UNIVERSITY OF SOUTH BOHEMIA IN ČESKÉ BUDĚJOVICE
FACULTY OF SCIENCE

BILIRUBIN ESTERIFICATION USING ALIPHATIC ALCOHOLS

BACHELOR THESIS

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Annotation

The structure of bilirubin resembles the one of phycocyanobilin, which is a pigment of cyanobacteria and algae used to capture light energy and phytochrome used by plants to sense light. We have chosen bilirubin as a model system to verify the possibility of chemical modification and to test its ability to form self-assembling aggregates with other suitable pigments. In this work, the bilirubin molecule is analysed by HPLC and the conditions for better analysis are optimized. Next, bilirubin is esterified under acidic conditions and the results are tested with HPLC method.

Affirmation

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Kristýna Poslední

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List of Abbreviations

HPLC.....High Performance (Pressure) Liquid Chromatography

TLC.....Thin Layer Chromatography

DMSO.....Dimethyl sulfoxide

CHINA... Mobile phase: methanol-acetonitrile-2% acetic acid (72:25:3)

CNS.....Central Nervous System

Columns:

XDB.....eXtra Dense Bonding

ODS.....Octadecylsilane (C18)

SB.....StableBond

1 Introduction

1.1 Photosynthesis and artificial antennas

Photosynthesis is an important biochemical process, where the light energy of sun is changed to the energy of chemical bonds. High-energetic organic substances are formed from simple inorganic substances such as carbon dioxide and water. It is the main source of the energy on the earth.

There are two steps of photosynthesis – light and dark reactions. The light reactions need light to convert the chemical energy to the form of ATP and NADPH. The first step is to capture the photon by the molecule of photosynthetic pigment. Depending on the type of organism, this pigment can be (bacterio)chlorophyll, carotenoid or an open chain tetrapyrrole called bilin [1].

The excitation energy is transported fast through the light-harvesting complexes – antennas to the reaction centres. There the excitation energy is used for a separation of charge followed by an electron flow through various components of electron transport chain. Next, the energy released during the electron transport is used to form an electrochemical gradient on the photosynthetic membrane and for the formation of ATP and NADPH (or NADH in the case of bacteria). These are later used for the synthesis of the organic substances in the dark part of the photosynthesis. [2]

Nowadays there are attempts to invent artificial photosynthetic systems including antennas, which could collect the sunlight more efficiently. The artificial antennas should cover a large spectral range and the excitation energy should be transferred fast to the reaction centres. This means that there should be short distances between antenna pigments. They should be chemically and photochemically stable. These requirements can fulfil for example some special nanostructures from native or synthesized porphyrin molecules (tetrapyrrole creating a large ring). [3, 4]

The tetrapyrrole-like structure of bilirubin is very similar to phycocyanobilin [5], a pigment of certain photosynthetic microorganisms used to capture light energy. Bilirubin could probably be used for the synthesis of artificial antennas if the molecule were to be modified.

1.2 Bilirubin

Bilirubin is an organic orange-brown substance largely found in human and most mammals' bodies as a bile pigment. In case of increased bilirubin level in human body it is responsible for the yellow colour of bruises and urine. In jaundice the high levels cause the yellow discoloration.

Originally thought as only animal pigment, bilirubin was also found in plants of genus Strelitzia (concretely in S. nicolai and S. reginae) [6].

1.2.1 Structure and formation of bilirubin in the body

Bilirubin is formed in the disposal process of old red blood cells in the spleen, where haemoglobin is released and decomposed to haem and globin. Haem consists of four substituted heterocyclic pyrrolic rings (tetrapyrrole), bound to a central iron atom and thus creating a large porphyrin ring.

When catabolised, haem is broken down into a green pigment biliverdin which is subsequently reduced to a free unconjugated bilirubin (bilirubin α). It is then covalently linked to albumin through an amide bond between one of its two propionic acid side chains and ε-amino group of lysine residue on albumin [7] (δ-bilirubin).

The bilirubin-albumin complex is transferred from spleen to the liver and albumin is released. One or two glucoronic acid molecules bind to propionic acid side chains of bilirubin and thus the mono- or diconjugated bilirubin (bilirubin β and bilirubin γ [8]) is formed. [9]

The conjugated double bonds are noticeable in the molecular structure of bilirubin (figure 1). Thanks to them the molecules of bilirubin are easily excitable and so its colour proves to be strong.

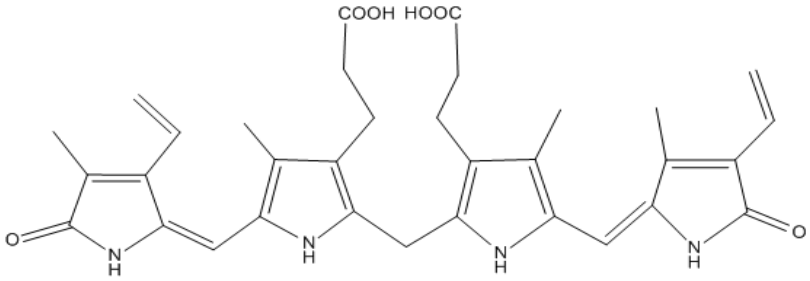

Figure 1: Structure of naturally occurring bilirubin (isomer IXα), $C_{33}H_{36}N_4O_6$, Mw = 584.36 g mol^{-1};
IUPAC name: 3-[2-[[3-(2-carboxyethyl]-5-[(Z)-(3-ethenyl-4-methyl-5-oxopyrrol-2-ylidene)methyl]-4-methyl-1H-pyrrol-2-yl]methyl]-5-[(Z)-(4-ethenyl-3-methyl-5-oxopyrrol-2-ylidene)methyl]-4-methyl-1H-pyrrol-3-yl]propanoic acid

1.2.2 Solubility of bilirubin

Bilirubin can be divided into direct and indirect according to its reaction with diazonium salt (van den Bergh reaction) to form a coloured complex. Since conjugated bilirubin is soluble in water, it can react with salt and thus it can be called direct. [10]

The unconjugated or indirect bilirubin is insoluble in water at physiologic pH. Bilirubin was considered to be a lipophilic substance thanks to its affinity to lipids in CNS. However the solubility in apolar solvents is rather low but increases with the polarity of the solvent. In the bilirubin structure, there are several strongly polar groups – two dioxy pyrryl and two carboxyl groups. Bilirubin is found to be much more water-soluble in alkaline environment where the dianion can form water soluble sodium salts. [10]

It was found that bilirubin is not soluble in carbon hydrides and alcohols and it is low soluble in carbon tetrachlorides, diethylether, acetone and ethylacetate. The maximal solubility is in DMSO and chlorooform [11].

When bound to albumin, the reaction of bilirubin with diazonium salt almost does not take place until the albumin is released from the bilirubin using accelerators such as caffeine. [10]

Tab. 1: Solubility of bilirubin in different solvents. The table is taken from literature [11].

Solvent	Solubility	Solvent	Solubility
	μM		*μM*
n-Hexane	0	Methanol	0
Cyclohexane	0	Ethanol	0
n-Heptane	0	1-Propanol	0
Liquid paraffin	0	2-Propanol	0
		1-Butanol	0
Benzene	22	1-Pentanol	0
Toluene	50	Cyclohexanol	0
Xylene	45		
Pyrrole	150	Ether	1
Pyridine	580	Ethylene glycolmonomethyl ether	15
Dichloromethane	1,800	Acetone	10
Chloroform	2,500	Methyl ethyl ketone	22
Carbon tetrachloride	40	Methyl propyl ketone	25
		Methyl isobutyl ketone	15
Formamide	2,300	Diisobutyl ketone	8
N,N-Dimethylformamide	800		
Dimethyl sulfoxide	>10,000	Ethyl acetate	9
Water, acid	0	Olive oil	1.3
Water, alkaline	>10,000		

1.2.3 Isomers

Isomers are compounds which have the same molecular formula but different structure. They can be divided into three types: constitutional, where the connectivity between atoms is important; configurational with the different arrangements in space as for example R/S or E/Z isomers; and conformational where the spatial arrangement depends only on bond rotations, for example the "seat" and "boat" conformations of cyclohexane. [12]

Normally, the unconjugated bilirubin α present in plasma is folded as ZZ (trans) conformation with six hydrogen bonds maintaining its stability. When exposed to light, the (4Z,15Z)- double bonds isomerise to (4Z,15E)-, (4E,15Z)- or (4E,15E)- cis bilirubin configurations. After the isomerisation, bilirubin becomes more polar and is more soluble in water. [13]

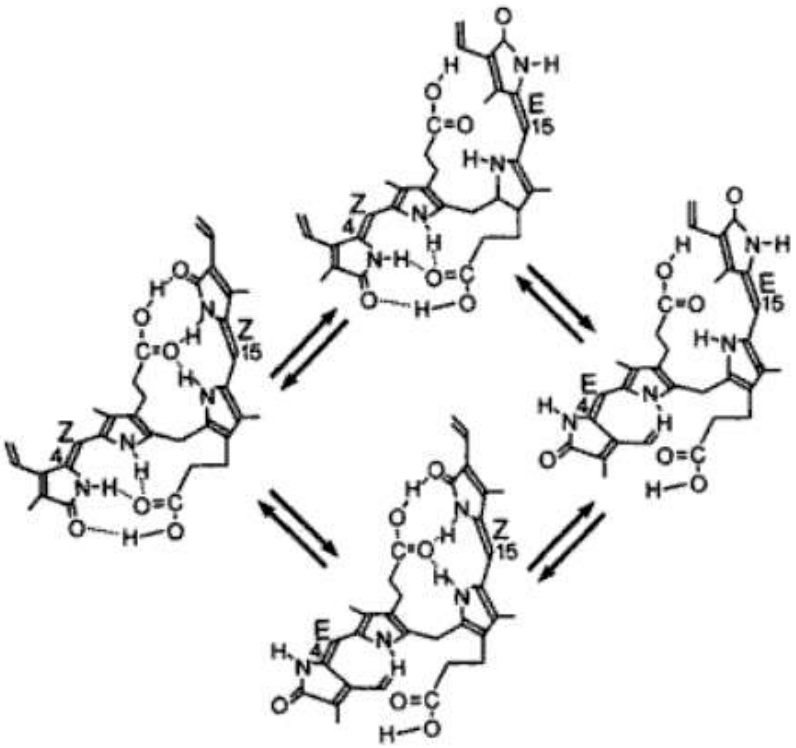

Figure 2: Configurational isomers of bilirubin between carbons 4-5 and 15-16 (from left clocwise: ZZ, ZE, EZ, EE) [14]

There are also constitutional isomers, which are mainly formed during the irradiation. These can be for example cyclobilirubin isomers (lumirubin) formed when irradiated with green light. [13] The different constitutions of bilirubin are numbered with Roman numerals.

The natural isomer is (4Z15Z)-bilirubin IXα but the commercial bilirubin preparations also contain other isomers.

Bilirubin used in this work contained α isomers of bilirubin III (absorption maximum at 455-458 nm; figure 3), IX (absorption maximum at 453-455 nm; figure 1) and XII (absorption maxima at 449-453 nm). [8]

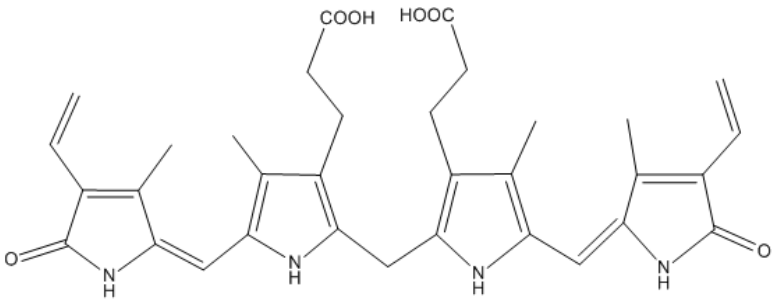

Figure 3: bilirubin-IIIα isomer

1.3 Esterification

As the name suggests, esterification is a reaction where ester is formed. The widely used method for formation of an ester is a reaction of carboxylic acid with an alcohol (mostly Fischer esterification, see figure 4).

In the nucleophilic acyl substitutions carboxylic acids are most reactive under acidic conditions because the negatively charged carboxylate ion does not allow the attack of a nucleophile.

In order for a reaction to proceed in direction to form an ester, the excess of water favours the hydrolysis under acidic conditions. [15]

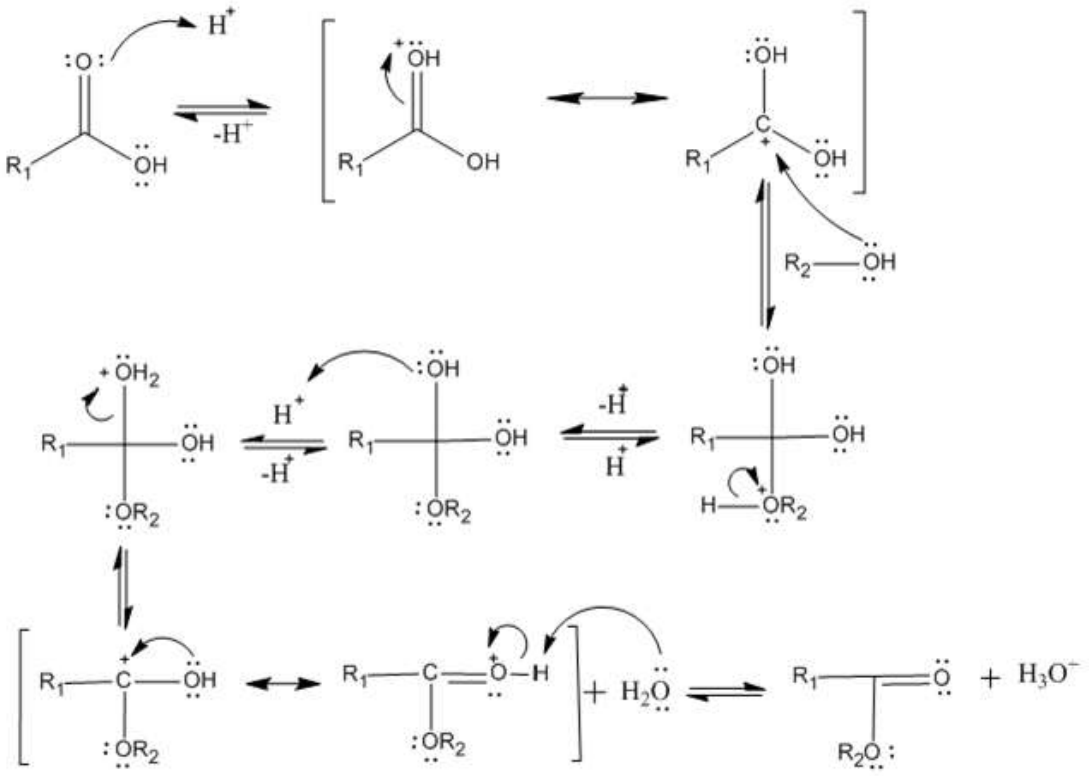

Figure 4: Reaction mechanism of the Fischer **e**sterification

In the case of bilirubin, different alcohols can bind to the propionic acid side chains. Bilirubin can be either mono- or di-esterified.

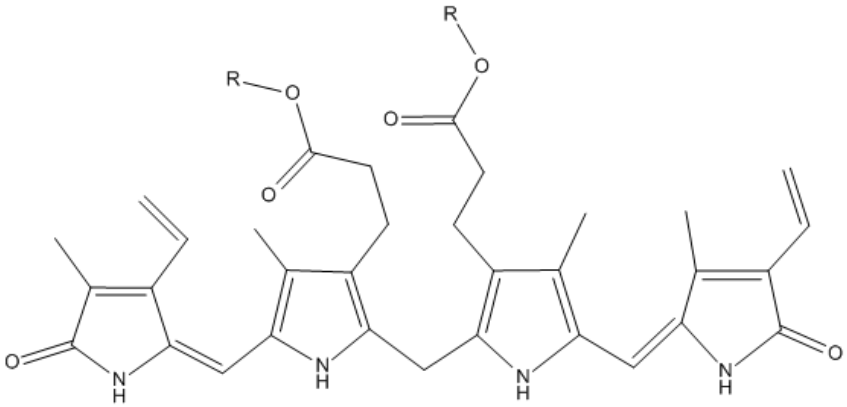

Figure 5: Bilirubin diester

2 Practical work

2.1 Aim

The main objective of this work is the development and optimization of bilirubin identification by means of HPLC method. Further bilirubin are esterified with differently long aliphatic alcohols (methanol, ethanol, butanol, heptanol and octanol) in the acidic environment. The esters are analyzed using HPLC.

2.2 Materials and methods

2.2.1 Sample product formation

Bilirubin used in all the experiments is purchased from Sigma-Aldrich Company as an orange-brown powder with purity ≥ 98 %. It is a mixture of α-isomers III (absorption maximum: 455-458 nm), IX (453-455 nm) and XII (449-453 nm). The bilirubin is stable when stored in the dark at -20 °C.

Since bilirubin has high extinction coefficient (between 52 and 65 cm^{-1} mM^{-1} at about 450 nm for the three different isomers presented [8]) only less than 0.003 g of bilirubin is dissolved in 1 mL of DMSO or chloroform. The sample is vortex-mixed for 20 seconds and the esterification is performed by addition of the alcohol under acidic conditions. Alcohols used for the esterification are: methanol, ethanol, butan-1-ol, heptan-1-ol and octan-1-ol. The ratio of dissolved bilirubin to alcohol is done at ratio 1:1 but also 1:4 is experimented with.

The acidic environment is obtained using concentrated sulphuric acid with the resulting concentration of 0.5 % or 2 % in the sample.

After mixing all the reagents, the samples have to be incubated so the reaction can proceed. Different times of the incubation are tested according to the length of alcohol added. For methanol and ethanol it is about two hours, for butan-1-ol three and for heptan-1-ol and octan-1-ol five hours. After the incubation some precipitation can occur and so the samples are centrifuged at 10 000 × g for 1.5 min. The supernatant is used for further analysis by HPLC. All steps are done at room temperature under dim light to avoid bilirubin photodegradation.

2.2.2 HPLC

High performance liquid chromatography

Chromatography is a technique to separate the individual components of sample to determine their presence and concentration. The method is based on the different distribution coefficients between sample components dissolved in mobile and the stationary phase. A mobile phase (in liquid chromatography it is a liquid) carries sample through a stationary phase (solid) which holds some of the components longer than the others and so the components are separated. [16]

In HPLC, the stationary phase consists of very small particles (3-10 μm in diameter) which provide more uniform flow and less distance for the solutes to be transported in the mobile phase to interact with the stationary phase. They are also more physically stable and therefore the mobile phase can be pushed with pumps at pressure of about 3 000 psi (200 atmospheres) through the column, which increases the flow rate and speeds up the whole separation process. [16]

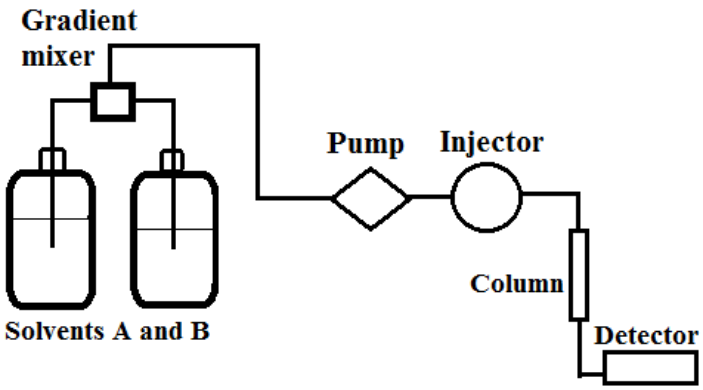

Figure 6: Simplified HPLC instrumentation (gradient system)

For the chromatographic characterization of the sample products, the Waters HPLC system equipped with Waters 600 Controller with a Gradient pump, an injector and a photodiode array detector (Waters 996) is used. The data are processed in a Millennium 32 Software. The data are exported at the absorbance of 450 nm and graphs in this work are drawn in Microsoft Excel 2007.

Reversed-phase HPLC (stationary phase: non-polar, mobile phase: polar) is performed with these different columns:

- Agilent Eclipse XDB-C8: 4.6 mm ID × 250 mm, (5μm)
- Agilent Zorbax ODS: (C18) 4.6 mm ID × 200 mm, (5 μm)
- Waters Symmetry C18: 4.6 mm ID × 75 mm, (3.5 μm)
- Agilent Zorbax SB-C18: 4.6 mm ID × 150 mm, (5 μm).

Following mobile phases (isocratic systems) were used:

- Methanol
- CHINA (Methanol-acetonitrile-2% acetic acid (72:25:3)) [17].

The flow rate is set to 1 mL/min.

Different volumes are injected: 3; 5; 10; 50 μL

Before injecting the sample, the column is equilibrated for five minutes with the mobile phase used during the bilirubin analysis. The chromatograms were recorded at 450 nm.

3 Results and Discussion

3.1 Esterification

At the beginning of study, we first had to search for optimal conditions of the esterification reaction. Several parameters could have been altered, such as sulphuric acid concentration, amount of alcohol, reaction temperature and duration of the reaction. In our study three parameters of the four mentioned were checked. Since all the results using different parameters were tested by HPLC the data are shown in later chapters.

In experiment where longer aliphatic alcohols were used, the samples became slightly blue-green during the incubation, which was probably caused by partial oxidation of the bilirubin to blue-green biliverdin. The intensity of the blue-green colour was stronger with increasing time.

3.2 HPLC results

3.2.1 Injection volume of bilirubin

The first task was to develop and optimise the HPLC method for separation and analysis of free bilirubin. Bilirubin was dissolved in DMSO and injected into the HPLC column Agilent Zorbax SB-C18. The mobile phase methanol-acetonitrile-2% acetic acid in the ratio 75:22.23:2.67 was used. In the beginning 50 μL were injected, however the resulting chromatogram was found to be rather disrupted and no clear single peak was observed (figure 7, violet line). The large widespread peak with maximum around 6th minute is most likely also a bilirubin peak. The reason for formation of the first peak of bilirubin was an interaction of the stationary phase of column with too much of solvent injected together with the sample. It was found out that lower injection volumes improve the chromatogram.

Besides the peak disruption at injection of higher volumes, the increasing absorbance and peak area with increasing sample volume injected is clearly visible. As a result of these experiments the lower volumes (5 μL) were chosen as an optimal volume of sample injection.

In the article [17] bilirubin is also analysed with the same column, but it is dissolved in $CHCl_3$ and the mobile phase CHINA contains higher percentage of acetonitrile (in article

[17]: 25 % our: 22.3 %). The polarity of mobile phase influences the time of retention. In reversed phase chromatography, the retention decreases with increasing polarity of the mobile phase (acetonitrile percentage in our case). These differences are the reasons why bilirubin peak from the article [17] is shifted to sixth minute.

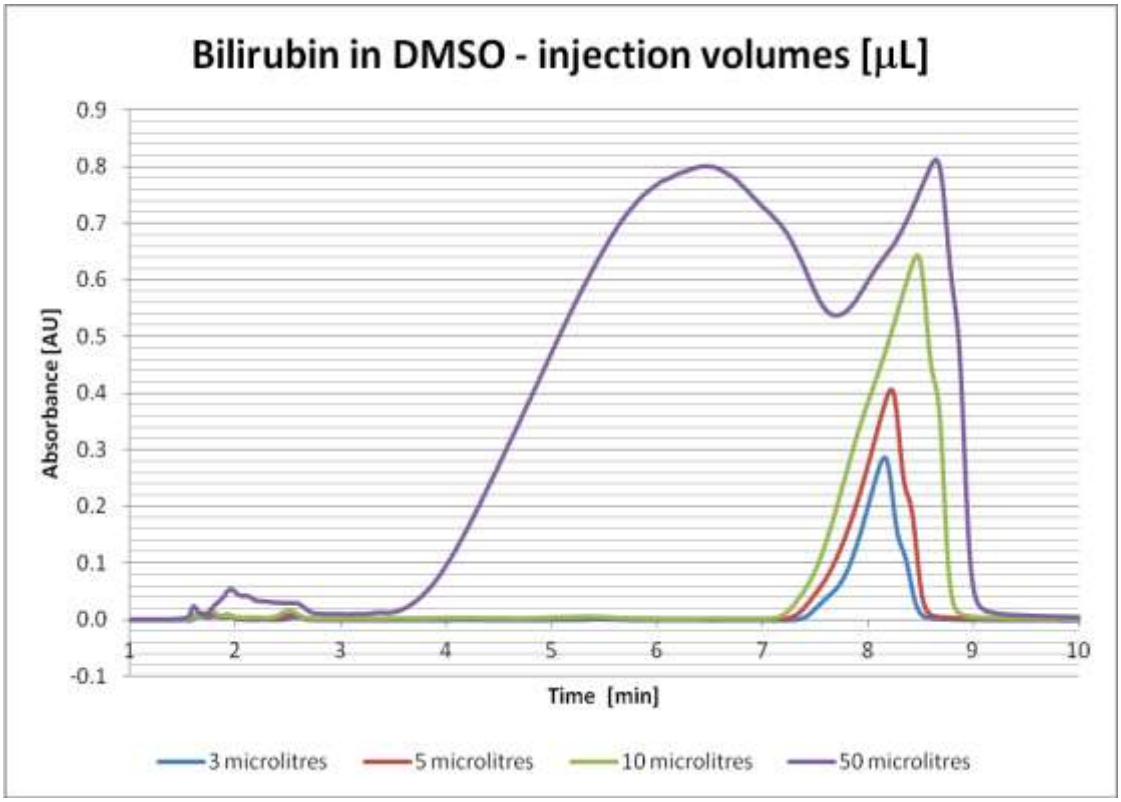

Figure 7: Influence of injection volume, mobile phase: methanol-acetonitrile-2% acetic acid (75:22.23:2.67) and the column: Agilent Zorbax SB-C18: 4.6 mm ID × 150 mm, (5 µm)

3.2.2 Short-time temperature stability of bilirubin

The influence of temperature on a thermostability of the dissolved bilirubin was also studied. A dissolved bilirubin in DMSO was heated to 40, 60 and 80 °C for 5 minutes and then 5 μL of bilirubin were injected in the HPLC at the same conditions as above. According to the chromatogram at figure 8 it is obvious that the temperature does not have any influence on the bilirubin stability at least during a short time to heat exposure.

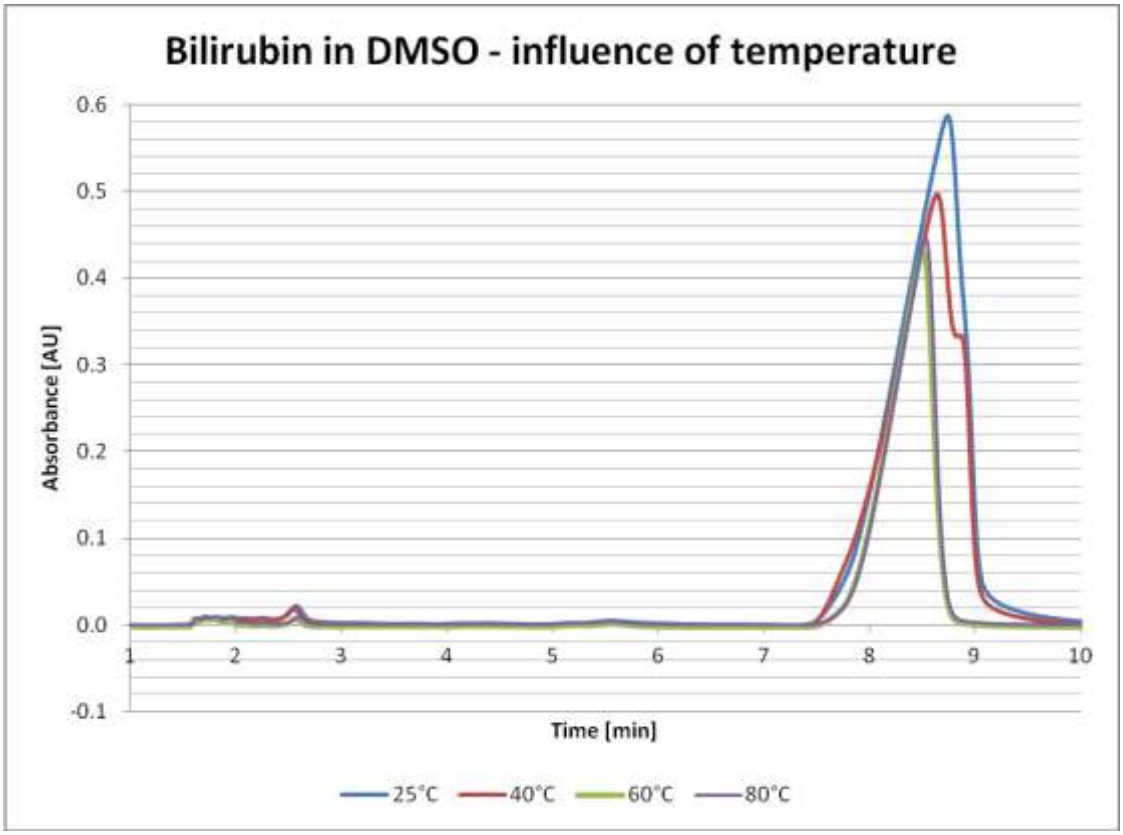

Figure 8: Influence of the temperature, mobile phase: methanol-acetonitrile-2% acetic acid (75:22.23:2.67) and the column: Agilent Zorbax SB-C18: 4.6 mm ID × 150 mm, (5 μm), injection volume: 5 μL

3.2.3 DMSO versus chloroform as solvent for bilirubin

Solubility of bilirubin varies among many organic solvents [11]. In the next step, we have compared DMSO as our original bilirubin solvent with another also efficient bilirubin solvent (figure 9). As it was already shown in table 1, the second best solvent is chloroform. According to the published results the area under the chromatographic peak of bilirubin was lower in the case of saturated solution in chloroform compared to DMSO revealing better solubility of bilirubin in DMSO. In this experiments a shorter (75mm) column was used (Waters Symmetry C18) with CHINA as a mobile phase. Due to the shorter column the retention times of bilirubin are lower which brings the advantage of faster analysis.

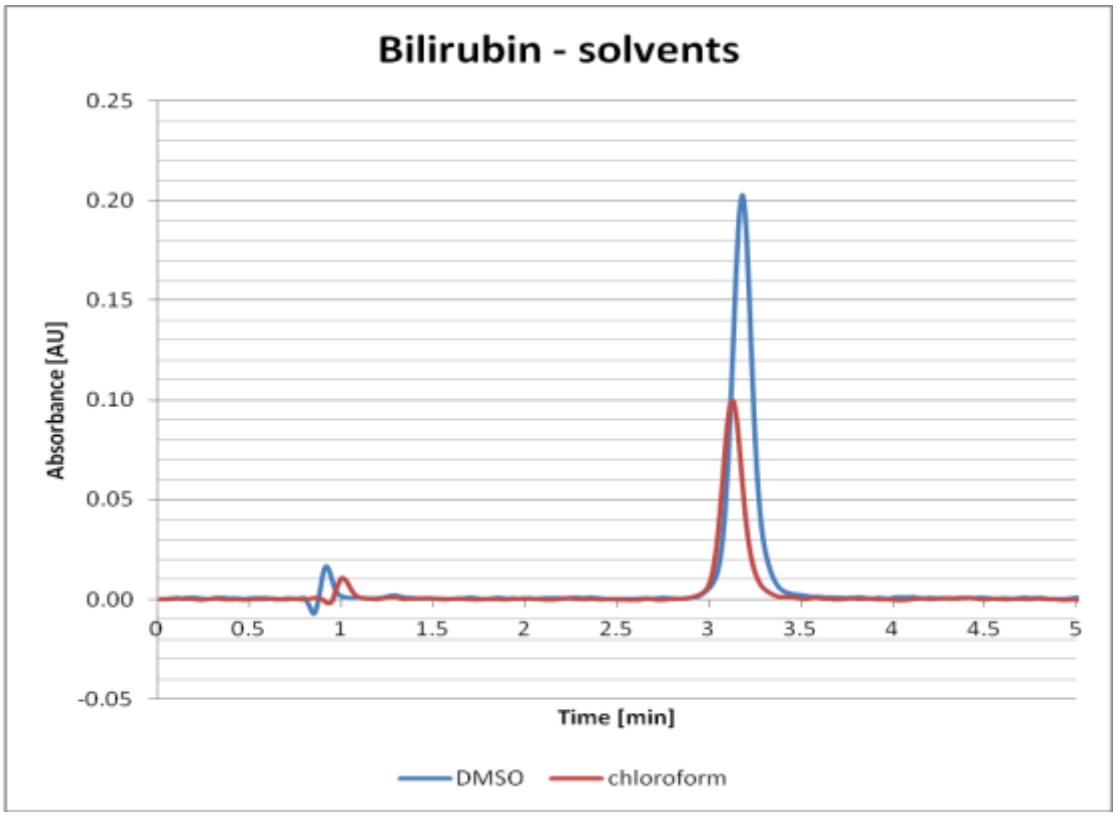

Figure 9: Solvents DMSO vs chloroform, mobile phase: CHINA and the column: Waters Symmetry C18: 4.6 mm ID × 75 mm, (3,5 μm), injection volume: 3 μL

3.2.4 Mobile phases for HPLC bilirubin separation

The chromatogram in figure 10 shows the difference when using only pure methanol or CHINA (methanol-acetonitrile-2% acetic acid (72:25:3) [17]) as mobile phases. The peaks are wider because at this stage we have injected 50 μL of the sample. When using methanol, a big peak of unknown compound between third and fourth minute was observed. Water could not be used in the mobile phases because it would later prevent the esterification reaction.

After more rather unsuccessful attempts to improve the chromatogram when using pure methanol as mobile phase, the CHINA mixture was chosen due to the chromatogram profiles as a suitable mobile phase.

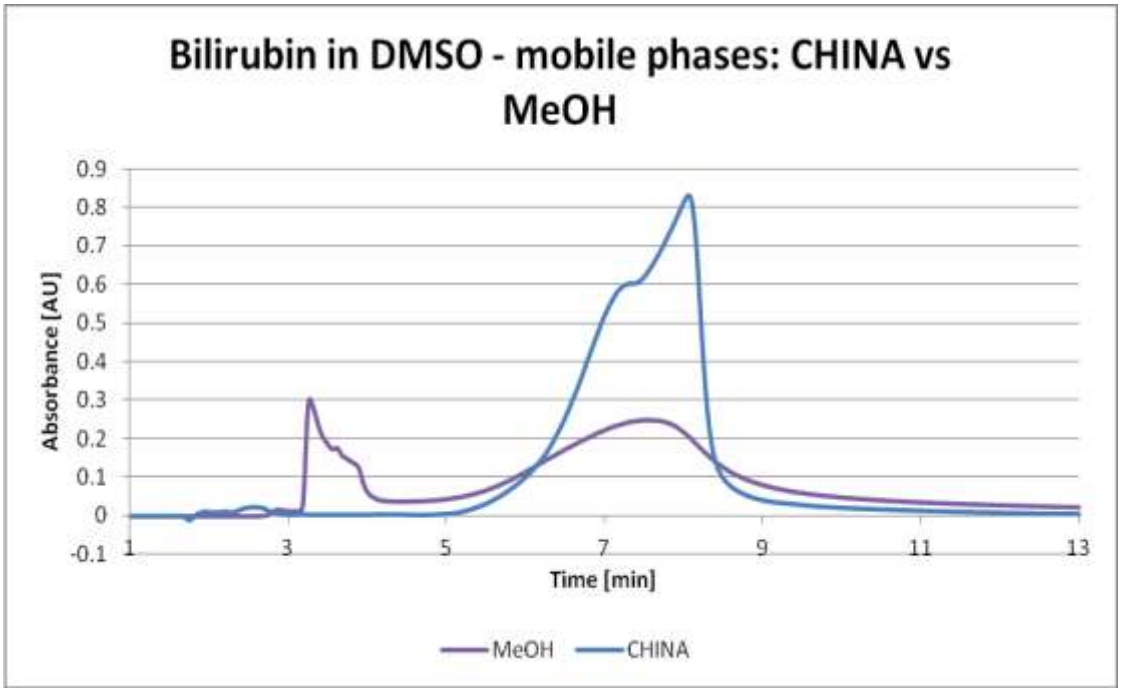

Figure 10: Column: Agilent Zorbax ODS: (C18) 4.6 mm ID × 200 mm, (5 μm), injection volume: 50 μL

3.2.5 HPLC column selection

Different columns were tested when optimizing the method of HPLC bilirubin separation. At first the Agilent Zorbax ODS and Agilent Eclipse XDB-C8 were compared. The C8 column showed unresolved and deformed peaks. Agilent Zorbax ODS showed better results but the best results were obtained when using Agilent Zorbax SB-C18 (figure 7). For a fast analysis of bilirubin the short column Waters Symmetry C18 was used (figure 9). This column would not be later suitable in the analysis of esters because the resolution of the peaks would be very low (the comparison of the data not shown).

3.2.6 Bilirubin esterification

After the chromatographic method was optimized, the esterification reactions were carried out in acidic environment (0.5% or 2%). In the chromatogram shown in figure 11, the esterification of bilirubin with alcohols under acidic conditions is depicted. In this case, the resulting concentration of sulphuric acid in the sample is 2 % and the ratio of alcohol to dissolved bilirubin in DMSO 1:1 is used.

The blue line shows chromatogram of esterified bilirubin with methanol, red with ethanol, green with butanol, violet with heptanol, black with octanol and the control bilirubin sample is the orange line. The esterified products of bilirubin conjugated with different alcohols can be observed with shorter retention times. Around 2.7 minutes there is a high peak of the bilirubin dimethylester, at 3rd minute is a peak from the ethanol sample and at 3.5 minute there is a peak of butanol sample. The peaks from heptanol and octanol are moved to longer retention times between 5 and 6.5 min. The increase of the retention times with the increase of the length of the esterified aliphatic alcohol is expected, because longer alcohols are more non-polar and so they are more retained in the stationary phase.

Some isomers of bilirubin have been formed during the incubation of the samples with reaction mixture as a cause of more factors. These factors can be light expansion, longer storage at room temperature, incubation time, or too acidic environment.

The peaks with the maximum around 7.6 min are peaks of pure bilirubin from all the samples.

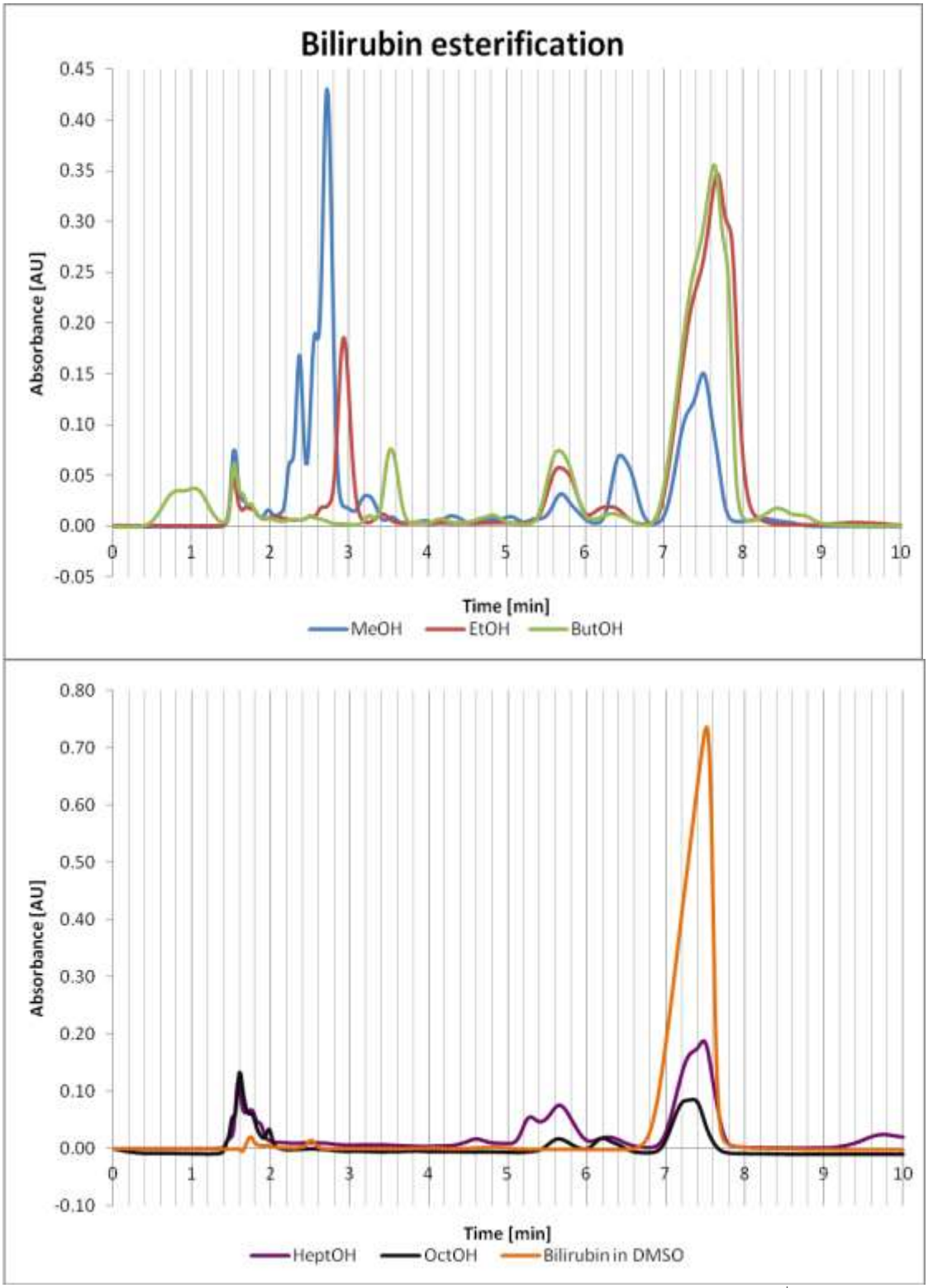

Figure 11: Bilirubin dissolved in DMSO, mixed with alcohol in ratio 1:1, acidity: 2% H^+, column: Agilent Zorbax SB-C18: 4.6 mm ID × 150 mm, (5 μm), mobile phase: CHINA, injection volume: 5 μL

3.2.7 Effect of sulphuric acid concentration in the reaction mixture

When 0.5% concentration of acid was used, the peaks looked very similar to the 2% but the esters concentrations were lower and the bilirubin concentration higher. The comparison of some of the results is shown in figure 12. More acidic environment in the reaction mixture obviously shifts the equilibrium of the esterification reaction to the products.

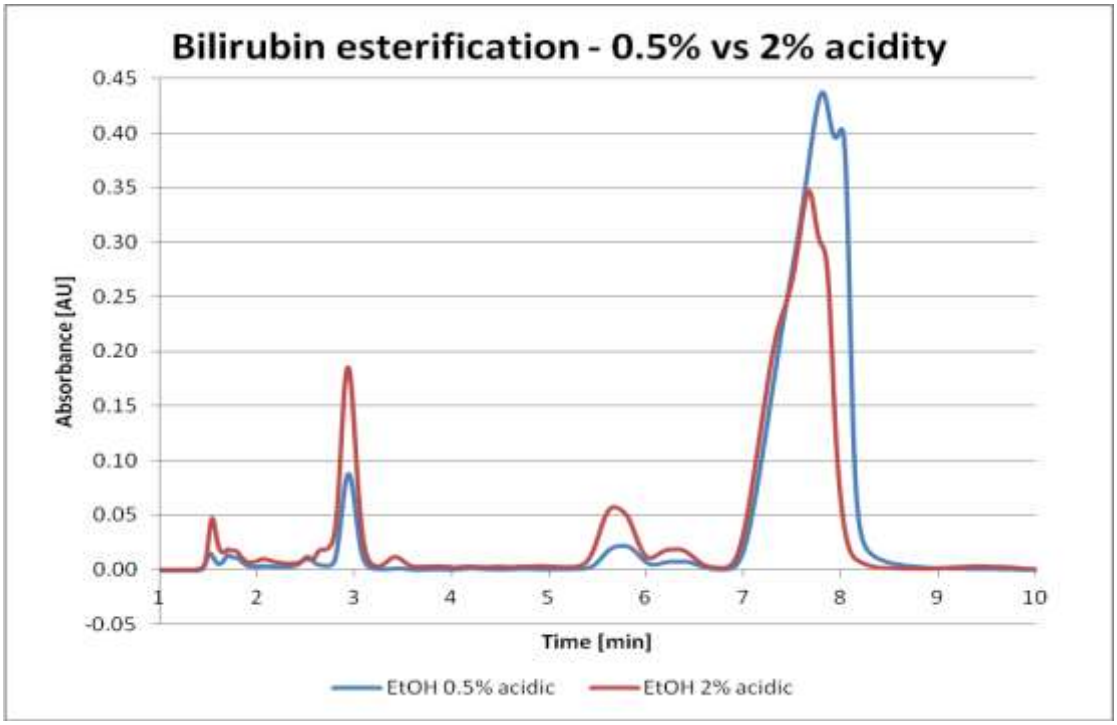

Figure 12: Bilirubin dissolved in DMSO, mixed with alcohol in ratio 1:1, acidity: 2% H^+, column: Agilent Zorbax SB-C18: 4.6 mm ID × 150 mm, (5 μm), mobile phase: CHINA

3.2.8 Effect of alcohol and dissolved bilirubin ratio

When the ratio of alcohol to dissolved bilirubin is set to 4:1, the retention time of the peaks are the same as in the ratio 1:1. However, as it can be clearly seen in figure 13 and when compared with figure 11, the esterification is more efficient. Again there is a noticeable shift between the esters: methyl ester between second and 3rd minute, ethyl ester at the 3rd minute and butyl ester at 3.5 minute. At 0.5% acid concentration the isomers are formed, even though not as many as in the case before. At 2% there were more isomers

formed during the incubation, which shows the influence of the acidity on the isomers formation.

For example, because the incubation time of butanol reaction mixture was longer than the incubation times of shorter alcohols, it was thus longer influenced by environment than the short alcohols, and so more isomers were formed.

Again when using 2%, the pure bilirubin peak is lower than the ester peak.

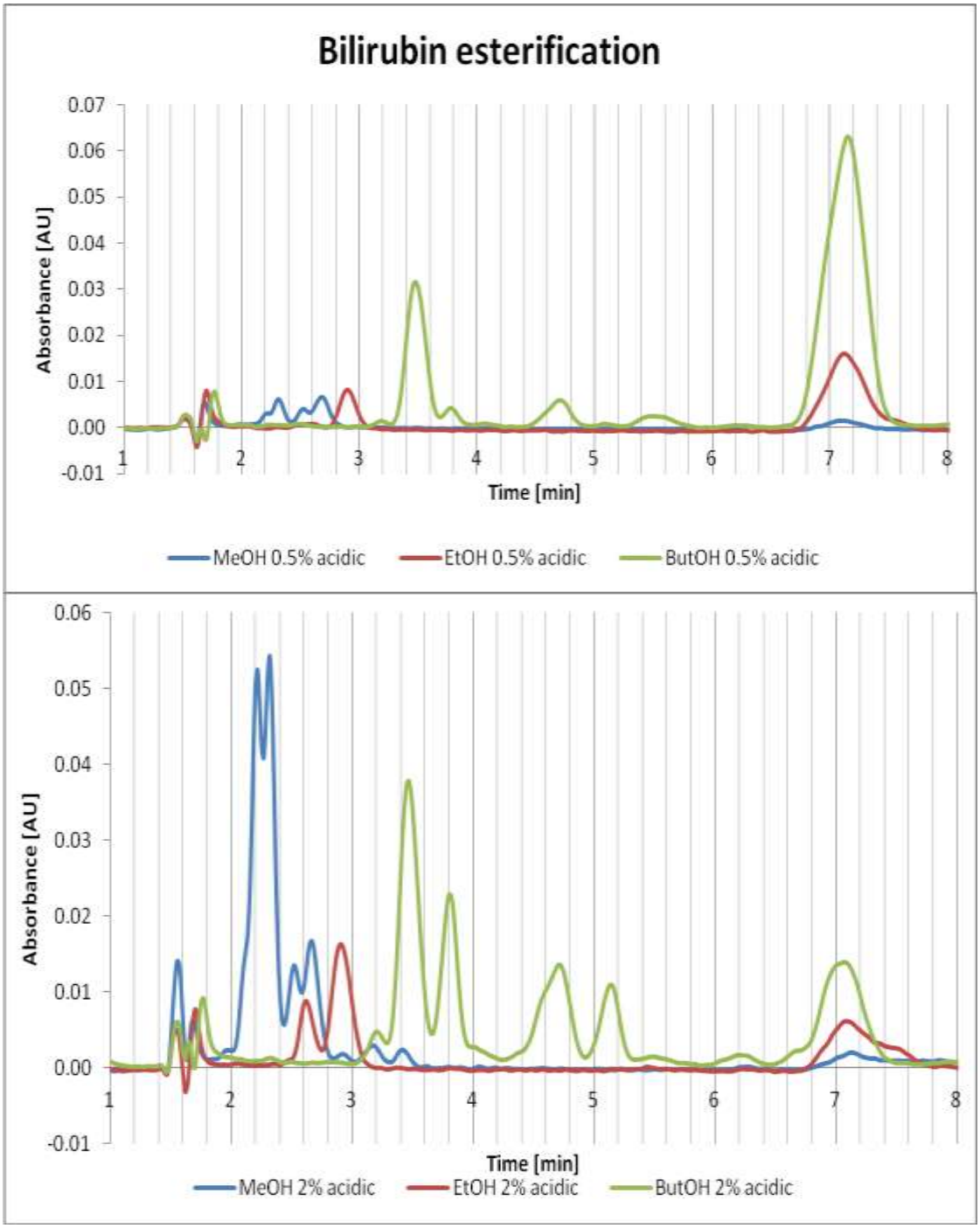

Figure 13: Bilirubin dissolved in DMSO, mixed with alcohol in ratio 2:8, acidity: 2% H^+, column: Agilent Zorbax SB-C18: 4.6 mm ID × 150 mm, (5 μm), mobile phase: CHINA

4 Conclusions

During my work, I was able to introduce and optimise the separation of bilirubin using HPLC and reverse phase column. The influences of temperature, different injection volumes, solvents, mobile phases, and columns were analysed.

Using CHINA as mobile phase became more efficient than just pure methanol. The best chromatograms were obtained using DMSO as a solvent and when smaller volumes of the sample were injected. No disturbances of bilirubin stability were found during the short time expositions of dissolved bilirubin to the temperatures up to 80 °C.

In the next step I have checked and found conditions for esterification of bilirubin with different alcohols. Longer esters formed in the esterification reaction are more retained in the stationary phase during the reversed phase chromatography. It was found that high excess of alcohol and more acidic conditions promote the esterification reaction.

5 References

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