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Phycobilin pigments as building blocks of artificial light-harvesting structures

Bachelor thesis

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Annotation

Phycobilin mixture was extracted from *Thermosynechococcus elongatus* cyanobacteria and purified with TLC method. Pure phycocyanobilin was esterified by Fischer esterification by several alcohol types. The results were analysed by TLC and HPLC methods.

Affirmation

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Kateřina Malinová

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Abstract

Phycobilins are linear tetrapyrrole pigments found in the light-harvesting photosynthetic system in cyanobacteria which help cyanobacteria absorb light in poor light conditions. In this work, phycocyanobilin was extracted from cyanobacteria *Thermosynechococcus elongatus* and purified by TLC. Purified phycocyanobilin was esterified by Fischer esterification with different alcohol chain lengths (methanol, 1-butanol, 1-pentanol and 1-hexanol). The results of the reaction were analysed by TLC and HPLC. HPLC confirmed that esterification was successful and gave relatively high yields. Due to the remaining acidity in the sample, the products of the reaction transesterified during storage in methanol. After stabilisation of reaction products, the esters may be tested for incorporation into self-assembling aggregates.

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1. Introduction

1.1. Phycobilins

Phycobilins are pigments which serve as light-harvesting pigments in photosynthetic apparatus of cyanobacteria, red algae and cryptophytes (Beale and Cornejo, 1991). Phycobilin molecule has a linear structure composed of four pyrrole rings (Fig. 1.1., Fig. 1.2.). These molecules are further incorporated into phycobiliproteins, which are chromoproteins binding phycobilins with covalent bonds. This covalent bond is typically a thioether bond between the first carbon on the vinyl chain attached to the pyrrole ring A and a cysteine residue on the peptide chain (Fig 1.2.) (Šetlík, 1998). The peptide chain interactions with phycobilin molecule affect the absorption properties of individual phycobilins more than small differences in individual phycobiliprotein structures. Four types of phycobiliproteins are known according to the position of their absorption maxima. Allophycocyanin has absorption maxima between 650 and 680 nm, phycocyanin (PC) has absorption maxima between 620 and 636 nm, phycoerythrocyanin (PEC) around 575 nm and phycoerythrin (PE) between 545 and 565 nm (Šetlík, 1998).

Even though the phycobilin molecule is commonly drawn as a linear structure, disconnected ring-like shape in spatial dimension is a more accurate description (Šetlík, 1998). This shape may be affected by the amino acid residues from the protein attached. The absorption maxima are significantly affected by the shape of the molecule. There are several phycobilin chromophores of phycobiliproteins known. Blue phycocyanobilin (PCB), studied in this work and red phycoerythrobilin are the most abundant chromophores of known phycobiliproteins (Šetlík, 1998).

Very similar molecules to phycobilins are bilirubin and biliverdin. Like phycobilins, they are both linear tetrapyrroles. Biliverdin is the first by-product after metabolism of haem, which comes from cleavage of haemoglobin, resulting in the formation of haem and globin. After biliverdin is formed, biliverdinreductase reduces methylidyne group, and brownish bilirubin is formed. Bilirubin is the final metabolite of all mammal's metabolism. Phycocyanobilins used in this work are among all phycobilins structurally the most similar to human bilirubin (Kamišová, 2013).

Cyclic tetrapyrrole structures called porphyrins also exist (Fig. 1.3.). Other structures, such as chlorophylls or haems, are derived from this structure (Šetlík, 1998).

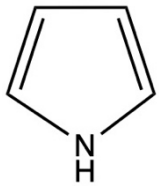

Fig. 1.1.: *Pyrrole ring (original from Šetlík (1998), drawn in ChemDraw software).*

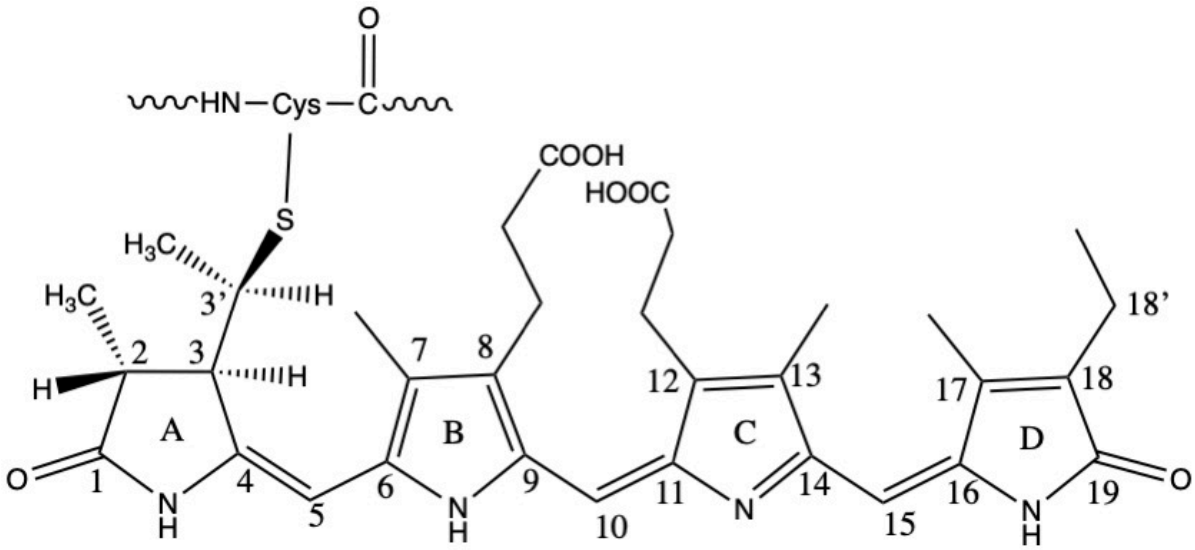

Fig.1.2.: *Phycocyanobilin with a protein attached by thioether bond (original from Šetlík (1998), drawn in ChemDraw software).*

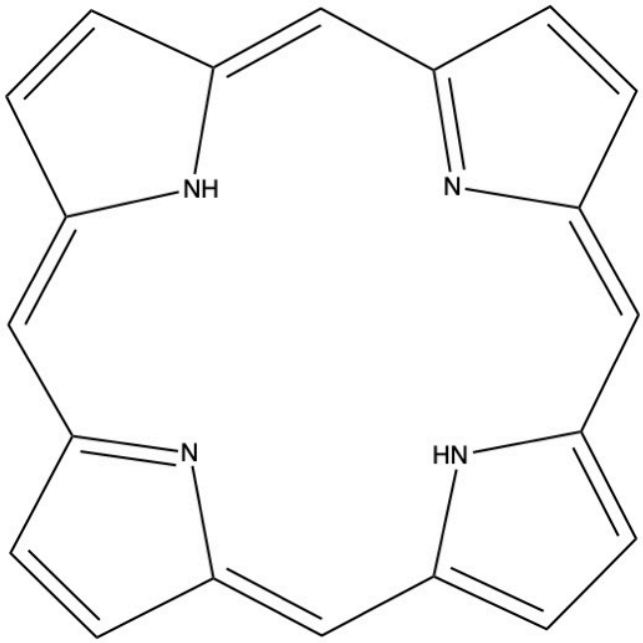

Fig. 1.3.: *Tetrapyrrole ring (original from Šetlík (1998), drawn in ChemDraw software).*

1.2. Phycobilin extraction from phycobiliproteins

To release the bilins from phycobiliproteins, a thioether bond between the tetrapyrrole bilin structure and cysteine residues must be cleaved. The most common method is cleavage by alcoholysis, which may be done in several ways.

The first method is a methanolic extraction described in óCarra (1966). As a first step, the algal sample was extracted with methanol several times to remove water and most of the other pigments such as chlorophylls and carotenoids. Then, the sample was heated in a methanol solution with ascorbic acid. Filtered extracts were evaporated and then redissolved in a small amount of methanol. To this solution, a little amount of peroxide-free ether and HCl were added in order to extract other pigment residuals into the ether layer. Solid sodium acetate was added to neutralise the HCl. The pigment was again extracted into ether and washed once again with sodium acetate and water solution (1:1. v/v). Finally, bilin was extracted from the water phase into chloroform and then dried with nitrogen.

Another method is extraction by alcoholysis in Soxhlet extractor. Fu (1979) used dried, purified biliprotein folded into a filter paper and extracted in a Soxhlet apparatus put over a heating element with methanol and 1-butanol. Bilins were cleaved by the alcohol reflux. The pigment extracts were dried and prepared for further use. Evaporation of butanol was supported by the addition of water, forming an azeotropic mixture.

The third method, also called methanolysis, described in Beale and Cornejo (1991), uses dimethyl sulfoxide for removing free pigments from harvested algae cells. After washing of extracted cells in water and trichloroacetic acid in the dark, the cells were suspended in methanol containing $HgCl_2$ and kept in the dark for 24 h. After this time the remaining free pigments were removed by washing in dimethyl sulfoxide : acetone mixture. Cells debris was then removed from the methanol solution containing phycobilins. In order to remove $HgCl_2$, beta-mercaptoethanol was added to the methanol solution forming a white precipitate. The white precipitate was centrifuged and methylene chloride : 1-butanol solution and water were added to the methanol solution to form two phases. The upper one was discarded, and the lower one, containing bilins, were purified by DEAE-sepharose gel chromatography and reverse-phase HPLC.

Another method, described in ÓhEocha (1963) was the only method, which did not use alcoholysis for phycobilin preparation. The dry phycobiliprotein mixture was poured into concentrated HCl for half an hour. After this, the distilled water was added (hydrolysate : water, 1:4), and the mixture was treated with sodium acetate until the pH value of the diluted

solution reached values between 6-7. The centrifuged supernatant was extracted several times in different environment. Firstly the hydrolysate was extracted into peroxide-free ether, then concentrated HCl was added and extraction with chloroform was followed. The last step included washing with distilled water. Cleaved phycobilin obtained was dried in vacuum. ÓhEocha (1958) also described the hydrolysis of phycobiliprotein in concentrated HCl at 80-85 ºC under nitrogen with immediate extraction to chloroform. This method was slightly modified by Kalkuš (2018) and was also used as a starting point in this work.

1.3. Self-assembling aggregates

Self-assembling aggregates are predominantly pigment made structures which do not carry protein molecules. These aggregates are commonly made from bacteriochlorophylls (Alster et al., 2010). These assemblies are found in certain species of photosynthetic bacteria species to harvest light and thus provide energy for biochemical reactions in photosynthesis. The aggregates are capable of collecting light very efficiently. For this reason, researchers are interested in making the aggregates artificially (Smith, 1983). If the aggregation is enhanced by addition of lipids or carotenoids, which are nonpolar compounds, the aggregation is spontaneous, and the aggregates are spectroscopically highly similar to those found in nature (Furumaki, 2014). Usually, bacteriochlorophylls (BChl) c, d, e and bacteriopheophorbides c, d are forming aggregates (Furumaki, 2014). Up to around 200000 molecules of BChl may form an aggregate (Furumaki, 2014). The huge number of BChl molecules are highly effective in capturing the light effectively. There are several methods to prove that the aggregates were formed. In the UV-vis spectroscopy, the aggregation causes a significant red shift of the red absorption peak of the spectrum. The aggregation is led by the hydrogen-bond formation between the metal (Fig. 1.4.), in case of bacteriochlorophylls it is a magnesium atom, and the keto or carbomethoxy groups in the ring (Furumaki 2014).
The aggregation is also supported by hydrophobic interaction of the nonpolar tails of BChl and is therefore enhanced by the addition of other nonpolar molecules, e.g. carotenoids or lipids. (Klinger, 2004).

1.3.1. Self-assembling aggregates and phycobilins

Self-assembling aggregates are viewed as molecules with high future potential as bio-inspired light-harvesting structures for direct production of electricity in the form of

photovoltaic devices or to power other processes by the harvested light energy. These devices were studied and are considered to be a unique solution, which is pollution-free and relatively cheap (Li, 2015). There is a noticeable gap in absorption spectra of the BChl aggregates between 500-600 nm (Fig.1.5.), which could be filled by incorporation of phycobilins. It is theoretically possible to implement the unmodified phycobilin to the BChl aggregate, however, the mechanism of BChl aggregation involves the forces associated with the nonpolar chains of the molecules and of the accessory pigments. Therefore the esterification of phycobilins with nonpolar hydrocarbon chains, which we aim to do in this work, may facilitate the aggregation.

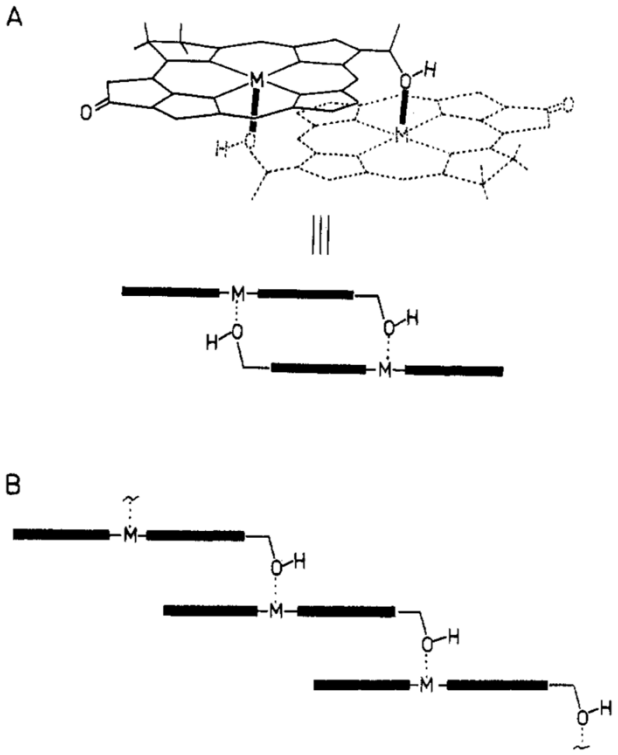

Fig. 1.4.: *Schematic representation of aggregates responsible for red-shift optical spectra: (A) possible dimer species; (B) more likely oligomeric aggregates. (Smith, 1983)*

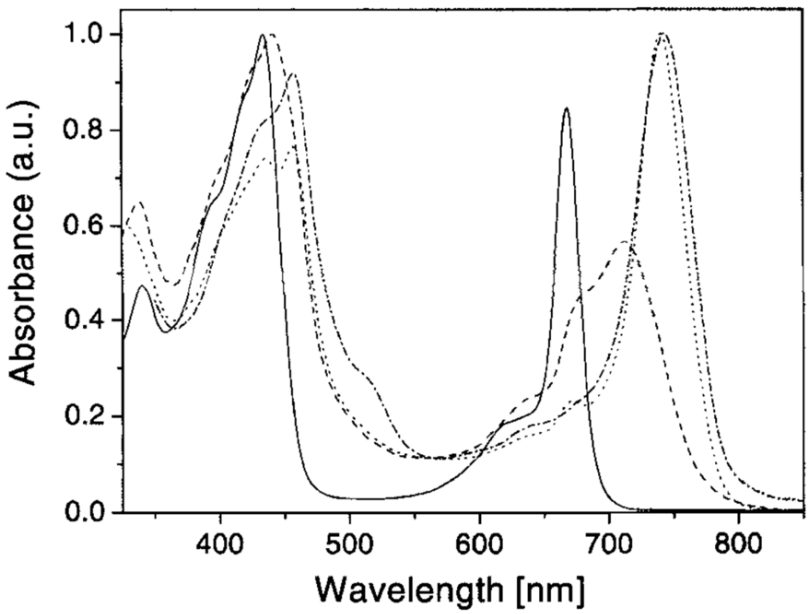

***Fig.1.5.**: Absorption spectra of BChl c in methanol (solid line); in Tris-HCl at pH=8.0 (dashed line); in monogalactosyl diacylglycerol and Tris-HCl at pH=8.0 (dotted line); for comparison spectrum containing BChl a and carotenoids were added (dashed-dotted line) (Klinger, 2004).*

1.4. Analytical methods and their principles

Several analytical methods were used in this work, and their principles are described in the following sections.

1.4.1. Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) is one of the most widely used chromatography methods. It has many advantages, such as providing results within a short period of time or a big number of samples possible to analyse in one turn. It is an affordable and easily repeatable method (Wall, 2005).

The basic principle of TLC is that two phases, the mobile and the stationary phase, have opposite polarities. After the application of the sample on the plate, the capillary forces take the mobile phase upwards, and the separation of analytes is achieved thanks to their different chemical affinities to the mobile and stationary phases (Wall, 2005).

The stationary phase, sometimes called a sorbent, can be made from several materials, for example, silica gel or aluminium oxide, the least common ones are cellulose or polyamide. The stationary phase is usually laid on aluminium or glass plates. The stationary phase should be properly chosen in consideration of the properties of compounds separated. The universal

stationary phase is silica gel, which was also used in this work. Silica gel has free hydroxyl groups, which give the material the absorptive properties and allow the separation of a wide range of materials. Silica gel has specific requirements for storage. For accurate results, the hydration of silica gel is optimal at 11-12% of water (Wall, 2005). When the TLC plate is exposed to high humidity, it can negatively affect the results, and it requires a process called pre-activation, usually exposure to high temperatures. The silica gel is present in the form of small particles, which are covered by pores. The pore size is given in angstroms (10^{-10} m) and commonly used are 40, 60, 80 or 100 Å. The resolution and migration rate depend on the pore size. In this work, silica gel 60 was used.

When choosing the mobile phase, the analytes should be completely soluble in it. The solvent should also have a low boiling point close to easily evaporate from the TLC plate at the room temperature. A polar solvent is not recommended to use due to its bad wetting properties. The analyte is spotted onto the TLC table with the aid of small capillary or a pipette tip. The result of TLC analysis is affected by the size and the shape of the spot, so the spotting should be done precisely and carefully.

Once the analyte is applied onto the TLC plate, and the chromatography is done, the distance of migration of the analyte and the mobile phase may be used to calculate a retention factor (Rf), which may help to identify the samples. Rf value is calculated as the distance travelled by the analyte divided by the distance travelled by the solvent from the origin (Wall, 2005).

1.4.2. Absorption spectroscopy

The next method used in this work for bilin sample analysis was absorption spectroscopy. Absorption spectroscopy determines how much a sample absorbs light. The method serves either to identify previously described molecules by their spectra or to estimate the concentration of absorbing chromophores. It is based on a comparison of intensities of a beam of light with certain wavelength passing through a solution in a cuvette and a reference beam. Absorbance instrument scheme is shown in Fig. 1.6. Absorbance and analyte concentration are estimated by using Lambert-Beer law (Eq. from Nilapwar, 2011):

$$A = \log 10 \frac{I_0}{I} = \varepsilon dc$$

Where:

A is absorbance, I_0 is incident light, I is transmitted light, ε is molar absorption coefficient, d is path length, and c is molar concentration.

Depending on the range of wavelengths analysed and the technique used, we differentiate several kinds of spectroscopy. Among the most used are UV-visible and infrared spectroscopy. The UV-visible region of spectra is between 200-780 nm. In this work, the range for measuring was between 230-850 nm, so a little part of the infrared spectrum was included as well. The apparent and absorbed colours of the material are mutually complementary. For example, if the sample fully absorbs white light, to the human eye, it will appear as black.

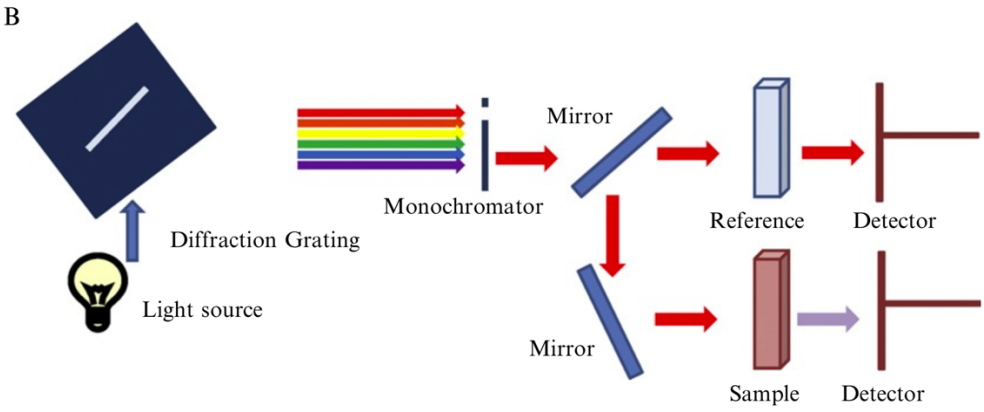

***Fig. 1.6.**: Typical optical layout of a double beam spectrophotometer (Nilapwar, 2011).*

1.4.3. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) was used for more complex analysis. It is one of many types of liquid chromatography which in general serve for the separation of molecules dissolved in a solvent (Belanger, 1997). During past years HPLC became to be one of the most important analytical methods. The basic principle of HPLC is the separation of a sample (mobile phase) and a stationary phase (column). The mobile phase carries the dissolved sample into the column, where the sample starts to separate according to its interaction with the stationary phase. Then the fractions leave the column at different times, called the elution times. Each of the fractions is then detected by a detector (Drbal a Křížek, 1999). Scheme of an HPLC instrument is described in Fig. 1.7. At the beginning of each analysis, the sample is injected in the injector with micro-syringe. Nowadays, mostly automatised injectors with sample carousels are used. The sample goes to the column, where the sample is separated. The pump carries the solvents from reservoirs and changes the gradient (the composition of the solvent), and it is also responsible for regulating solvent flow and pressure in the column. The most important part of each HPLC instrument is the detector. It is placed at the end of the column and analyses the sample as it elutes from the column. Nowadays exist various types of detectors, such as absorbance and fluorescence detectors or mass-spectroscopy detectors. The detector sends the data into the data system, which makes interprets data in chromatograms (Belanger, 1997).

Four major types of HPLC exist according to properties of the stationary phase: adsorption, partition, ion-exchange and size-exclusion chromatography. Among all of these, the adsorption chromatography is used the most. There we distinguish between two modes of action: normal phase and reversed phase. In normal phase method, the stationary phase is a polar substance, and the mobile phase is a nonpolar substance. In reversed phase, the column is nonpolar (hydrocarbon), and the mobile phase is polar (water, methanol). However, changing the polarity of the mobile phase in order to elute the compounds may be achieved by using more than one solvent, normally 2-3 are used in gradient methods. Gradient methods typically use a progression of solvent polarities to improve separation or shorten analysis time of a diverse range of analytes. When choosing a solvent, some factors to keep in mind are molecular size, proper eluting strength, polarity and viscosity and also solubility of analytes in the solvent of choice (Belanger, 1997).

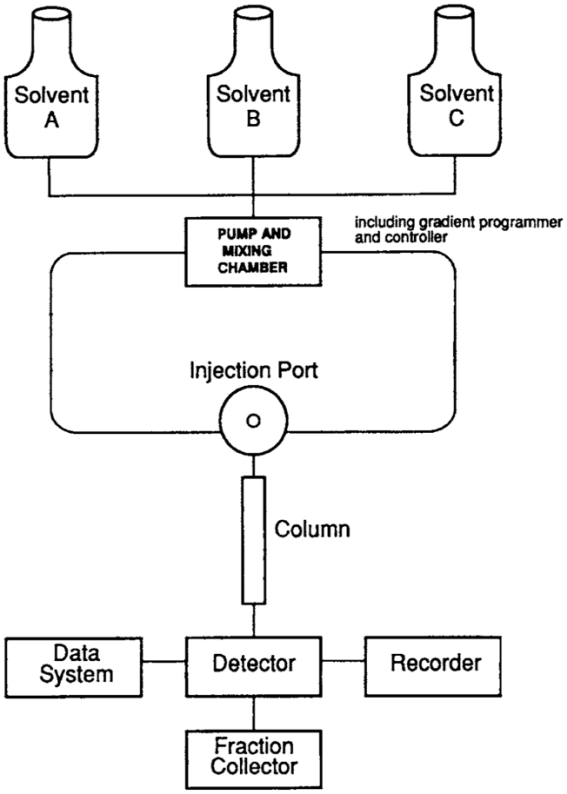

Fig. 1.7.: *Main components of HPLC (Belanger, 1997).*

1.5. Esterification

Esters are substances with a derived carboxylic acid group where at least one hydroxyl group is replaced by an alkoxy group. Esters are one of the oldest and largest groups known and are substances with a wide range of properties, many of them have attractive fruity smells and flavours (Clayden, 2012). They have wide application in chemical, food or pharmaceutical industry and many more (Gilles, 2015). Even fats are esters, and they are derived from glycerol and thus having three ester groups. Esterification, in general, is the name of a chemical reaction where at least two reactants form an ester as a product of a reaction. Several esterification reactions are known (Clayden, 2012)

1.5.1. Fischer Esterification

Fischer esterification is one of the most common esterification reactions. It is very easily doable, relatively fast, affordable and its yields are quite high. In Fischer esterification, a carboxylic acid reacts with an excess of alcohol in the presence of acid as a catalyst (Fig. 1.8.). The acidic conditions allow forming an oxonium cation with later attaching of the alcohol and cleavage of H^+. The acidic environment also helps to cleave the water and the reaction results

in the forming of ester. It is a typical equilibrium reaction, and its biggest disadvantage is the reversibility of the reaction. The reaction yield also may be increased by increased temperature (Clayden, 2012).

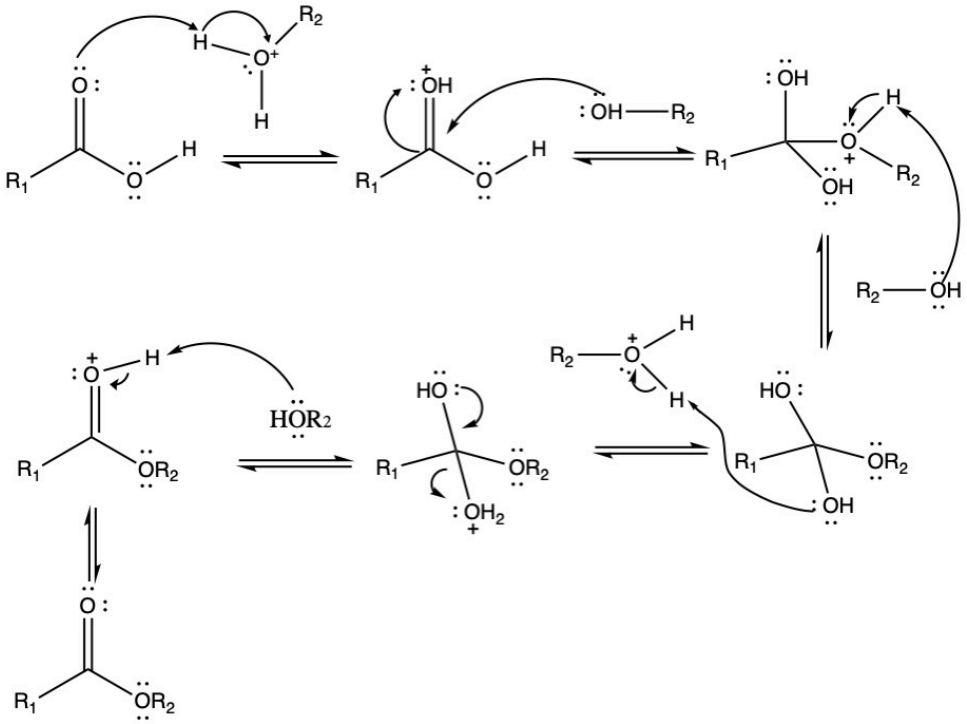

Fig. 1.8.: *Fischer reaction mechanism (original from McMurry (2008), drawn in ChemDraw software).*

1.5.2. Yamaguchi esterification

Yamaguchi esterification (Fig, 1.9.) is used for the synthesis of complex esters or macrolactones (Dhimitruka, 2006). The reaction is done in two steps. The first step is the reaction of the Yamaguchi reagent (2,4,6-trichlorobenzoyl) and the carboxylic acid in the presence of triethylamine (Yamaguchi, 1979) or pyridine (Wang, 2010). Triethylamine or pyridine are employed as the agents deprotonating the carboxylic acid. This results in the formation of an intermediate anhydride product. In the second step, the anhydride is mixed with alcohol in the presence of 4-dimethlyaminopyridine (DMAP), and it results in ester formation (Wang, 2010). Sometimes the reaction may be catalysed by hafnium (IV) salts (Dhimitruka, 2006). The main disadvantage of this reaction is poor atom economy, in other words, it is highly inefficient. It is not convenient to use this method for synthesising large amounts of esters (Yamaguchi, 1979).

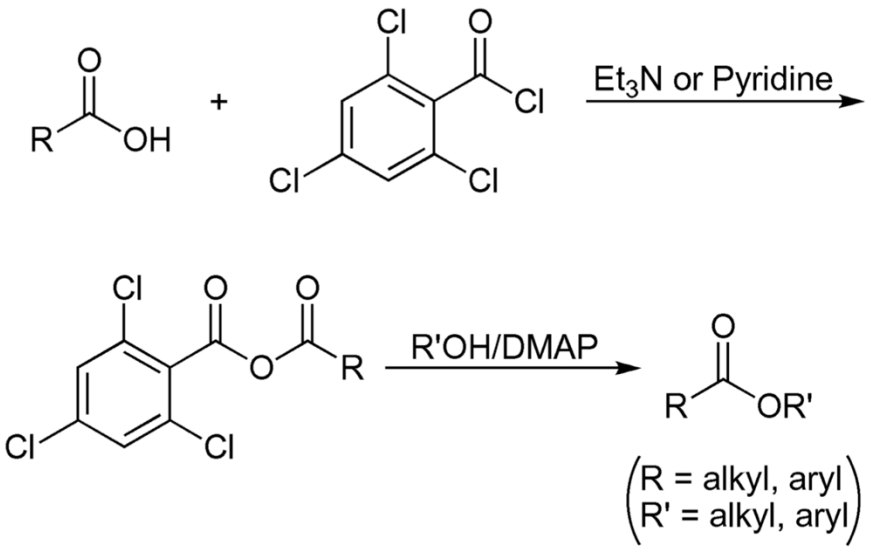

Fig. 1.9.: *Yamaguchi esterification reaction. (Wang, 2010).*

1.5.3. Steglich esterification

Steglich esterification is used for the preparation of bulky esters or thioesters of carboxylic acids (Fig. 1.10.) (Steglich, 1978). The reaction of secondary or tertiary alcohol and the carboxylic acid is catalysed by 4-dimethylaminopyridine (DMAP). DMAP is commonly known as a Steglich catalyst, and in Steglich esterification enhances the acylation of carboxylic acid (Wang, 2010). The whole reaction runs in the presence of N,N'-dicyclohexylcarbodiimine (DCC), which suppresses side product formation (Steglich, 1978). DCC accelerates the DCC-activated esterification and provides good yield at room temperature (Steglich, 1978). Gilles (2015) also used $CeCl_3$ as a catalyst. The whole reaction must run in non-aqueous conditions, otherwise, a reaction between water and DCC would occur (Steglich, 1978). If the reaction of DCC, which is a strong dehydrating agent, with water happened, dicyclohexylurea would be formed (Stevens, 1967).

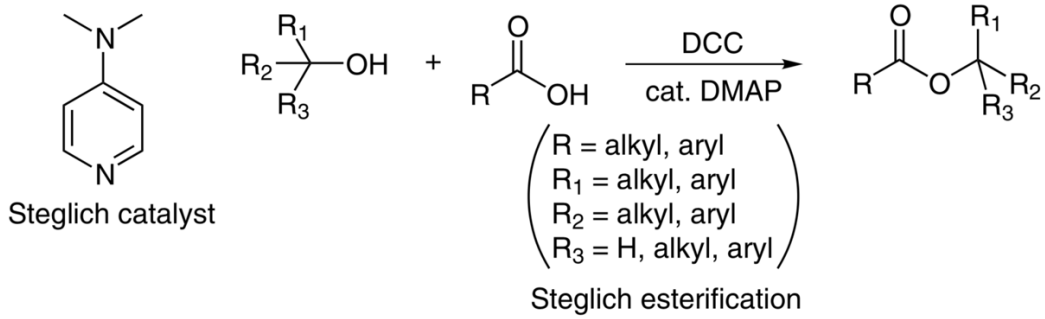

Fig. 1.10.: *Steglich esterification reaction (Wang, 2010).*

2. Aims of the work

This work had two main aims. The first one was to develop and optimise the current method of isolation of pure bilins from *Thermosynechococcus elongatus* cyanobacteria. The second aim was to esterify the harvested bilins with alcohols with different chain length.

3. Materials and Methods

Chemicals used in this work:

- acetone (purity ≥ 99%, Sigma-Aldrich)

-1-butanol (purity p.a., Sigma-Aldrich)

-chloroform (purity p.a., Sigma-Aldrich)

-diethyl ether (anhydrous, 99,8%, Aldrich)

-hydrochloric acid (purity p.a., 35%, Lach-Ner)

-hexane (purity: for HPLC, VWR)

-1-hexanol (anhydrous, ≥ 99%, Sigma-Aldrich)

-H_2SO_4 (purity: p.a., 96%, Lach-Ner)

-isopropanol (purity p.a., VWR)

-methanol (purity: for HPLC, VWR)

-1-pentanol (purity p.a., Lach-Ner)

3.1. Isolation of phycobiliproteins from *Thermosynechococcus elongatus* cyanobacteria

An initial material used for isolation were cells of *Thermosynechococcus elongatus* cyanobacteria. The cells were grown in WC growth medium (Guillard, 1972) in batch culture mode in 1 L Erlenmeyer flasks in the total volume of 2 L and kept in an incubator in temperature of 48 °C with weak illumination by a halogen incandescent lamp. Cells were harvested twice per week by centrifugation (7 min, 8000 × g) on the Avanti J-25 centrifuge (Beckman, Coulter, USA) with J-10 rotor. The pelleted cells were stored for further work under -80 ºC or immediately ruptured. For the cell rupturing procedure, the pellet was suspended in 10 mL of a buffer solution (0.3 M KH_2PO_4; 0.2 M Na_2EDTA, pH = 7.0) and properly homogenised. The suspension was ruptured (5 min, 15000 psi) by the air-powered disruptor EmulsiFlex C5 (Avestin, Canada). The suspension was then centrifuged (30 min, 25000 × g, Sigma 3-30KS) afterwards to remove cell wall debris and membranes. The whole procedure was done in dim light conditions.

The supernatant was then used as a source of phycobilin proteins. The supernatant was mixed with acetone to precipitate proteins (supernatant : acetone, 1:5), shaken properly and centrifuged (5 min, 6500 × g). The precipitated phycobilin protein was dried under vacuum in a desiccator. The rest of the mixture were phycobilins, and they were released by acidic hydrolysis by adding 10 mL of 35% HCl to the dried protein mass in the fume hood (ÓhEocha, 1958). Several hydrolysis procedures were done in this work. The specific details of each procedure are listed in subheadings below. The hydrolysis was terminated by addition of distilled water (hydrolysate : H_2O; 1:4), and the solution was centrifuged (10 min, 7500 × g) to remove remaining undissolved particulate matter. With the pellet formed, it is possible to repeat the hydrolysis in order to increase the yield. The pigment mixture was then repeatedly extracted into chloroform in a separatory funnel (chloroform : supernatant; 1:10) until little or no pigment appeared in the chloroform phase. Chloroform was evaporated under vacuum, and a dry bilin mixture was acquired (Kalkuš, 2018).

3.1.1. Hot hydrolysis

The biliprotein with concentrated HCl was put into a water bath on a hot plate, water bath temperature was set to 80 ºC. After 90 min, the hydrolysis was stopped (Kalkuš, 2018).

3.1.2. Cold hydrolysis

After the addition of concentrated HCl, the falcon tube with the mixture was kept in the dark for 24 hours or one week (H. Lokstein, personal communication). For better yield, the pellets remaining after this time were put together and another week-lasting cold hydrolysis was performed.

3.2. Purification of phycobilins

To purify the bilin sample further, the dry bilin mixture was dissolved in 250 µL of chloroform and applied on TLC silica gel plates. The detailed procedure of the TLC is described in paragraph 3.4. The mobile phase used was chloroform : methanol : hexane (17:4:1, v/v). After 15 minutes, the separation was finished, and the TLC plate was let to dry. Then the major blue strip containing phycocyanobilin was scraped out of the plate, transferred into an Eppendorf tube, dissolved in 1mL methanol and centrifuged in a table centrifuge (Eppendorf, Germany) (3 min, 14000 rpm) to separate the dissolved pigment from solid silica particles. After centrifugation, the upper blue solution with pure phycobilin was carefully transferred into a new Eppendorf tube and dried in vacuum.

3.3. Fischer esterification

To prepare esterified bilins, two Eppendorf tubes with the dry bilin sample were needed. All samples were dissolved in 250 µL of alcohol. To assess the possibility of esterification by different chain lengths, methanol, 1-butanol, 1-pentanol and 1-hexanol were used. One of the tubes served as a control, and 5 µL of concentrated 96% H_2SO_4 were added the other tube to carry out the reaction. Two reaction times (30min, 3 hours) were tested. The temperature on

the heating plate was set to 90 °C for all alcohols except methanol, which run at 55 °C due to its low boiling point. To terminate the reaction, 1000 µL of deionised water were added to the reaction tube. The pigment was then extracted from the reaction mixture by addition of 500 µL of diethyl ether. The mixture was properly shaken and subsequently centrifuged on a table centrifuge (2 min, 14000 rpm). The upper organic phase containing the pigments was carefully transferred into a new Eppendorf tube and dried with nitrogen gas flow.

3.4. Thin-Layer Chromatography (TLC)

The TLC was carried out on silica gel plates (TLC Silica gel 60, 5 ×10cm, Merck KGaA, Germany). Two mobile phases were used and compared in this work. The first method was used as developed and described in Kalkuš (2018). The mobile phase of the Kalkuš method is chloroform, methanol and hexane in 17:4:1 (v/v) ratio. The other method (Havaux, 2001) is used for the analysis of plant pigments, and the mobile phase was hexane and isopropanol in 100:10 (v/v) ratio. 42 mL of the mobile phase was poured into the TLC chamber of a size 10 ×10 cm. A filter paper of size 10 × 10 cm was inserted into the TLC chamber to get the environment there saturated with mobile phase vapour which improves the separation. In the meantime, two lines were made on the upper and the lower side of the plate 1 cm from the edges. A sample description was made above the upper line. The analysed samples were applied to the lower line. Afterwards, the TLC plate was put into the TLC chamber, and the separation started. After the analysis was finished, the TLC plate was removed from the TLC chamber, and the plate was let dry in low light and photographed. For later use the plates were put in a zip-lock bag and stored in a freezer (- 20 °C).

3.5. Absorption spectroscopy

Absorption spectra were detected in the range of 230 to 850 nm in quartz cuvettes by a UV-1800 spectrometer (Shimadzu, Japan), using fixed 1 nm slits. The analyte was dissolved in chloroform or methanol. Spectra were plotted in SigmaPlot software.

3.6. HPLC

The HPLC analysis was carried out using the method of Jeffrey et al. (2005) on a Waters Alliance HPLC system. The system consists of Waters e2695 Separations module and Waters 2998 PDA detector. The stationary phase was a reversed-phase column (Waters NovaPak C18 3.9 × 300 mm, 4.0 µm). The separation was done using a gradient method. Mobile phases used are listed in Tab. 3.1.

Solvent A	MeOH : 0.5 M ammonium acetate – 80 : 20, v/v, pH 7.2
Solvent C	acetonitrile : water – 90 : 10
Solvent D	ethyl acetate 100%

Tab. 3.1.: *Jeffrey et al. composition of mobile phase (Troppmair, 2018)*

Injection volume was 100 µL. The flow rate was set to 1 mL per minute. The analyte was dissolved in 150 µL of methanol prior to injection. Gradients of mobile phases used in the total run time of 25 min are listed in Tab. 3.2. The system was controlled, and data were acquired by using Millennium 31 software (Waters, USA).

Time (min)	% of solvent A	% of solvent C	% of solvent D
0.00	100.0	0.0	0.0
4.00	0.0	100.0	0.0
18.00	0.0	20.0	80.0
25.00	0.0	20.0	80.0

Tab.3.2.: *Gradient of mobile phases in used HPLC methods. (Troppmair, 2018).*

4. Results

4.1. Purification of bilin mixture from biliproteins

As the first step of this work, raw bilin mixture was prepared. The method of Kalkuš (2018) was used as a starting point and is denoted here as hot hydrolysis. To improve the result, a modified method using room temperature hydrolysis was also tested. The hydrolysis was performed on dried acetone precipitate of water-soluble cell fraction. To compare the hydrolysis methods, the source material was split into three aliquots. One tube underwent hot hydrolysis using the method described in 3.1.1. Cold hydrolysis, described in 3.1.2., was performed in the other two tubes for 24h and one week, respectively. Following the hydrolysis, the free pigment was collected into chloroform phase in a separatory funnel (Fig. 4.1.). No difference in the behaviour of the mixtures from cold or hot hydrolyses was observed at this stage. Absorption spectra of the chloroform phases of the three tested conditions were also very similar. Representative absorption spectra of the chloroform phase (Fig. 4.3.) show a broad band around 550-600 nm, indicative of a presence of bilins. The spectrum also shows a sharp peak at 361 nm, with a side shoulder at 416 nm, and a broader peak at 656 nm. In the upper phase, no bilin was detected (Fig. 4.3.). Obtained chloroform extracts were analysed by TLC (Fig. 4.2.) using the Kalkuš method. The TLC showed several coloured bands. The major components of the mixtures were, going from the bottom to the top, a purple band followed by a large blue band, several greenish bands and several yellow bands. Based on the work of Kalkuš (2018), the purple and blue bands were assigned as phycoerythrobilin and phycocyanobilin. Based on their colour, position in the separation pattern and UV/VIS spectra from TLC (not shown) the green bands were assigned to be chlorophylls or pheophytins. These are likely responsible for the sharper features of the absorption spectra (Fig.4.3.) around 416 and 656 nm. Likewise, the yellow bands were likely carotenoids. The identity of these components was not analysed further.

The separation patterns did not differ significantly between the hot and cold hydrolysis. We concluded that both types of hydrolyses were similarly effective. A disadvantage of the hot hydrolysis method is imperfect temperature control at the beginning and the end of the experiment and limited amount of material undergoing the hydrolysis in our conditions. Cold hydrolysis of one-week duration was selected as the best method for further work for its easier handling and easier planning of laboratory time and thus the possibility of obtaining higher amounts of pigment. In TLC analysis of the cold hydrolysis, the blue and purple bands were

separated more clearly, so later during further purification (described in paragraph 3.2.) it was easier to collect the blue band and avoid the contamination with a purple pigment. As also mentioned by Kalkuš (2018), there was a possibility of repeating the process of hydrolysis of the unhydrolysed pellet after centrifugation to obtain more pigment and thus to increase the effectivity. As can be seen in the TLC analysis (Fig. 4.2.), the amount of bilin (blue band) is similar in either hot, cold (24 h), repeated cold and week cold hydrolyses. The centrifuged pellet which underwent 24h cold hydrolysis was hydrolysed for 6 more days once again. All the samples were examined on TLC together, and it was clear that the repeated hydrolysis still yielded a significant amount of bilin.

Fig. 4.1.: *Typical result of the chloroform extraction*

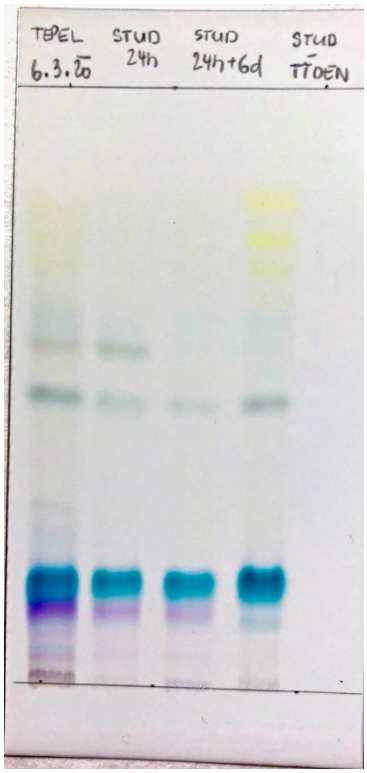

Fig. 4.2.: *TLC analysis of the results of hot, cold (24 h), repeated colds (24 h + 6 d) and one week cold (from left to right) hydrolyses.*

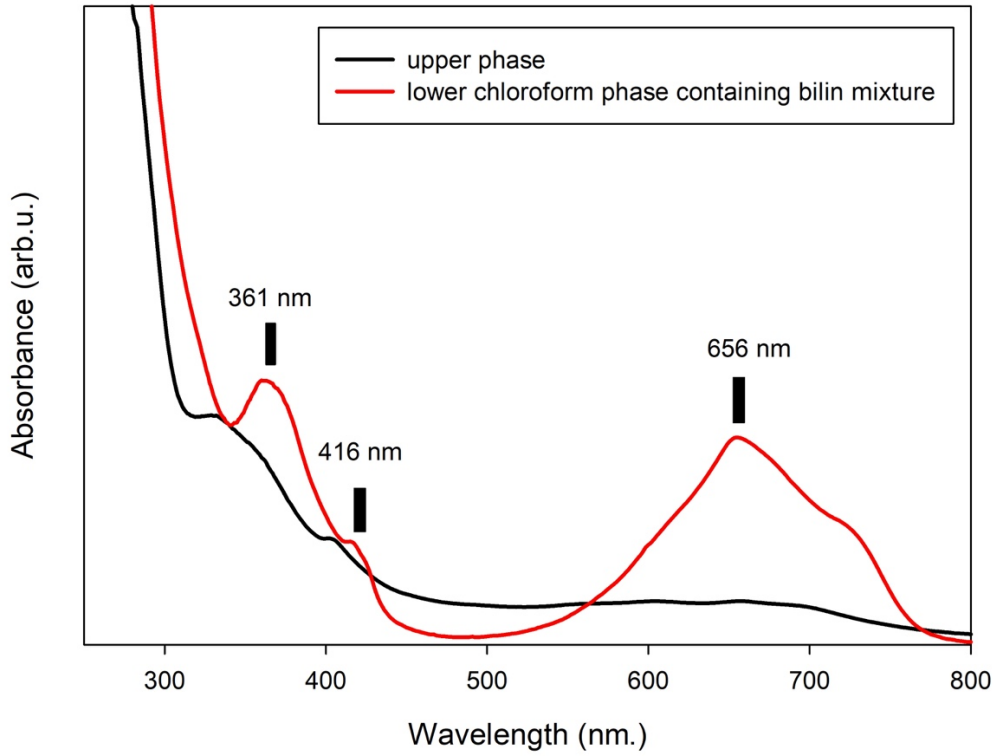

Fig. 4.3.: *Absorption spectra from chloroform extraction of upper phase and lower phase containing bilins and other pigment contaminants.*

4.2. Esterification of bilin mixture

Bilin mixture obtained in the previous steps was esterified with alcohols using the Fischer esterification method as described in Kalkuš (2018). In comparison to Kalkuš (2018), we used several alcohols with different chain lengths, namely methanol, butanol and hexanol.

In order to enhance the reaction yield, the temperature of the reaction was set to 90 °C for all alcohols except methanol, which run at 55 °C, because of the low boiling point of methanol. Each reaction was done twice, with different duration, 30 min and 3 h. The products of the reaction were separated between diethyl ether and water phase. The results are presented in Fig. 4.4. The reaction mixture formed two distinct phases with the exception of the methanol reaction. This is probably due to the relatively good miscibility of diethyl ether with methanol and methanol with water. Thus, the addition of more water was required in the case of methanol reaction in order to obtain two phases. The other esters transferred well into the upper organic phase, which was indicated by its blue colouration. The upper phase was collected, dried under nitrogen gas and later used for further analysis.

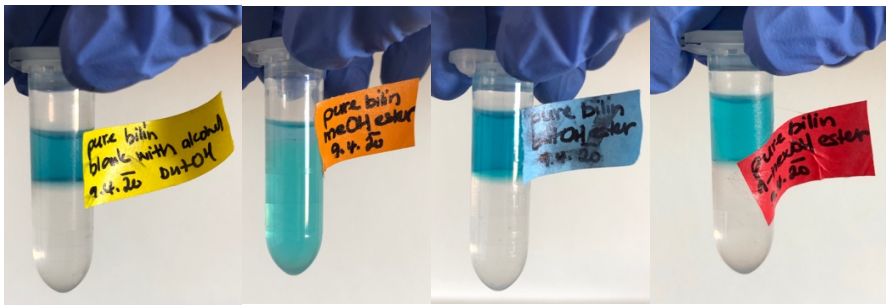

Fig. 4.4.: *Reaction mixtures after addition of water and diethyl ether. From the left: bilin mixture blank with butOH with no H_2SO_4 and no heating; methyl ester, 1-butyl ester, 1-pentyl ester, 1-hexyl ester*

4.2.1. TLC analysis of the results of esterification of bilin mixture

Collected reaction products of 30 min and 3 h reactions were analysed with two TLC methods (Fig. 4.5.), Kalkuš (2018) and Havaux (2001).

The results of the TLC analysis of 30 min esterification reaction are shown in Fig. 4.5. and the results of 3 h esterification are presented in Fig. 4.6. On both, 30 min and 3 h esterification, The Kalkuš method shows a huge difference between non-esterified bilin mixture blank and its esters on both, 30 min and 3 h, esterification reactions. The blue bands of esters were approximately at the same height and had similar intensity. In the case of 30 min esterification, the impurities appeared as purple bands beneath the blue ones. The Kalkuš method indicated that most of the pigment was esterified, but the analysis method was not selective enough with regards to the length of the esterifying chain. In order to increase the selectivity of TLC, a method used for nonpolar plant pigments described in Havaux (2001) was tested. The Havaux method shows the differences not only between non-esterified bilin mixture and its esters but also among the esters themselves. The longer the chain of the alcohol was used, the higher the band was found. However, lots of pigment stayed immobile at the bottom in this method.

Fewer impurities appeared below the main blue bands in the TLC analysis of the 3 h esterification product done by Kalkuš method (Fig. 4.6. left). However, more bands appeared below the highest band in case of butyl ester and hexyl ester. They had lower intensity than the band located in the top.

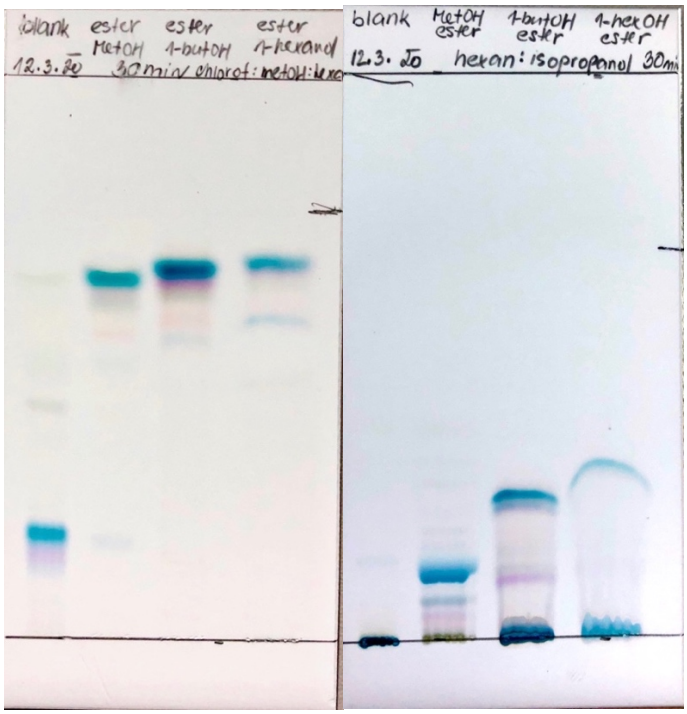

Fig. 4.5.: *Analysis of the products of the 30 min hot esterification of bilin mixture by TLC. Methods used were Kalkuš (2018) on the left and Havaux (2001) on the right. Order of the samples on each place from the left: bilin mixture blank, methyl ester, 1-butyl ester, 1-hexyl ester.*

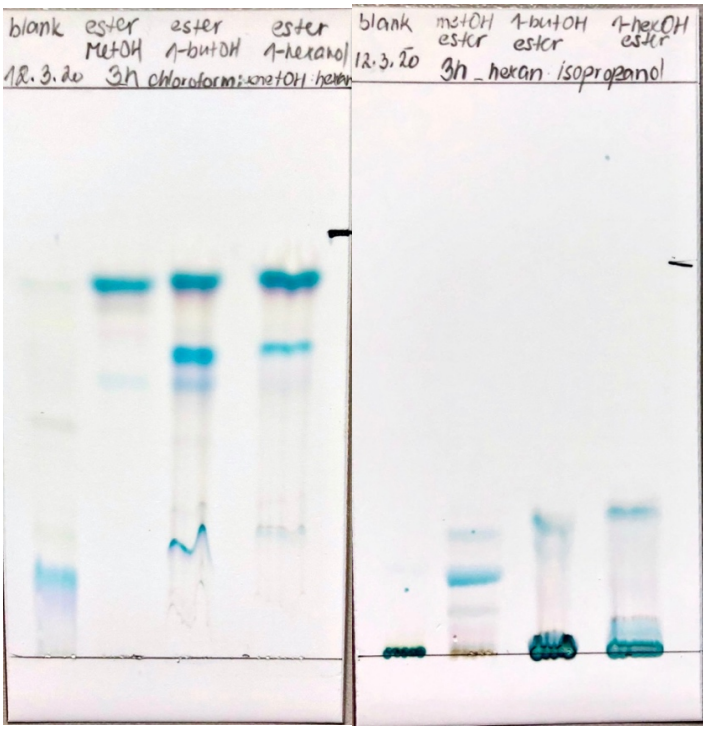

Fig. 4.6.: *Analysis of the products of the 3 h hot esterification of bilin mixture by TLC. Methods used were Kalkuš (2018) on the left and Havaux (2001) on the right. Order of the samples on each place from the left: bilin mixture blank, methyl ester, 1-butyl ester, 1-hexyl ester.*

4.3. Further purification of bilin mixture

The bilin mixture was further purified in order to obtain pure phycocyanobilin. Using a pure bilin without significant amounts of unknown contaminants was important for any further work. Chloroform extract from cold, one-week lasting hydrolysis was applied to a TLC plate in a long line and separated by the Kalkuš method (Fig. 4.7). The major blue stripe (in the red frame on Fig. 4.7.) was carefully scraped from the TLC plate. The material was dissolved in methanol and centrifuged to separate the silica gel particles. The absorption spectra (Fig. 4.8.) showed a simpler shape than for the mixed extract with two major maxima at 361 nm and 682 nm, which agree with published phycocyanobilin absorption spectra (óCarra, 1980). The obtained material was dried in vacuum and used further for esterification experiments.

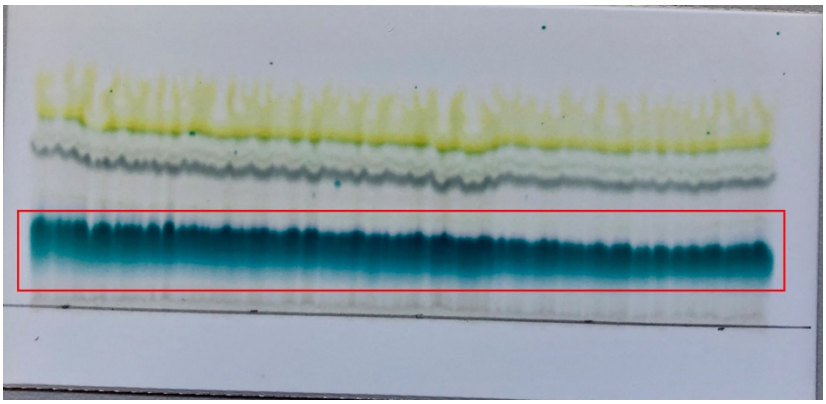

Fig. 4.7.: *Bilin mixture purification by TLC, Kalkuš (2018) method. The blue band in the red frame is phycocyanobilin which was collected and later used for esterification.*

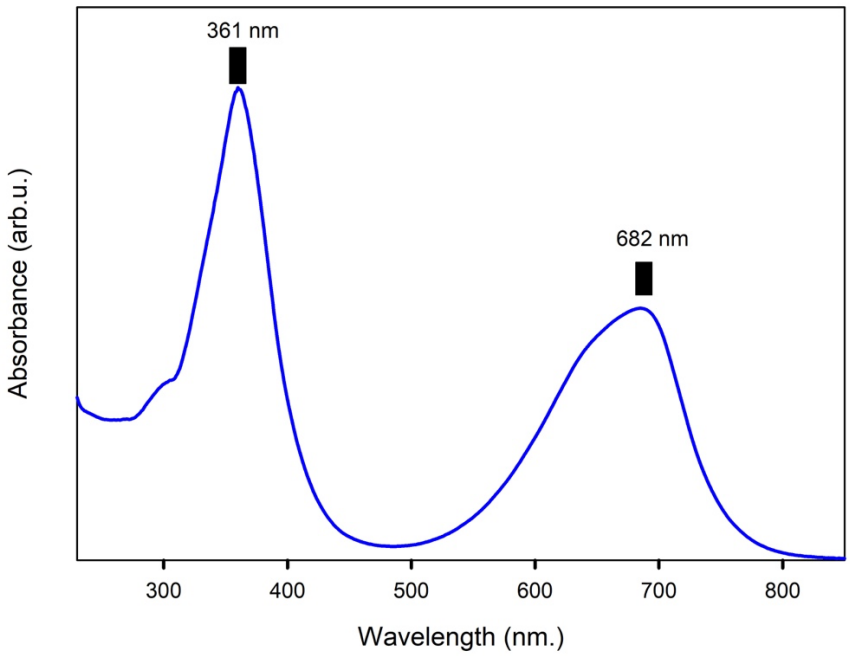

Fig. 4.8.: *Absorption spectra of phycocyanobilin purified by TLC in methanol with labelled maxima.*

4.4. Esterification of pure phycocyanobilin

Pure phycocyanobilin obtained as in 4.3. was esterified with alcohols with different chain lengths, methanol, 1-butanol, 1-pentanol, 1-hexanol, using Fischer esterification, for 3 h. The procedure was the same as described in paragraph 4.2.

4.4.1. TLC analysis of the pure phycocyanobilin esters

Contents of the collected esterification reaction products were analysed with two TLC methods (Fig. 4.9.), Kalkuš (2018) and Havaux (2001). The TLC done with Kalkuš (2018) method showed a significant difference between phycocyanobilin and esters. All esterification products had larger mobility than the original bilin. However, the differences among esters themselves were again not observable. The only exception was the presence of a second band in the products of methanol and butanol reactions. Unfortunately, in the Havaux (2001) method, the pigments were not mobile enough, with the exception of the methanol products. The Havaux (2001) method, therefore, indicated that the reaction was not efficient enough for alcohols longer than methanol. Because the results of the two TLC methods were contradictive, we proceeded to analyse the reaction products by HPLC. Standard HPLC method for analysis of algae pigments was used with satisfactory results.

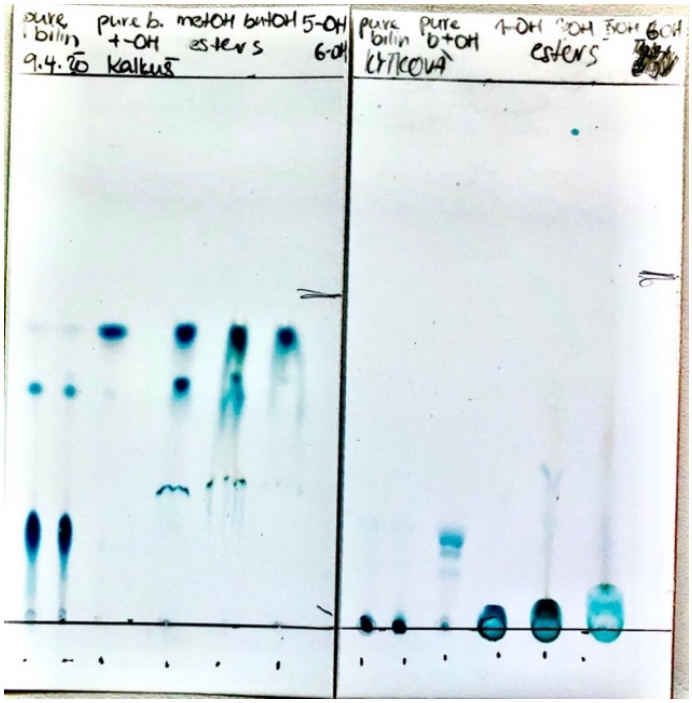

Fig. 4.9.: *Analysis of the products of the 3h hot esterification by TLC. Methods used were Kalkuš (2018) on the left and Havaux (2001) on the right. Order of the samples on each plate from the left: two blanks, methyl ester, 1-butyl ester, 1-pentyl ester, 1-hexyl ester.*

4.4.2. HPLC analysis of pure phycocyanobilin esterification products

The phycocyanobilin ester samples obtained in the previous step were further analysed by HPLC (Fig. 4.10.). The pure bilin sample (bottom chromatogram of Fig. 4.10.) was eluted immediately after about 2 minutes of the dead time of the used system. The majority of the pigment was eluted within the first 4 min of the analysis. The separation was not perfect, and several peaks could be observed close together within the first 4 min. The last peak was small and eluted at 6.38 min of the analysis. Major pigment peaks were visible at 2.85 min and 3.14 min.

In the case of the methyl ester sample, the retention times increased significantly. Two major peaks were eluted at 5.99 min and 6.37 min and were connected very closely. No further significant peaks were found. Before elution of peak 2, a very small minor peak was noticed.

Butyl ester elution times were further longer and its two major peaks eluted at 9.10 min and 9.38 min. Two additional small peaks were detected prior to and behind the major peaks (at 8.33 min and 10.85 min, respectively).

Pentyl ester retention times were again increased in comparison to the butyl ester sample and two major peaks eluted at 10.22 min and 10.49 min. The two secondary small peaks became comparatively higher and sharper and were eluted at 8.89 min and 12.4 min.

The last sample, hexyl ester, had the two major peaks elution times at 11.35 min and 11.58 min and therefore the retention times again increased as in the case of the previous samples. The two secondary peaks were less sharp and were smaller than in the case of pentyl ester.

In this analysis, the products of the esterification reaction had longer retention times than the free bilin. The retention times increased with the increasing length of the esterifying chain. The elution times of the two major peaks, which are labelled as peak 2 and peak 3 in Fig. 4.10., in each sample are shown in Tab. 4.1. Percentage of total chromatogram occupancy by two major and two minor peaks are also presented in Tab. 4.1. Peak 3 occupied from 57.06 - 74.21% of the total area (only the four greatest peaks were analysed here, this caused some errors, which we consider insignificant) for all the samples. Peak 2 occupancy varied from 18.34 - 31.13%.

The samples analysed in this work did not contain only one type of molecule. Two major peaks were found in the HPLC of bilins in all cases. Both peaks were molecules with the phycocyanobilin backbone as indicated by the absorption spectra in Figs 4.11. and 4.12. The

absorption spectra of major peaks are very similar regardless of the length of the esterifying chain. The absorption maxima were 361 nm and 640 nm for all esters. The pure phycocyanobilin sample had absorption maxima 361 nm and 654 nm in both peak 2 and peak 2 (Fig. 4.11. and 4.12.). Also, the minor secondary peaks found in HPLC of butyl-, pentyl- and hexyl- esters had absorption spectra of phycocyanobilin.

Absorption spectra of four (pure phycocyanobilin, pentyl and hexyl esters) or three (methyl ester and butyl ester) major peaks described in Fig. 4.10. are presented in Figs. 4.13.-4.17. All the peaks had very similar absorption spectra with maxima 361 nm a and 640 nm. The exception were peaks 1-3 of pure phycocyanobilin sample (Fig. 4.13.) with elution times 2.85, 3.14 and 4.16 min. These peaks had their absorption maxima shifted to 654 nm. The other exception was peak 4 in hexyl ester sample with an absorption maximum at 632 nm. The UV maximum of 361 nm remained the same for all the peaks.

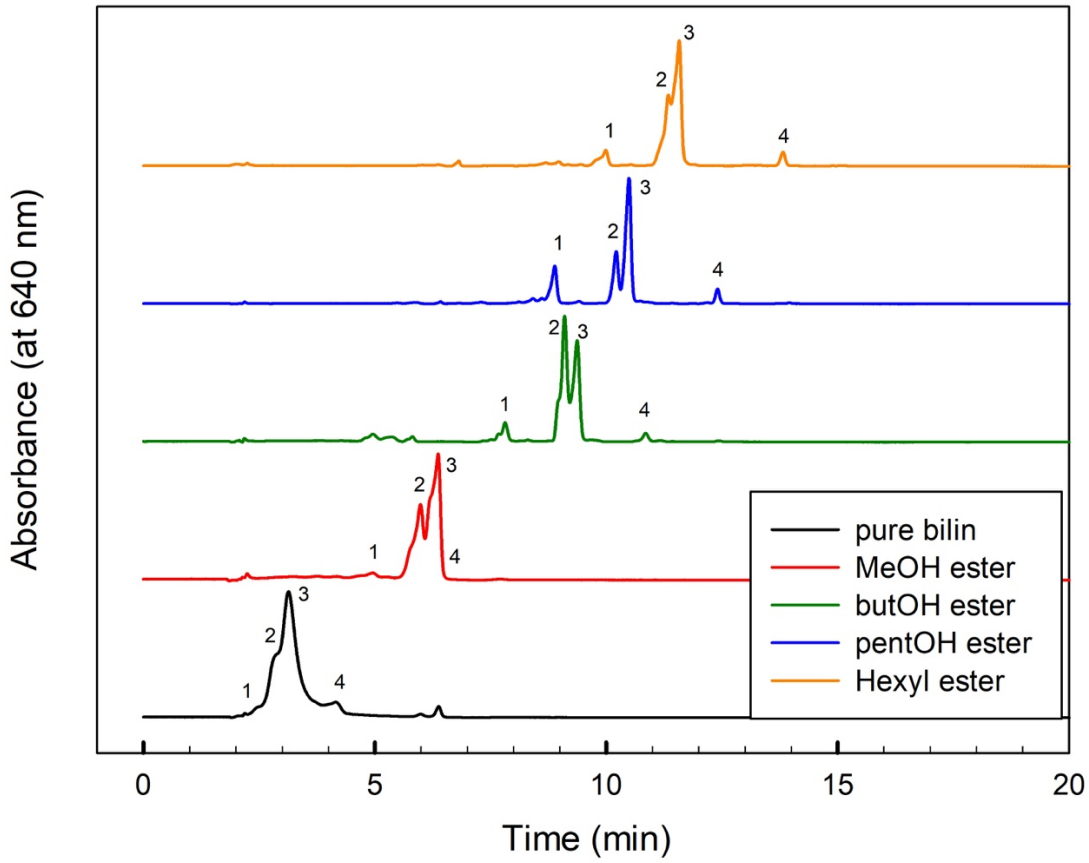

Fig.4.10.: *HPLC chromatograms of pure phycocyanobilin and its esters. Pure phycocyanobilin, methyl, butyl, pentyl and hexyl esters from the bottom, with four greatest peaks labelled.*

sample	peak 2 elution time (min)	peak 3 elution time (min)	peak 1 area (%)	peak 2 area (%)	peak 3 area (%)	peak 4 area (%)
pure bilin	2.85	3.14	1.33	18.34	74.21	6.11
methyl ester	5.99	6.37	3.03	36.32	60.33	0.32
butyl ester	9.10	9.39	11.92	22.62	61.87	3.59
pentyl ester	10.22	10.49	14.77	22.90	57.40	4.93
hexyl ester	11.35	11.58	7.69	31.13	57.06	4.13

***Tab. 4.1**.: Retention times of two major peaks in HPLC chromatogram and percentage area they occupy among four greatest peaks.*

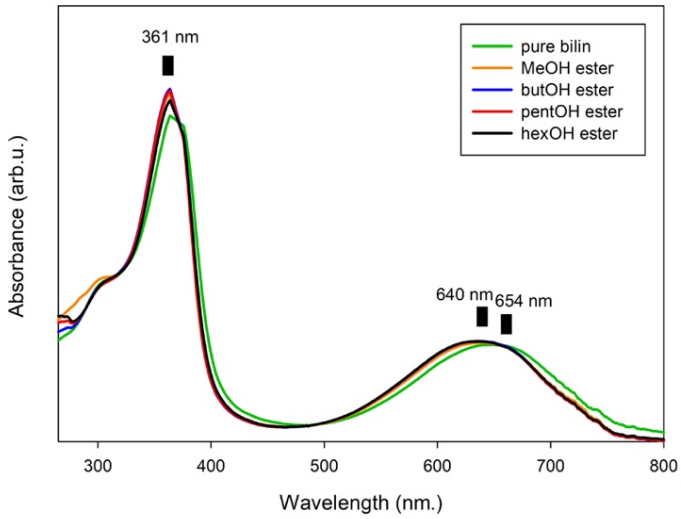

***Fig. 4.11.:** Absorption spectra of peak 2 in pure phycocyanobilin and its methyl, butyl, pentyl and hexyl esters in methanol solvent with labelled maxima*

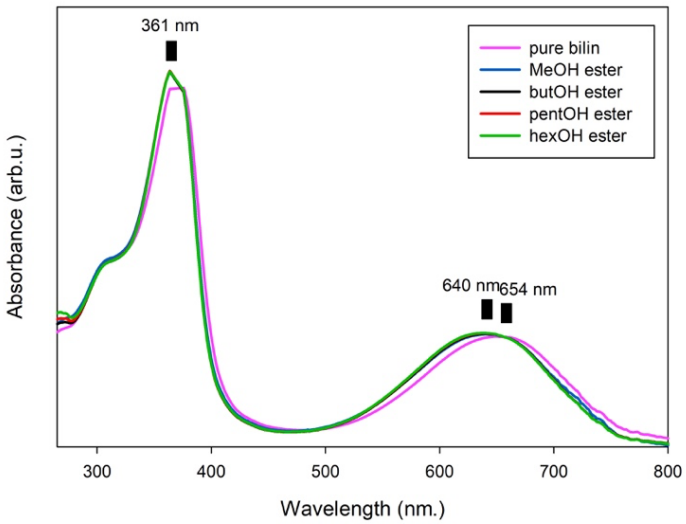

***Fig. 4.12.:** Absorption spectra of peak 3 in pure phycocyanobilin and its methyl, butyl, pentyl and hexyl esters in methanol solvent with labelled maxima*

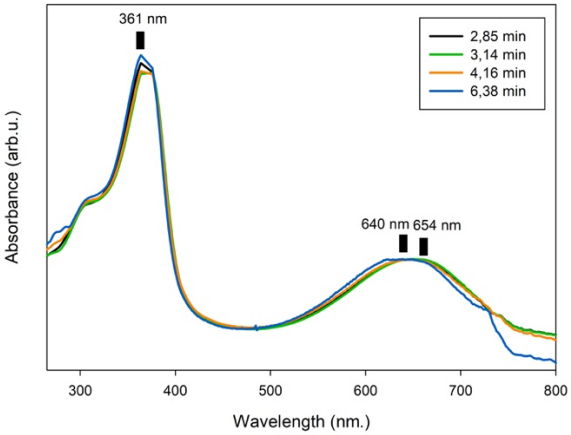

Fig. 4.13.: *Absorption spectra of four greatest peaks in HPLC analysis of pure phycocyanobilin in HPLC eluent with labelled maxima. Peak retention times are indicated.*

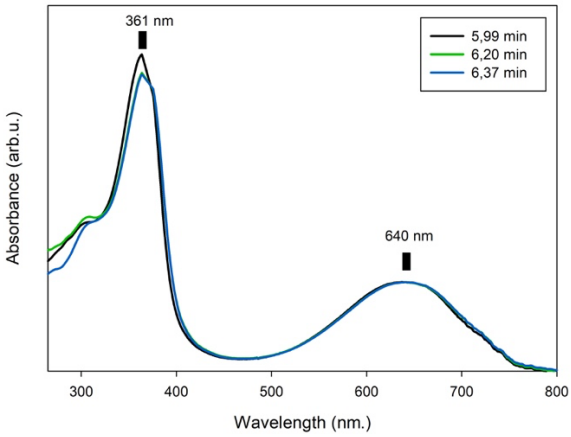

Fig. 4.14.: *Absorption spectra of three greatest peaks in HPLC analysis of phycocyanobilin methyl ester in HPLC eluent with labelled maxima. Peak retention times are indicated.*

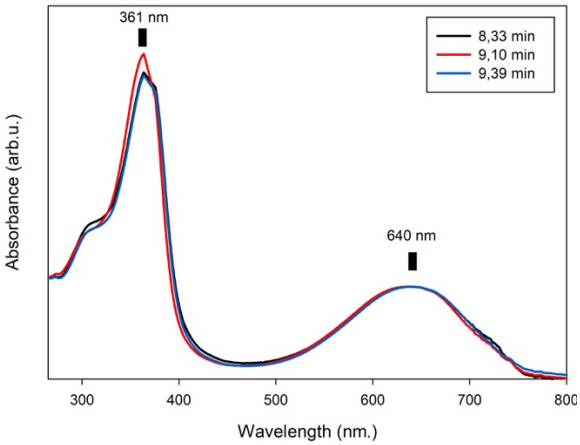

Fig. 4.15.: *Absorption spectra of three greatest peaks in HPLC analysis of phycocyanobilin butyl ester in HPLC eluent with labelled maxima. Peak retention times are indicated.*

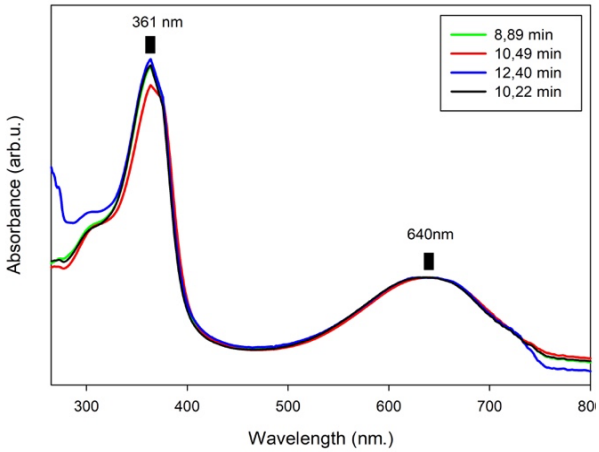

Fig. 4.16.: *Absorption spectra of four greatest peaks in HPLC analysis of phycocyanobilin pentyl ester in HPLC eluent with labelled maxima. Peak retention times are indicated.*

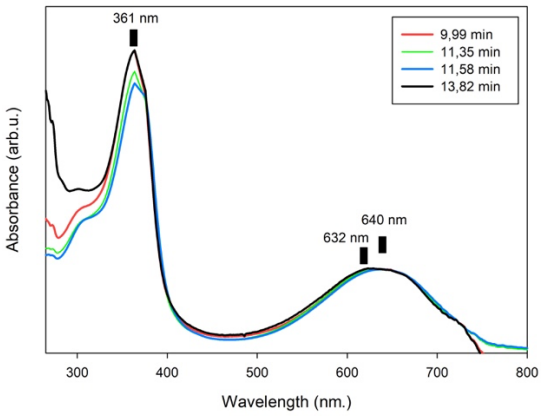

Fig. 4.17.: *Absorption spectra of four greatest peaks in HPLC analysis of phycocyanobilin hexyl ester in HPLC eluent with labelled maxima. Peak retention times are indicated.*

4.5. Stability of esterification products

The HPLC analysis described in the previous section was repeated after one week to analyse the stability of the reaction products. Pure bilin and its methyl, butyl, pentyl and hexyl esters dissolved in methanol were kept in the dark at 4 °C for one week and then analysed by an identical HPLC method.

The results (Fig. 4.18.) show that most of the original non-esterified pure phycocyanobilin remained unchanged. The major peaks were eluted at 2.76 min and 3.11 min, which are very similar to the elution times of the original sample. However, two small peaks with elution times at 5.99 min and 6.37 min also appeared. These elution times completely agree with elution times of original methyl ester of pure bilin done earlier and described in paragraph 4.4.2.

As can also be seen in Fig. 4.18., the elution times differed from the first analysis for all esters with the exception of the methyl ester. The major peaks (peak 1 and peak 2 in Fig. 4.18.) of all esters were eluted at 5.99 min and 6.37 min (M_1 and M_2 in Fig. 4.18.). These elution times again agree with elution times of methyl ester. Thus, we conclude that the experimental procedure, as described in this work, is not sufficient to produce stable esters of phycocyanobilin.

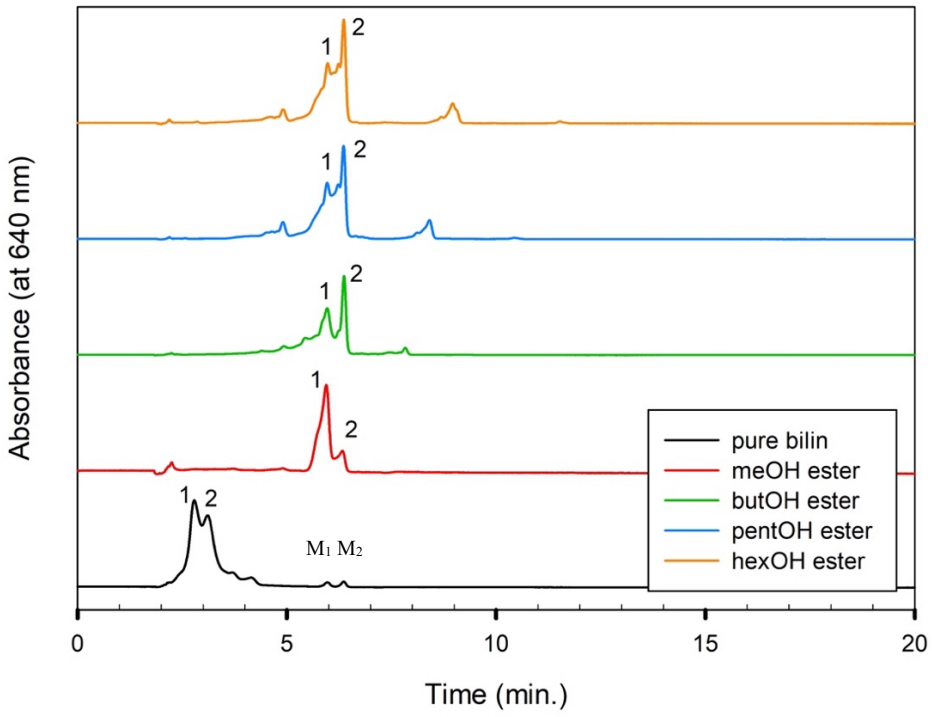

Fig. 4.18.: *HPLC chromatograms of pure phycocyanobilin and its esters- hexyl-, pentyl-, butyl- and methyl ester and pure bilin from the top after one-week storage in methanol with four major peaks labelled.*

5. Discussion

In this thesis, phycocyanobilin was prepared from cells of a cyanobacterium *Thermosynechococcus elongatus*. Although our initial extraction method (Kalkuš, 2018) worked reasonably well, we have decided to replace it with cold hydrolysis run for several days at room temperature. The cold hydrolysis made it possible to plan the work better as it was not necessary to safeguard the hot reaction as used by Kalkuš (2018). Nevertheless, the work was limited by low bilin availability, and much time was spent on bilin extraction rather than on the esterification reactions and their analysis. Future work should obtain a more abundant source of raw bilin mixture or pure phycocyanobilin to enable more experiments and more thorough analysis. One such source could be purified phycocyanin, a protein with phycocyanobilin used as a fluorescent dye. Using this pure source would be however expensive as a portion of 25 mg phycocyanin costs about 500 EUR [1].

Another source of phycocyanobilin could be Spirulina which is a widely sold dietary supplement product containing dried cells of the cyanobacterium *Arthrospira platensis*. The cost of the Spirulina product is about 350 EUR per 25 kg [2]. However, all the extraction and purification work would still need to be done. Boussiba and Richmond (1979) described another approach of purification. They isolated phycocyanin by hydroxyapatite chromatography. Further polyacrylamide electrophoresis confirmed that no protein contamination was present.

The raw pigment extract obtained after hydrolysis contained significant amounts of contamination by carotenoids and porphyrins (Fig. 4.3.) despite starting with water-soluble cell fraction which should be enriched in phycobiliproteins and should only contain minimum amounts of photosynthetic membranes. To solve this problem, we purified phycocyanobilin from the raw pigment extract by TLC (Fig. 4.7.). This enables obtaining small quantities of purified phycocyanobilin within about one hour of time. The phycocyanobilin gained in this way was still not completely pure. Only the main phycocyanobilin band was collected, but still, several bands were visible in TLC and several peaks in HPLC analyses (Fig. 4.9. and Fig. 4.10). This indicated that the purification was still not perfect or that the pigment was being modified or degraded after the purification. Higher sample purity could potentially be obtained by using quantitative HPLC for purification or DEAE-sepharose gel chromatography as in

[1] https://www.sigmaaldrich.com/catalog/product/sigma/p2172
[2] https://www.amazon.co.uk/Organic-Spirulina-Powder-Nukraft-Available/dp/B07PN1F27D?th=1

Beale and Cornejo (1991). Our HPLC method provided insufficient separation of pure phycocyanobilin (see results in section 4.4.2. and below).

The products of the esterification but also free bilin have good solubility in ether, no matter the chain length of the esterifying alcohol. The Kalkuš TLC method had a very limited difference in mobility among the esterified samples (Figs. 4.5., 4.6., 4.9. left), but this method showed good apparent esterification yields. The Havaux method showed significant differences in the mobility of the samples (Figs. 4.5., 4.6., 4.9. right). However, it proposed low yields in contrast with the results obtained by Kalkuš method. The only exception was the methyl ester, which shows good esterification yield in Havaux analysis. The other pigment contaminants were also esterified in 30 min hot esterification of non-purified bilin mixture because the mobility of the purple bands appearing below the main blue band of phycocyanobilin and its esters changed accordingly (Fig. 4.5.).

The immobility of the sample in the Havaux method may be caused by unsuitability of the method for the analyte but also by accidental contamination of the plate during manipulation or by overloading the sample. Isopropyl alcohol in the mobile phase may react with H_2SO_4 residues and form unwanted propene (Clayden, 2012). However, this method was not primarily developed for phycobilin analysis. Rüdiger and óCarra (1968) developed a TLC mobile phase for analysing bilin dimethyl ester, containing benzene, petroleum ether and methanol (50:10:6, v/v), which should be considered as a potential mobile phase also tested for other bilin esters.

In summary, the esterification reaction was successful in modifying the bilin molecules, which was later confirmed by HPLC analysis. However, the two used TLC methods did not agree on the yield of the esterification reactions. This discrepancy was not solved during this work. The HPLC analyses (section 4.4.2. and below) nevertheless provided a consistent picture of the results of the reactions.

Evaporation of esterifying alcohols after the termination of the reaction was very difficult except in the case of methanol. Butanol, pentanol and hexanol ester samples were dried under nitrogen gas stream, and it took around 30-40 min until the sample could be further used. Fu (1979) used addition of water while drying the residues of 1-butanol to form an azeotropic mixture which evaporates easier than pure 1-butanol. This approach may be helpful in shortening the time of evaporation of other alcohols after esterification.

The duration of the esterification reaction, 30 min or 3 h, had no significant effect on the result. There were no apparent differences in yield of the major blue band on both, Kalkuš and

Havaux, TLC methods. In the case of 3 h esterification, there was slightly more pigment in the less mobile blue band below the main band on Kalkuš TLC analysis (Fig. 4.6. left).

Further HPLC analysis of esterified pure phycocyanobilin provided clear evidence of increasing retention time when longer alcohols were used for esterification. Four major peaks were visible in HPLC analysis. Most of the pigment was within the second and third peak. Absorption spectra of these four major ester peaks show almost identical chromophore backbone (Figs. 4.13.-4.17.). The ratio of the two greatest peaks was varying (Tab. 4.1.), but no link was observed between peak ratio and esterifying chain length. The phycocyanobilin molecule is not fully symmetrical. Thus, the two closely separated peaks (denoted 2 and 3 in Fig. 4.10.) could be two different monoesters. Formation of diester is also possible, however, probability of its occurrence should decrease with increasing alcohol length. No such peak was present in our HPLC data. The only dimethyl ester was described by Rüdiger and óCarra (1968) by Fischer esterification or by Gossauer (1977) by chemical synthesis from monothiosuccinate and pyrrole derivative.

The bilin esters obtained in this work were not stable when stored in methanol and apparently converted to methyl esters. This is likely caused by remaining acidity after hydrolysis and Fischer esterification. In the work of ÓhEocha (1963 and 1966) the hydrolysate was washed six times with water and neutralised by sodium acetate until the pH reached values between 6 and 7. Likewise, esterification products were stabilised by repeated washing with water in the work of Rüdiger and óCarra (1968). These missing washing and neutralisation steps may be potentially a source of error and modification of products.

After achieving stable phycocyanobilin esters, the effect of different length of esterifying chains on incorporation into artificial aggregates of BChl c may be tested. Most of the pigment in our work was in one of two major HPLC peaks (88.4% in average was within the two major peaks), so the esterification product yield is high, and it can be directly used for first experiments and proof-of-concept work.

6. Conclusions

Phycobiliprotein mixture was isolated from *Thermosynechococcus elongatus* cells, hydrolysed by cold acid hydrolysis, and phycocyanobilin was then purified from the mixture by TLC.

Purified phycocyanobilin was successfully esterified by alcohols with different chain lengths. Although the TLC analysis was inconclusive, the HPLC analysis provided clear results. Obtained esters contained at least three kinds of product with almost identical UV-vis absorption spectra. After optimising the storing conditions, the obtained samples can be tested for incorporation into artificial aggregates of Bchl c.

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8. Attachments

ISOLATION OF PHYCOCYANOBILIN MIXTURE

(original from Kalkuš (2018). Modified.)

1. Centrifuge (7 min × 8000 g) cyanobacteria grown in WC medium (approx. 2 L).

2. Divide the pellet formed at 4 parts, one for immediate use, three remaining store at -81 °C.

3. Solve the pellet in 10 mL buffer (0.3 M KH_2PO_4; 0.2 M Na_2EDTA; pH=7.0) and homogenize.

4. Disrupt the suspension with EmulsiFlex C5 (Avestin, Canada), (5 min, 15 000 psi). Centrifuge the disrupted material (5 min, 6000 × g).

5. Keep the s theupernatant in dark before further use.

6. Centrifuge the supernatant (30 min, 25 000 × g). Discard the pellet.

7. Mix the supernatant with acetone (supernatant : acetone, 1:5) so a blue precipitate is formed. Centrifuge (5 min, 6000 × g) to pellet the precipitate. Discard the supernatant, dry the precipitate in vacuum or desiccator.

8. Suspend the blue precipitate by adding 10 L of 35% HCl, until the precipitate is dissolved.

9. Put the precipitate into the water bath at 80 °C for 90 min (HOT hydrolysis) **OR** keep the tube with the precipitate in the dark for 24 h/one week (COLD hydrolysis).the

10. After desired time, stop the reaction by adding distilled water (hydrolysate : water, 1:4) and centrifuge (10 min × 7500 g).

11. If desired, repeat the hydrolysis (steps 8-10) for higher yield.

12. Extract the supernatant in a separatory funnel into chloroform (chloroform : supernatant, 1:10). Repeat this step until no pigment goes into the chloroform phase and let the chloroform evaporate in the vacuum in the dark.

FISCHER ESTERIFICATION OF PHYCOBILINES BY METHANOL, 1-BUTANOL, 1-PENTANOL AND 1-HEXANOL

1. All work should be done in dim light or darkness. Dissolve the purified phycobilin in 1 mL of chloroform.

2. Prepare five Eppendorf tubes. Label one as a blank, on t,he other four write the alcohols used.

3. Add 200 µL of phycobilin dissolved in chloroform into each Eppendorf tube. Let the samples dry in vacuum.

4. Once the samples are dried, continue by adding 250 µL of alcohol (methanol, 1-butanol, 1-pentanol and 1-hexanol) into each tube according to the labels.

5. Add 5 µL of 96% H_2SO_4 into each Eppendorf tube. Put the methanol sample onto the heating plate set to 55 °C and the other samples onto the heating plate set to 90 °C.

6. Run the reaction for 3 hours.

7. Terminate the reaction by adding 1 mL of redistilled water. Add 500 µL of diethylether into each Eppendorf tub e. The methylester sample requires adding more redistilled water (up to 7 mL).

8. Centrifuge the samples at 14000 rpm for 2 min.

9. Carefully pipette the lower water phase out. Discard the lower phase.

10. Dry the ester samples under the nitrogen gas steam (takes a while).

11. Proceed to further analysis.