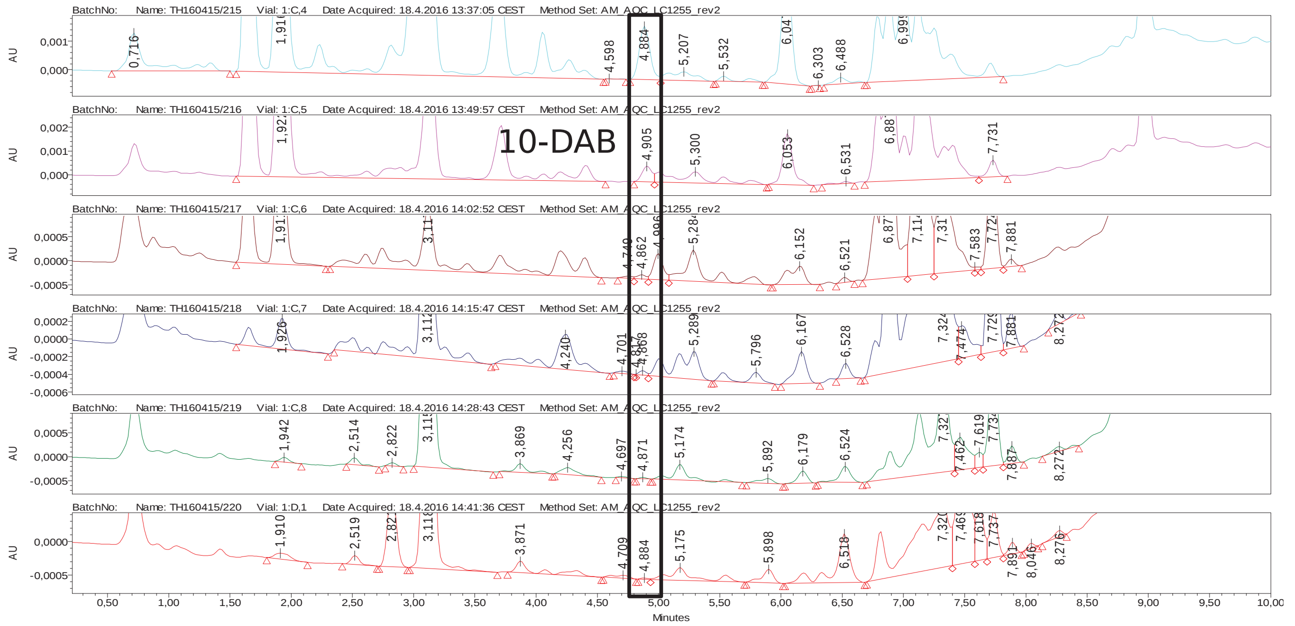


Figure 43: HPLC analysis of fractions 15-20, MIBK/acetone/water 2:3:2, 800 mg.

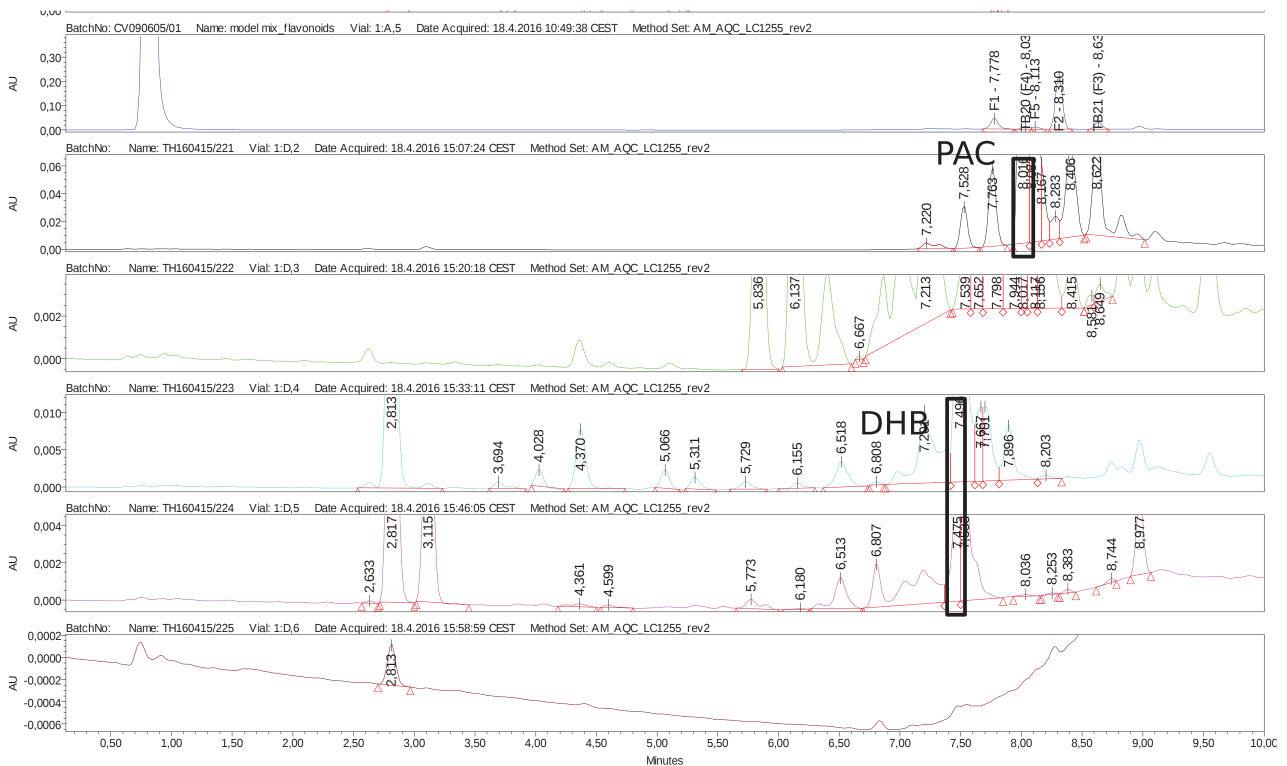


Figure 44: HPLC analysis of flavonoids, fractions 21-25, MIBK/acetone/water 2:3:2, 800 mg.

Table 21: Main fractions target molecule purity, MIBK/acetone/water 2:3:2, 800 mg.

Analysis	10-DAB purity (%)	DHB purity (%)	PAC purity (%)
RE	2.56		
Fr.11	5.75		
Fr.12	52.41		
Fr.13	58.13		
Fr.14	17.54		
Fr.15	2.46		
Fr.16	0.87		
Fr.17	0.2		
Fr.18	0.14		
Fr.19	0.09		
Fr.20	0.03		
Fr.21			17.93
Fr.22			
Fr.23		36.99	
Fr.24		7.17	

Discussion: Initial stationary phase retention by using MIBK/acetone/water 2:3:2 was 67.0 %. Injection of 800 mg spiked Rafinated extract in mixture of UP/LP caused big bleeding of stationary phase - about 20 %. The most polar 10-DAB started to elute between 40-75th minute (200-380 ml) in reverse phase mode. Subsequent mode change to normal mode, the first fraction after the switch contained PAC and 3rd and 4th DHB. 10-DAB main fractions HPLC purity were about 55 % (see Table 21). Fractions with PAC had purity about 18 % and DHB about 40 %. The mass percentage of IMP found in fractions 4-9 was calculated as 24.8 % (198.2 mg out of total of 800 mg).

In all three experiments (50 mg, 200 mg and 800 mg) the initial SP_{ret} was between 60-73 % which is satisfactory (no tendency to raise or decrease with increasing mass injection). Residual SP_{ret} was always at least 45 % meaning that this SS is suitable for taxanes separation.
With increasing mass injection 10-DAB started to eluate in sooner fractions - fraction 14 (for 50 mg), fraction 13 (for 200 mg) and fraction 12 (for 800 mg).
Bleeding was distinctively increasing with mass injection increase and in 800 mg injection it was probably caused by slight system overload.

6.6.4 Toluene/acetone/water 4:2:4, 50 mg RE

Reverse phase mode purification

HPCCC semi-preparative CCC coil (128.5 ml)

Solvent system: toluene/acetone/water 4:2:4 (UP about 50 volume %)

Rotation: 1200 rpm

Flow: 5 ml/min

UV detection: 210 nm, 220 nm, 230 nm, 250 nm

Initial SP_{ret}: [128.5+1.6+4–40/128.5]x100=**73.2 %**

Sample: *Taxus baccata* Rafinated extract (evaporated dry rest) – 50 mg dissolved in 1.5 ml upper and 1.5 ml lower phase.

Injection: loop volume 4 ml

Fraction volume: 10 ml; waste from 60th min

Residual SP_{ret}: 56/128.5=**43.6 %**

Procedure: Reverse mode only. HPLC pump channels purging: A=UP, B=LP; CCC column stationary phase (A) filling; F=5 ml/min; rotation setting; mobile phase equilibrium (B); initial SP_{ret} calculation; 3 ml sample was injected to 4 ml loop, analysis started in reverse mode (B channel pumping); fraction collection; 60th min rotation stopped and, of analysis → the rest of SP collected – residual SP_{ret} calculation; all fraction bleeding calculation (bleeding observed in fractions 3-7, see Graph 13); fractions 1-31 analyzed by HPLC (Figure 46-Figure 50). Graph 14 shows elution of impurities and target molecules in fractions. In Table 23 purity of target molecules is described.

HPLC sample preparation: 4 ml of most fractions was evaporated with N_2 and diluted with 1 ml MeOH:ACN 1:1. But due to excessive bleeding in some vials only less volume was analyzed, see Table 22. This means that vials 2-7 contained less than 4 ml of the LP but still were evaporated and diluted.

Table 22: HPLC analysis sample preparation - vials with less volume.

Vial No.	Volume analyzed (ml)	Notes
4	2	LP – white opaque
5	1.7	LP – white opaque
6	2.1	LP – white opaque
7	2.7	LP – yellow opaque
8	4	yellow opaque
9	4	white opaque
14	4	white opaque
15	4	white opaque

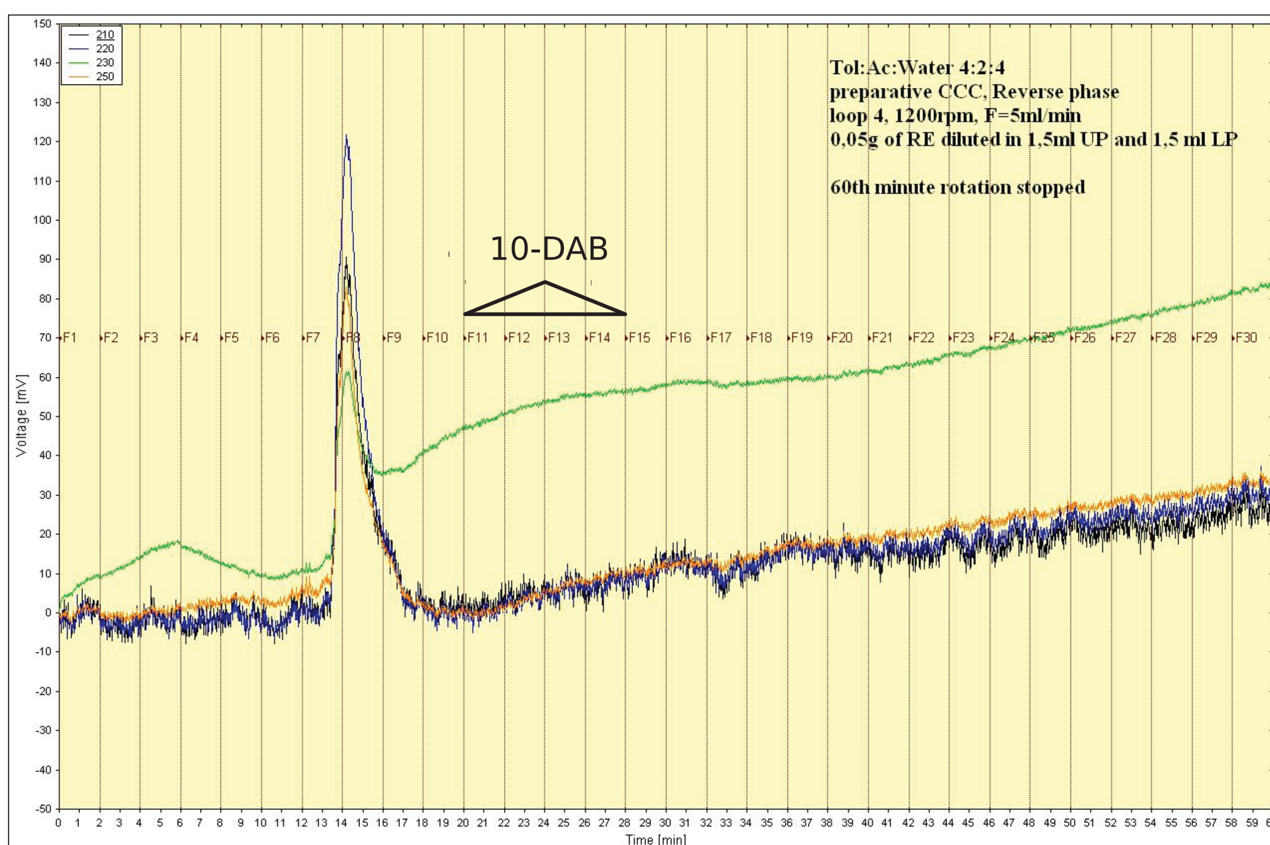
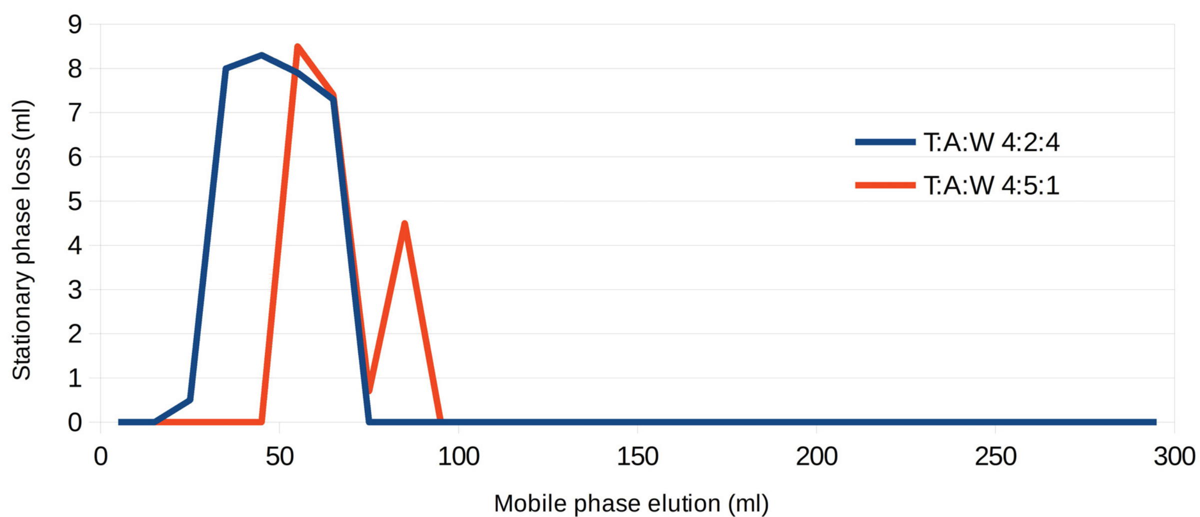


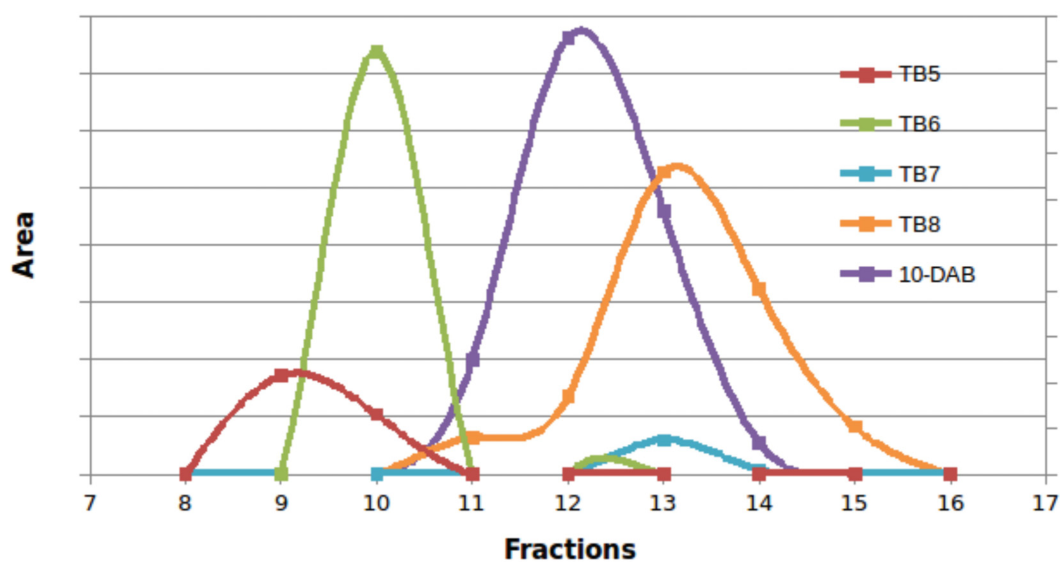
Figure 45: HPCCC UV detector profile for toluene/acetone/water 4:2:4, 50 mg, reverse phase mode.

Bleeding monitoring

The separation was done with 50 mg, no additional DHB or PAC added.
The comparison of toluene:acetone:water SS ratios – 4:5:1 and 4:2:4 was graphically calculated.



Graph 13: Bleeding monitoring for toluene:acetone:water different ratios, reverse mode.



Graph 14: CCC chromatography profile for toluene:acetone:water 4:2:4, 50 mg, only reverse mode.

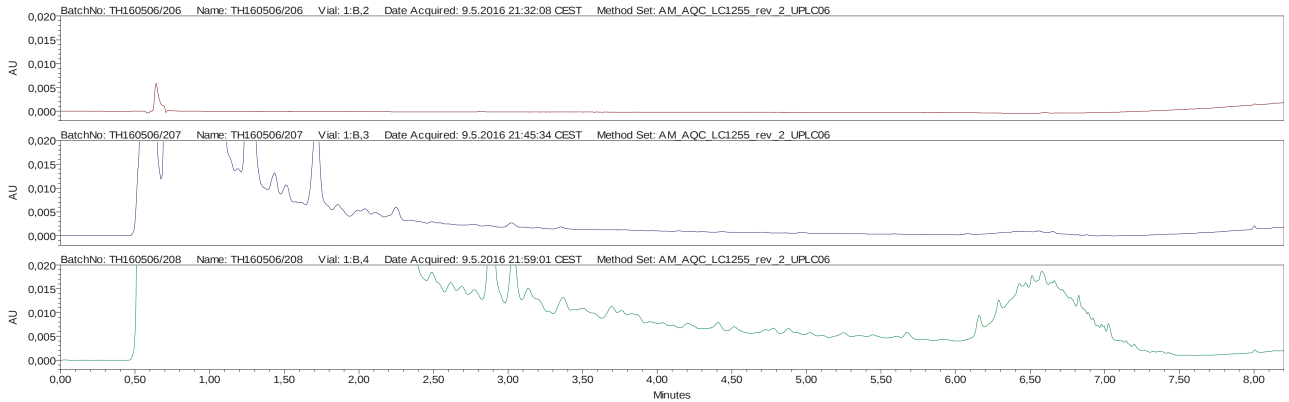


Figure 46: HPLC analysis of fractions 6-8, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode.

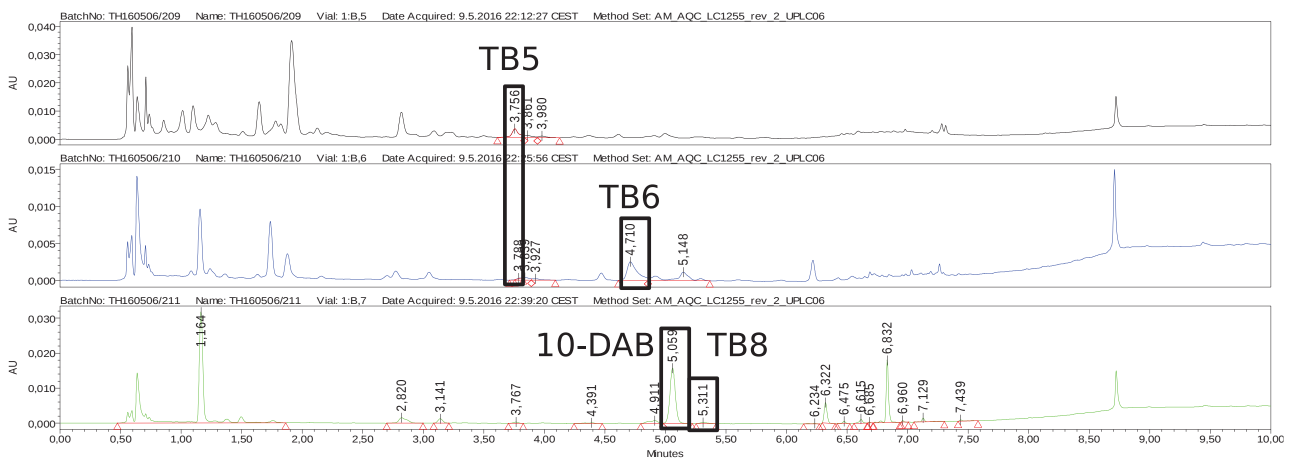


Figure 47: HPLC analysis of fractions 9-11, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode.

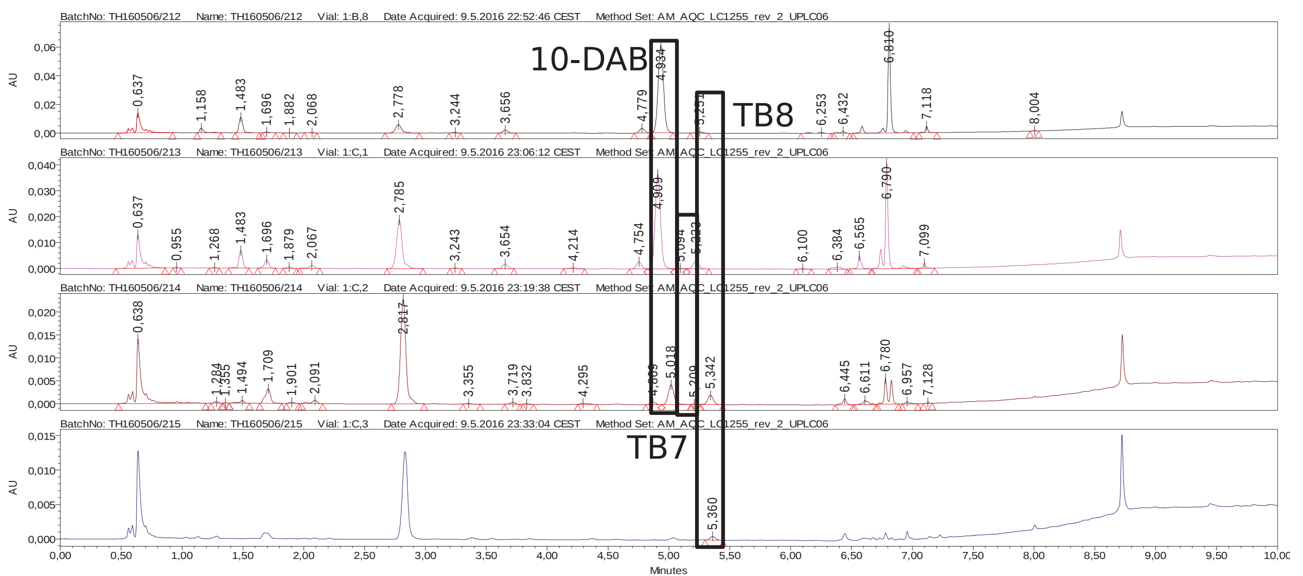


Figure 48: HPLC analysis of fractions 12-15, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode.

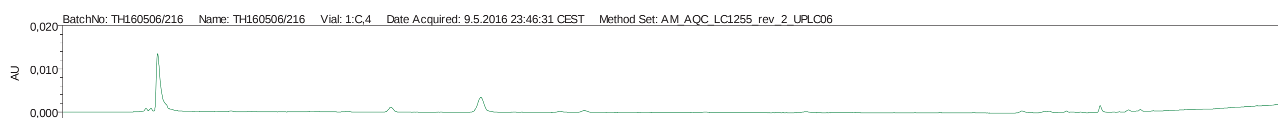


Figure 49: HPLC analysis of fraction 16 as an example of fractions 16-30, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode.

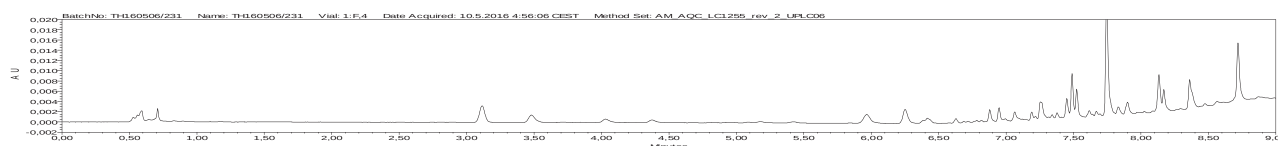


Figure 50: HPLC analysis of fraction 31 - lipophilic impurities, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode.

Table 23: Main fractions target molecule purity, toluene/acetone/water 4:2:4, 50 mg.

Analysis	10-DAB purity (%)
Fr.11	20.72
Fr.12	43.19
Fr.13	31.82
Fr.14	7.76

Discussion: Solvent system toluene/acetone/water 4:2:4 was chosen for its quite suitable and low distribution coefficient (0.8237 for 10-DAB in reverse mode in preliminary testing) and for that reason it was expected that 10-DAB will be eluted very soon. The fraction volume was therefore set to 10 ml for better resolution and only reverse phase mode was performed to see when 10-DAB eluates. The results confirmed that 10-DAB eluted mostly in 12-13th fraction (=6th fraction in SS with fraction volume 20 ml) – in comparison with solvent system MIBK/acetone/water where the compound eluted between 13-15th fractions (fraction volume 20 ml).

Fractions 4-7 containing less evaporated volume did not contain 10-DAB and thus results were not skewed.

Initial stationary phase retention by using toluene/acetone/water 4:2:4 was 73.2 %. Injection of 50 mg Rafinated extract in mixture of UP/LP caused quite low bleeding of stationary phase. The most polar 10-DAB started to elute between 20-28th minute (110-140 ml) in reverse phase mode. Fractions also contained polar impurities. No dual mode was done and so no lipophilic impurities were separated – only collected as a waste after the rotation stop, you can see in HPLC fraction 31 (Figure 50). The most concentrated 10-DAB fractions HPLC purity was from 32 to 43 % (Table 23). The similar CCC was done with toluene/acetone/water 4:2:4; the results are in the following chapter.

6.6.5 Toluene/acetone/water 4:5:1, 50 mg RE

Reverse phase mode purification

HPCCC semi-preparative CCC coil (128.5 ml)

Solvent system: toluene/acetone/water 4:5:1 (UP about 85 volume %)

Rotation: 1200 rpm

Flow: 5 ml/min

UV detection: 210 nm, 220 nm, 230 nm, 250 nm

Initial SP_{ret}: [128.5+1.6+4–60/128.5]x100=**57.6 %**

Sample: *Taxus baccata* Rafinated extract (evaporated dry rest) – 50 mg dissolved in 1.5 ml upper and 1.5 ml lower phase.

Injection: loop volume 4 ml

Fraction volume: 10 ml; waste from 60th min

Residual SP_{ret}: 48/128.5=**37.4 %**

Procedure: Reverse mode only. HPLC pump channels purging: A=UP, B=LP; CCC column stationary phase (A) filling; F=5 ml/min; rotation setting; mobile phase equilibrium (B); initial SP_{ret} calculation; 3 ml sample was injected to 4 ml loop, analysis started in reverse mode (B channel pumping); fraction collection; 60th min rotation stopped, end of analysis → the rest of SP collected – residual SP_{ret} calculation; all fraction bleeding calculation (bleeding observed in fractions 6-9, see Graph 13); fractions 1-31 analyzed by HPLC (Figure 52-55). Graph 15 shows elution of impurities and target molecules in fractions. In Table 25 purity of target molecules is described.

HPLC sample preparation: 4 ml of every fraction was evaporated with N_2 and diluted with 1 ml MeOH:ACN 1:1. Also in this experiment vials 6 and 7 contained less than 4 ml of LP, see Table 24.

Table 24: HPLC analysis sample preparation - vials with less volume.

Vial No.	Volume analyzed (ml)	Notes
6	1.5	
7	2.6	white opaque
8	4	white opaque in the interface
9	4	white opaque in the interface
10	4	white opaque
11	4	white opaque

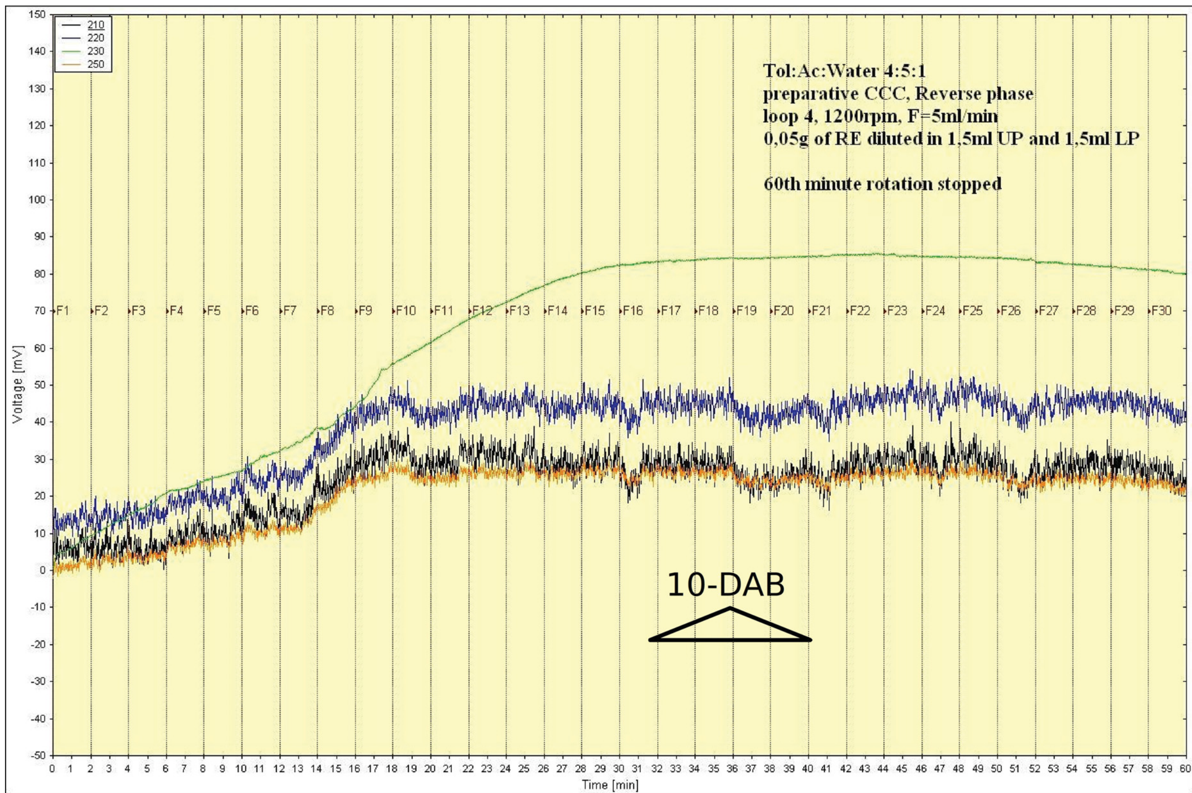
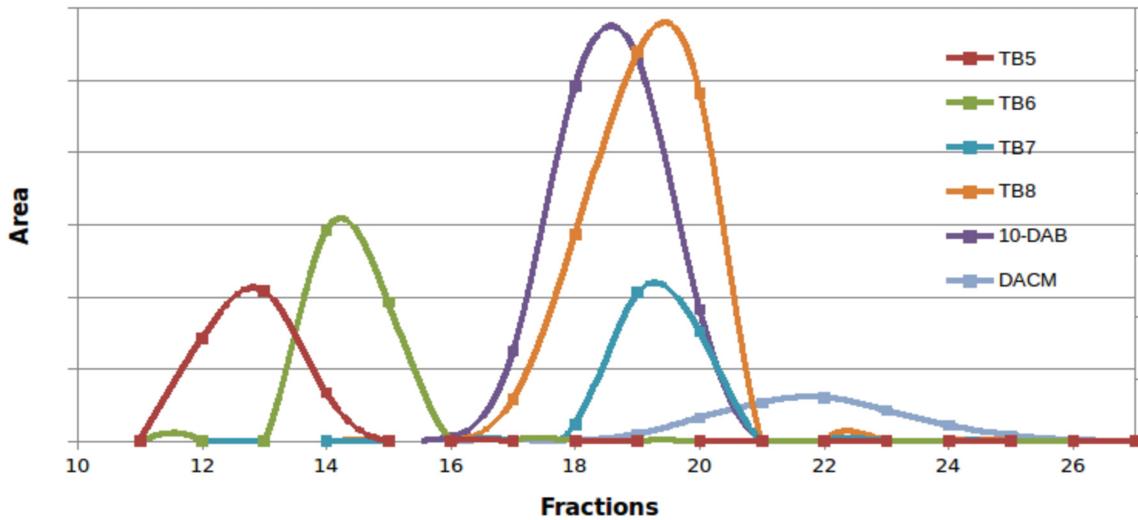


Figure 51: HPCCC UV detector profile for toluene/acetone/water 4:5:1, 50 mg, reverse phase mode.



Graph 15: CCC chromatography impurity and 10-DAB profile, 50 mg, only reverse mode.

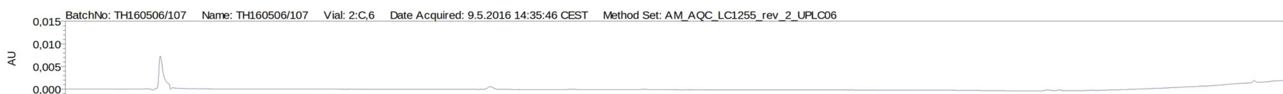


Figure 52: HPLC analysis of fraction 8 (fraction 8 as example of fractions 1-7), toluene/acetone/water 4:5:1, 50 mg, reverse phase mode.

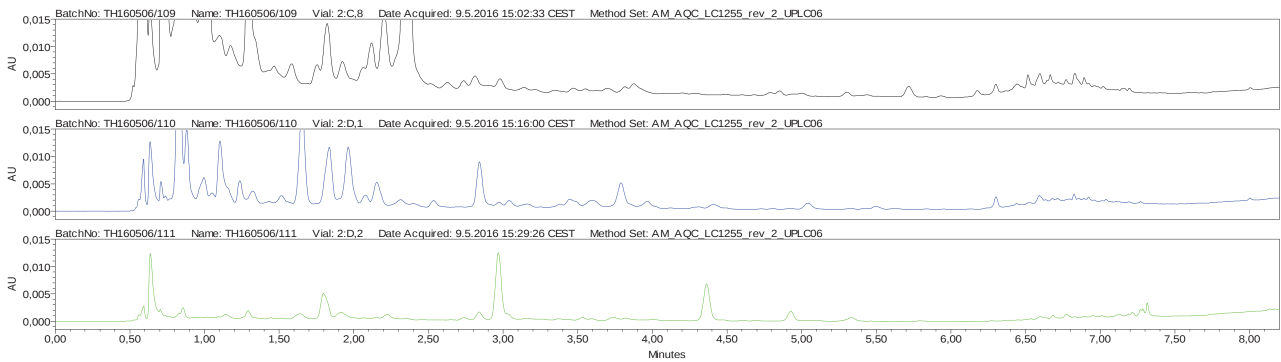


Figure 53: HPLC analysis of fractions 9-11, toluene/acetone/water 4:5:1, 50 mg, reverse phase mode.

TB5
TB6
10-DAB
TB7 TB8
DACM

Figure 54: HPLC analysis of fractions 12-20 (fractions 21-30 did not contain any important compounds), toluene/acetone/water 4:5:1, 50 mg, reverse phase mode.

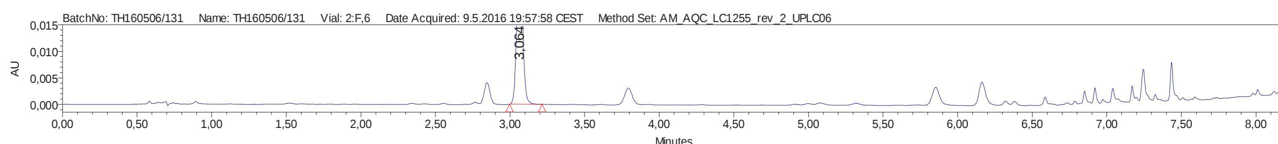


Figure 55: HPLC analysis of fraction 31, toluene/acetone/water 4:5:1, 50 mg, reverse phase mode.

Table 25: Main fractions target molecule purity, toluene/acetone/water 4:5:1, 50 mg.

Analysis	10-DAB purity (%)
Fr.16	1.01
Fr.17	17.84
Fr.18	40.28
Fr.19	39.10
Fr.20	25.83

Discussion: Solvent system toluene/acetone/water 4:5:1 was chosen for its suitable distribution coefficient (1.9173 for 10-DAB in reverse mode in preliminary testing). The fraction volume was set to 10 ml for good resolution and only reverse phase mode was performed to see when 10-DAB eluates. The results showed that 10-DAB eluted mostly in 18-19th fraction (= 9th fraction in SS with fraction volume 20 ml) – in comparison with solvent system MIBK/acetone/water where the compound eluted between 13-15th fractions (fraction volume 20 ml).

Initial stationary phase retention by using toluene/acetone/water 4:5:1 was 57.6 %. Injection of 50 mg Rafinated extract in mixture of UP/LP caused quite low bleeding of stationary phase. The most polar 10-DAB started to elute between 30-40th minute (160-200 ml) in reverse phase mode. Fractions also contained polar impurities. No dual mode was done and so no lipophilic impurities were separated – only collected as a waste after the rotation stop, see Figure 55. The most concentrated 10-DAB fractions HPLC purity was about 40 % (see Table 25).

SS toluene:acetone:water 4:2:4 showed SP_{ret} similar to SS MIBK:acetone:water 2:3:2 200 mg. Residual SP_{ret} in toluene:acetone:water 4:5:1 was only 37 % which is the lowest of all experiments in preparative scale.

7. CONCLUSION

Paclitaxel, 10-DAB and DHB present different polarity and therefore DUAL mode and washing technique had to be used. This technique had never been used in TEVA laboratory in Opava before.
Solvent system literature research provided list of conditions which were subsequently born in mind.

Phase diagrams were successfully implemented to solvent system selection. Test tube experiments were performed to find distribution coefficients. This data is very valuable for future plans and show theoretical dependencies on different solvents. For example solvent systems containing hexane are too lipophilic and give very low D. Very suitable solvents are toluene and MIBK, therefore they were used for upcoming tests. There are visible differences between acetone and methanol usage as miscible intermediate solvent. Although the results show some obscurity the data predicted suitable SS: MIBK/acetone/water 2:3:2 and toluene/acetone/water in various ratios.

Column mass overload dependence was studied and showed only slight distribution coefficient change. The main conclusion is that distribution coefficients coming from the analytical concentration test tube experiments could be used for preparative purposes.

SS containing MIBK/methanol/water gave large stationary phase bleeding therefore $CaCl_2$ was added subsequently. 1% $CaCl_2$ addition showed large bleeding too nevertheless it was not progressing.

Few CCC preliminary experiments with analytical amount of paclitaxel, 10-DAB and DHB were done. SS composition was made on the basis of test tube results and literature sources. SS containing MIBK with acetone or methanol gave UV visible peaks for all molecules. The resolution was low. Because of molecules wide polarity range DUAL mode was used.

The same conditions were executed with Rafinated extract. There was problem with sample dissolution. The results differs according to type of injection. In case of SP equilibrium the massive bleeding was observed from 6th to 8th fraction. This fractions were dark green/brown colour. From TLC it looks like that all material is co-eluted from sixth to eighth fractions. In this case there was probably no separation. In case of no SP equilibrium there was almost no bleeding and PAC retained dominantly in column.

Preliminary experiments processed on analytical column without proper optimization showed that satisfactory separation is possible, the liquid sample can be easily injected and therefore it is possible to move forward to preparative scale.

There is knowledge that the bleeding is dependent on rotation speed. Our next target was to see bleeding dependence on rotation speed. The best conditions were selected – 1200 rpm and 5 ml/min. There was no substantial difference between the flow rate

3 ml/min and 5 ml/min, therefore for much faster separation process the 5 ml/min flow rate was chosen as more advantageous.

Preparative scale HPCCC in optimized conditions was used for MIBK/acetone/water 2:3:2 with 50 mg, 200 mg and 800 mg of Rafinated extract. In these experiments the initial SP_{ret} was between 60-73 % which is satisfactory. Residual SP_{ret} was at least 45 %.
Injection of 50 mg RE caused quite low bleeding of SP, probably due to very low overload. 10-DAB as main peak started to elute in 15th fraction but did not leave the column completely and the rest of compound stayed in the column held on stationary phase. Therefore the dual mode switch should have postponed. The purity of 10-DAB in 15th fraction was 55.79 %.

For experiment with 200 mg RE the sample was spiked with 2 mg DHB and 2 mg PAC to see the separation in HPCCC machine. SP bleeding was still low. Most of 10-DAB eluted in fractions 14 and 15 but mode was again switched in after 15th fraction. There was a possibility of some 10-DAB still being held in the column. The purity in main fractions was 62.5 % and 67.2 %. PAC eluted immediately after dual mode switch in 16th fraction and its purity was about 50 %. The last to elute was DHB in 17th and 18th fraction with 60 % purity in 17th fraction.

800 mg RE was also spiked with PAC and DHB. This experiment showed big bleeding especially in first 5 fractions probably caused by slight overload. 10-DAB was eluting for a long time, majority of this compound eluted in 12th and 13th fraction with purity about 52-58 %. The dual mode was switched after 20th fraction and PAC eluted in 21st fraction. Its purity was 18 %. In fraction 23 DHB eluted and was 37 % pure.

SS toluene:acetone:water 4:2:4 and 4:5:1 was also tested because distribution coefficient of 10-DAB was suitable for experimenting. Toluene:acetone:water 4:2:4 showed SP_{ret} similar to MIBK:acetone:water 2:3:2 200 mg. Residual SP_{ret} in toluene:acetone:water 4:5:1 was only 37 % which is the lowest of all experiments in preparative scale.

During all experiments impurities were analyzed. Only TB5 and TB6 are more polar than 10-DAB and therefore were eluted at the front. After dual mode switch the most lipophilic impurities left the column together with PAC and after that less polar impurities and DHB were eluted. If the volume of fractions was lower there should be more accurate separation but this was not the target of this thesis.

To sum it up two SS – MIBK:acetone:water 2:3:2 and toluene:acetone:water 4:2:4 could be used in taxanes separation - not with HPCCC (this machine is very small and used for laboratory purposes and research) but transfer is possible to CPC which already is separating other natural materials in Teva in Opava.

8. ABBREVIATIONS

10-DAB	10-deacetylbaccatin
ACN	acetonitrile
CCC	countercurrent chromatography
CCD	countercurrent distribution
CPC	Centrifugal partition chromatography
D	distribution coefficient
DCCC	Droplet countercurrent chromatography
DHB	9-dihydro-13-acetylbaccatin III
DMSO	dimethyl sulfoxide
EA	ethyl acetate
EtOH	ethanol
FDA	Food and drug administration
HPCCC	High performance countercurrent chromatography
HPLC	High performance liquid chromatography
HSCCC	High speed countercurrent chromatography
IPC	in-process control
K_d	distribution constant=partition ratio, in CCC also distribution coefficient
LL	liquid-liquid
LP	lower phase
MeOH	methanol
MIBK	methyl isobutyl ketone
MP	mobile phase
MTBE	methyl *tert*.-butyl ether
NP	normal phase
PAC	paclitaxel
PE	primary extract
QC	quality control
QP	quality person
RE	rafinated extract
RP	reverse phase
SP	stationary phase
SP_{ret}	stationary phase retention
SS	solvent system
TLC	Thin-layer chromatography
UP	upper phase

9. TABLE INDEX

Table 1: Adverse effects of docetaxel and paclitaxel in 3-week clinical study.............12

Table 2: Summary of developed taxane analogues including potential compounds for the future cancer treatment. [10]...12

Table 3: Differences between HPCCC and HSCCC presented on example of separation of two isomers isolated from Chinese medicines. [26]..19

Table 4: HPCCC types offered by Dynamic Extractions Ltd. and their specification.....20

Table 5: HEMWat solvent system ratios...26

Table 6: The list of taxanes separations created after literature and Internet search.. 27

Table 7: Summary of test tube experiments containing distribution coefficients.........37

Table 8: Phase sampling..38

Table 9: Concentration dependence of distribution coefficient..................................39

Table 10: Monitoring of MIBK/methanol/water 3:3:4 SP loss without SP retarder.........40

Table 11: Difference in SP loss (start to end) expressed as a percentage...................41

Table 12: Monitoring of MIBK/methanol/water 3:3:4 SP loss with SP retarder.............42

Table 13: Difference in SP loss (start to end) expressed as a percentage...................43

Table 14: HPLC analysis of Rafinated extract..45

Table 15: HPCCC parameters of MIBK/acetone/water 2:3:2 tested on model mixture..47

Table 16: HPCCC parameters of MIBK/methanol/water 3:3:4 tested on model mixture. 48

Table 17: HPCCC parameters of MIBK/methanol/water 3:3:4 tested on Rafinated extract...51

Table 18: HPCCC parameters of MIBK/acetone/water 2:3:2 tested on Rafinated extract without phases equilibrium...54

Table 19: Main fractions target molecule purity, MIBK/acetone/water 2:3:2, 50 mg....61

Table 20: Main fractions target molecule purity, MIBK/acetone/water 2:3:2, 200 mg.. 65

Table 21: Main fractions target molecule purity, MIBK/acetone/water 2:3:2, 800 mg.. 70

Table 22: HPLC analysis sample preparation - vials with less volume.........................72

Table 23: Main fractions target molecule purity, toluene/acetone/water 4:2:4, 50 mg. 75

Table 24: HPLC analysis sample preparation - vials with less volume.........................76

Table 25: Main fractions target molecule purity, toluene/acetone/water 4:5:1, 50 mg. 79

10. FIGURE INDEX

Figure 1: Separatory funnel....15

Figure 2: Craig's countercurrent distribution apparatus consisting of 25 tubes (manually operated). [18]....16

Figure 3: Mechanical cycle of Craig apparatus. [18]....16

Figure 4: DCCC machine composed of series of tubes filled with SP and droplets of MP being pushed through them. [20]....16

Figure 5: Theoretical detail of CPC instrument. [34]....17

Figure 6: CPC instrument containing series of extration cells. [21]....17

Figure 7: Yoichiro Ito. [24]....18

Figure 8: Two axis creating planetary motion. [26]....18

Figure 9: Planetary motion. [26]....18

Figure 10: HPCCC machine developed by Dynamic Extractions. [28]....19

Figure 11: Theoretical countercurrent distribution of components with different distribution constants. [37]....23

Figure 12: Model ternary diagram of MIBK, acetone and water creating biphasic system....24

Figure 13: Changing the SS ratio for more preferential phase volume. [38]....25

Figure 14: Change of the distribution coefficient using ternary diagram....25

Figure 15: Paclitaxel....28

Figure 16: 10-DAB....29

Figure 17: DHB....30

Figure 18: Spectrum HPCCC instrument (by Dynamic Extractions) in detail....32

Figure 19: Example of HPCCC system set-up....33

Figure 20: Reverse and normal phase mode. [45]....34

Figure 21: HPLC analysis of Rafinated extract and mixture of 10-DAB, DHB and PAC..44

Figure 22: HPLC analysis of Model mix (black) and flavonoids (blue)....45

Figure 23: Model HPCCC chromatogram; measured in 4 different length waves (220 nm, 230 nm, 240 nm and 250 nm), in this case 14 fractions were collected....45

Figure 24: TLC one spot analysis used for MIBK/acetone/water 2:3:2....46

Figure 25: Ternary diagram for MIBK/acetone/water 2:3:2....46

Figure 26: HPCCC UV profile of model mixture in MIBK/acetone/water 2:3:2....47

Figure 27: Ternary diagram for MIBK/methanol/water 3:3:4....48

Figure 28: TLC one spot analysis....48

Figure 29: HPCCC UV profile of model mixture in MIBK/methanol/water 3:3:4....49

Figure 30: TLC support done for model mixture in MIBK/methanol/water 3:3:4..........50
Figure 31: HPCCC UV profile of Rafinated extract in MIBK/methanol/water 3:3:4........52
Figure 32:TLC support done for Rafinated extract in MIBK/methanol/water 3:3:4........52
Figure 33: HPCCC UV profile of Rafinated extract in MIBK/acetone/water 2:3:2 without phases equilibration..55
Figure 34: TLC support done for Rafinated extract in MIBK/acetone/water 2:3:2 without phases equilibrium..55
Figure 35: HPCCC UV detector profile for MIBK/acetone/water 2:3:3, 50 mg, dual mode..59
Figure 36: HPLC analysis of fractions 10-15 with important impurities, MIBK/acetone/water 2:3:2, 50 mg..60
Figure 37: HPCCC UV detector profile for MIBK/acetone/water 2:3:3, 200 mg, dual mode..63
Figure 38: HPLC analysis of RE, mixture of 10-DAB+DHB+PAC, fractions 4-10, MIBK/acetone/water 2:3:2, 200 mg..64
Figure 39: HPLC analysis of fractions 11-18, MIBK/acetone/water 2:3:2, 200 mg........64
Figure 40: HPCCC UV detector profile for MIBK/acetone/water 2:3:3, 800 mg, dual mode..67
Figure 41: HPLC analysis of flavonoids, RE, fractions 7-9, MIBK/acetone/water 2:3:2, 800 mg..68
Figure 42: HPLC analysis of fractions 10-14, MIBK/acetone/water 2:3:2, 800 mg........68
Figure 43: HPLC analysis of fractions 15-20, MIBK/acetone/water 2:3:2, 800 mg........69
Figure 44: HPLC analysis of flavonoids, fractions 21-25, MIBK/acetone/water 2:3:2, 800 mg..69
Figure 45: HPCCC UV detector profile for toluene/acetone/water 4:2:4, 50 mg, reverse phase mode..72
Figure 46: HPLC analysis of fractions 6-8, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode..74
Figure 47: HPLC analysis of fractions 9-11, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode..74
Figure 48: HPLC analysis of fractions 12-15, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode..74
Figure 49: HPLC analysis of fraction 16 as an example of fractions 16-30, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode..75
Figure 50: HPLC analysis of fraction 31 - lipophilic impurities, toluene/acetone/water 4:2:4, 50 mg, reverse phase mode..75
Figure 51: HPCCC UV detector profile for toluene/acetone/water 4:5:1, 50 mg, reverse phase mode..77

Figure 52: HPLC analysis of fraction 8 (fraction 8 as example of fractions 1-7), toluene/acetone/water 4:5:1, 50 mg, reverse phase mode..77

Figure 53: HPLC analysis of fractions 9-11, toluene/acetone/water 4:5:1, 50 mg, reverse phase mode...78

Figure 54: HPLC analysis of fractions 12-20 (fractions 21-30 did not contain any important compounds), toluene/acetone/water 4:5:1, 50 mg, reverse phase mode.. .78

Figure 55: HPLC analysis of fraction 31, toluene/acetone/water 4:5:1, 50 mg, reverse phase mode...79

11. GRAPH INDEX

Graph 1: Concentration dependence of distribution coefficient, HPLC evaluation.......38

Graph 2: Monitoring of MIBK/methanol/water 3:3:4 SP loss without SP retader...........40

Graph 3: MIBK/methanol/water 3:3:4 + 1% CaCl2 stationary phase loss....................42

Graph 4: Stationary phase bleeding of MIBK/methanol/water 3:3:4 tested on model mixture..49

Graph 5: Stationary phase bleeding of MIBK/methanol/water 3:3:4 tested on Rafinated extract..51

Graph 6: Stationary phase bleeding of MIBK/acetone/water 2:3:2 tested on Rafinated extract without phases equilibrium..54

Graph 7: Monitoring of stationary phase bleeding of MIBK/acetone/water 2:3:2, rotation speed optimization...56

Graph 8: Monitoring of stationary phase bleeding of MIBK/acetone/water 2:3:2, flow rate optimization..57

Graph 9: Bleeding monitoring at 1200 rpm for 6.6.1-3, dual mode............................59

Graph 10: CCC chromatography profile – 50 mg (only revese mode).........................60

Graph 11: CCC chromatography impurities and target molecules profile – 200 mg, dual mode..63

Graph 12: CCC chromatography impurities and target mulecules profile, 800 mg, dual mode..67

Graph 13: Bleeding monitoring for toluene:acetone:water different ratios, reverse mode..73

Graph 14: CCC chromatography profile for toluene:acetone:water 4:2:4, 50 mg, only reverse mode..73

Graph 15: CCC chromatography impurity and 10-DAB profile, 50 mg, only reverse mode..77

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