University of South Bohemia in České Budějovice
Faculty of science

Formation of complexes of astaxanthin with metal ions and their basic spectroscopic characterization

Bachelor thesis

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České Budějovice 2019

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Velanová, H., 2018: Formation of complexes of astaxanthin with metal ions and their basic spectroscopic characterization. Bc. Thesis, in English. - 38 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The complexes of astaxanthin with calcium, zinc and copper were formed and characterized with UV-Vis spectroscopy. Red shifts of the absorption maxima were observed and compared with data available in the literature. Acquired complexes were subjected to HPLC analysis, however only analysis of complexes with copper was succesful and individual compounds were identified. For the same complex transient absorption spectra were measured.

Declaration [in Czech]

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Acknowledgements

I would like to thank my supervisor Hristina Staleva-Musto for guiding me through the first half of the lab work and for getting me excited about the topic. I would also like to thank professor Tomáš Polívka for helping me with the interpretation of data from transient absorption spectroscopy, for giving me valuable notes on the thesis and last but not least for always being excited about the results even though the rest of us was rather sceptical. Big thanks also belongs to Radek Litvín who let me work in his lab and stayed late to run the endless HPLC analyses but I am most grateful that I could always turn to him when I had any trouble with the experiments, and there were a lot! I cannot forget to thank Mr. Fuciman for measuring the transient absorption with me and showing me how to fit the data.

There are obviously more people I would like to thank for helping me finish this thesis but a special thanks belongs to Michal Slaba whose graphical skills and patience saved me from stealing multiple figures from other publications.

Contents

1 Introduction

Organisms obtain their colour from natural pigments and the coloration is a very important feature of their everyday life. It plays a role in a number of ecological interactions, mediates both inter- and intraspecies communication last but not least it is important for humans as well in attracting attention and providing with nutrients (Eldahshan et al., 2013; De Carvalho et al., 2017). Pigments are also physiologically important, not only in plants where their essential role in light harvesting is well known for a long time, but also for their photoprotective and anti-oxidative properties.

1.1 Carotenoids

1.1.1 Structure, biosynthesis and occurrence

Carotenoids form one of the most important groups of pigments. According to Yabuzaki (2017) there are more than 1100 described carotenoids by now and the number is most likely to rise in the future. There are two classes of carotenoids: carotenes and xanthophylls which are oxygenated derivatives of carotenes. Carotenoids are C40 tetraterpenoids soluble in lipids. Their structure consists of long conjugated π-electron system, so called polyene chain, which is responsible for the ability to absorb light between 400 and 500 nm. This property ascribes carotenoids their typical yellow, orange and red colour (Goodwin, 1980). The polyene chain can be ended by cyclic groups (so called rings) and may be supplemented with oxygen groups forming xanthophylls. In nature, most of the carotenoids are present in all-trans configuration. In Figure 1 you can find some of the most well-known carotenoids both linear and non-linear, symmetrical and asymmetrical, with and without oxygen groups.

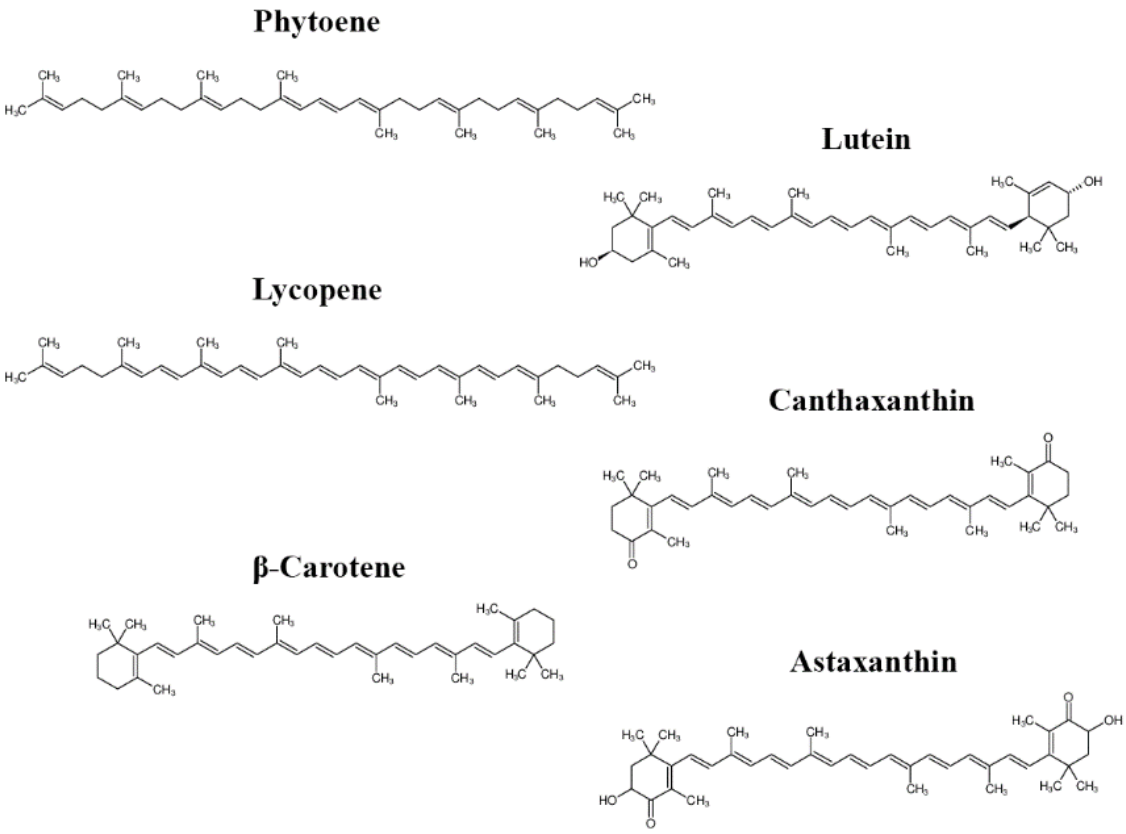

Figure 1. Examples of carotenoids.

The most widely used biosynthetic pathway of carotenoids is the cascade leading to β-carotene formation (see Figure 2). This pathway can be found in all carotenogenetic groups: bacteria, fungi, algae and plants. First of all, two molecules of geranylgeranyl diphosphate are linked forming phytoene, the first carotene of the pathway. Further conversion involves four desaturation and two cyclization steps. Other carotenoids can be derived further from β-carotene. Xanthophylls are oxidation products of α- and β-carotene formed enzymatically. Their most common oxygen groups are hydroxy, oxo and epoxy (Sandmann, 1994).

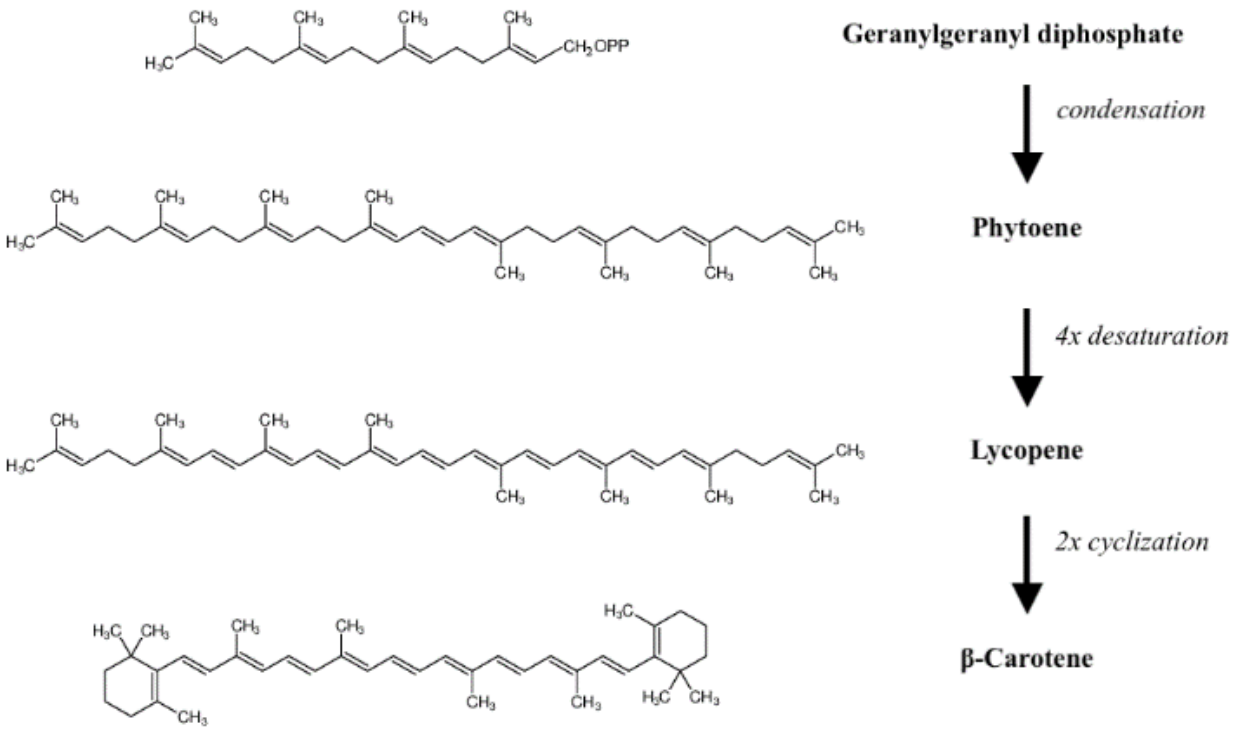

Figure 2. Biosynthetic pathway of carotenoids.

Carotenoids accumulate in bodies of many organisms but only some of them can synthesise them on their own. Among these belong heterotrophic bacteria and fungi as well as photosynthetic prokaryotes and eukaryotes. For both lower and higher plants carotenogenesis is essential in their photosynthetic activity (Sandmann, 1994). They absorb light in blue-green region covering the part of spectra where chlorophyll cannot absorb, enabling the plants to absorb photons of any wavelength in the visible light therefore enhancing photosynthesis.

1.1.2 Photophysical properties

For a basic description of excited state dynamics of carotenoids, a model including three states, namely S_0, S_1 and S_2, is used. S_0 is the ground state from which carotenoids can be excited to higher states. Because of the symmetry, one photon transition between S_0 and S_1 is forbidden while transition between S_0 and S_2 is allowed. The S_1 state is the lowest singlet excited state of carotenoids. The S_0-S_1 transition is forbidden thus carotenoid must be first excited to S_2 state and after that, through a process of internal conversion, the S_1 state can be populated. This process takes typically 50 to 300 fs. Because of the forbidden transition from ground state, S_1 state, also called the dark state, is invisible in absorption spectra, therefore other methods such as transient absorption must be used for its investigation. The S_2 state of carotenoids is populated by one photon excitation and it is highly absorbing due to strongly

allowed S_0-S_2 transition. This is the reason for the coloration of organisms containing carotenoids. Naturally occurring carotenoids absorb in blue-green region, therefore the S_0-S_2 transition occurs between 475 and 525 nm and this assigns carotenoids the yellow to red colour. Apart from these three states, there is another one occurring among carbonyl carotenoids, which possess a conjugated keto group. It is a dark state with a charge transfer character, so called intramolecular charge transfer (ICT). This state is highly dependent on solvent polarity, the more polar solvent the stronger ICT signal occurs. It has been found that the ICT state is coupled with the S_1 state but the exact relation between them is yet to be determined (Keşan, 2016; Polívka and Sundström, 2004; West, 2018).

1.1.3 Function

Carotenoids have many functions in different organisms. On the top of the function in energy transfer during photosynthesis they also protect organisms against oxidative stress, they help to fold proteins and even take part in signalling in plants and animals by changing the coloration (Britton et al., 2007). Carotenoids can form complexes with various substances (e. g. proteins and other pigments) and their chemical and physical properties are strongly influenced by their structure as well as by other molecules in the surrounding area. As an example, different isomerisation state of carotenoids depending on their location can be mentioned. The carotenoids located in in light-harvesting complexes occur in all-trans configuration while carotenoids in reaction centres possess cis configuration (Koyama and Fujii, 1999).

Let's focus on the role of carotenoids in photosynthesis. Carotenoids absorb light in blue-green region and transfer the excitation energy to the red-shifted chlorophylls via the singlet-singlet excitation energy transfer (EET). The EET from Carotenoid (Car) to Chlorophyll or Bacteriochlorophyll ((B)Chl) can occur from the two lowest excited states of carotenoids, S_1 and S_2, see Figure 3 (Mirkovic et al., 2016). In some organisms, energy transfer takes place only from the S_2 state whereas in others the EET takes place from both states increasing the efficiency of the transfer (Gradinaru et al., 2001).

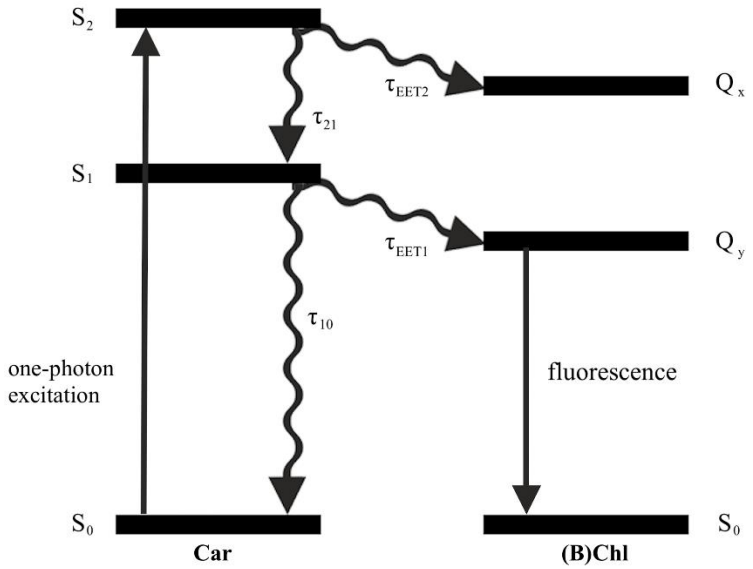

Figure 3. Scheme of excitation energy transfer in photosynthesis. Solid arrows represent allowed transitions, wavy arrows represent internal conversion. Figure created based on Mirkovic et al., 2017.

Second most well-known attribute of carotenoids is their photoprotective function particularly against singlet oxygen ($^1O_2$) and reactive oxygen species (ROS). Singlet oxygen is an extremely reactive molecule that is able to oxidize pigments, proteins and lipids in the membranes; hence causes lethality of living tissues. The ground state of oxygen is a triplet state ($^3O_2$). In order to create singlet oxygen, energy must be transferred to $^3O_2$ from a long-lived excited sensitizer molecule, in the case of photosynthetic organisms the sensitizer molecule is usually chlorophyll. It is achieved through the triplet-triplet energy transfer reaction (Mirkovic et al., 2016). The mechanism of energy transfer is described in equations 1-3 and in Figure 4.

$$^3(B)Chl + {}^3O_2 \rightarrow (B)Chl + {}^1O_2 \quad (1)$$

$$^3(B)Chl + Car \rightarrow (B)Chl + {}^3Car \quad (2)$$

$$^1O_2 + Car \rightarrow {}^3O_2 + {}^3Car \quad (3)$$

First equation shows the formation of singlet oxygen via energy sensitizer: triplet chlorophyll. Second equation describes the quenching of triplet chlorophyll by carotenoids to prevent the generation of $^1O_2$ and the third equation shows the ability of carotenoids to scavenge singlet oxygen directly (Mirkovic et al., 2016).

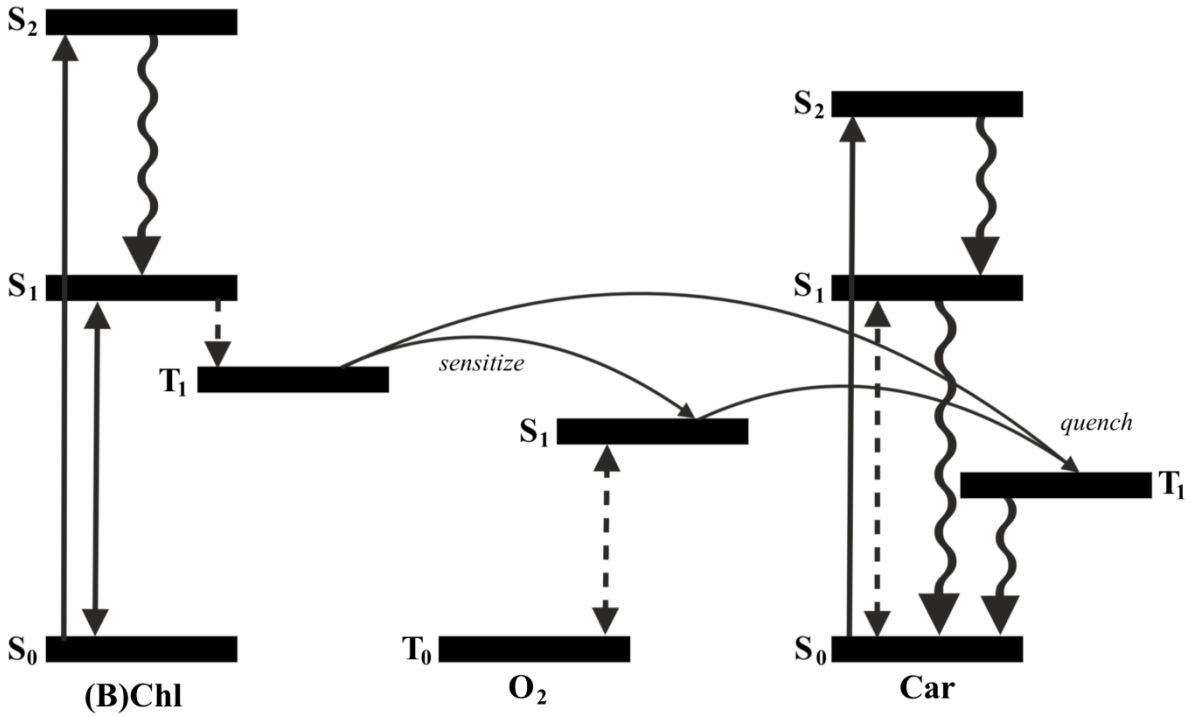

Figure 4. Scheme of energy transfer during photoprotection. Solid arrows represent allowed transitions, dashed arrows represent forbidden transitions and wavy arrows represent internal conversion. Figure created based on Mirkovic et al., 2017.

1.2 Astaxanthin

Astaxanthin is a carotenoid which belongs to xanthophyll family because apart from carbon and hydrogen it also contains oxygen atoms in the form of hydroxy and keto group, therefore it belongs to the group of carbonyl carotenoids. Astaxanthin consists of two terminal ionone rings linked by a polyene chain and it is one of the symmetrical carotenoids (see Figure 5). Astaxanthin can be found in nature in many shapes it exists in stereoisomers, geometric isomers and in free or esterified forms (Higuera-Ciapara et al., 2006). Free astaxanthin is relatively hard to find in nature, instead most of it is conjugated with proteins or esterified with fatty acids (Kidd, 2011). The molecular formula of astaxanthin is $C_{40}H_{52}O_4$ and its molecular weight is 596,84 g/mol.

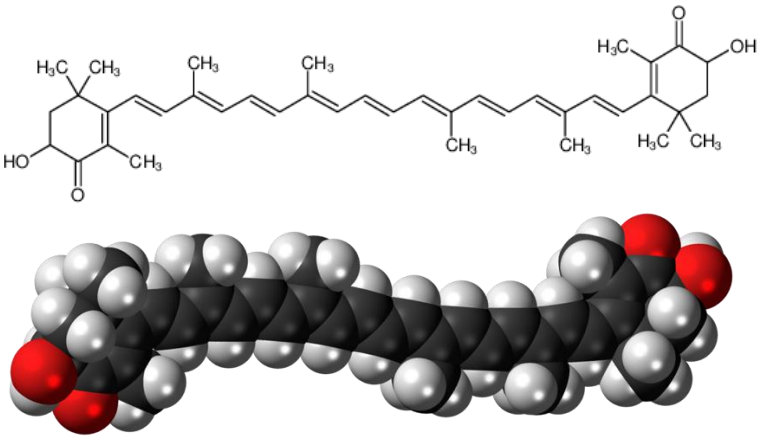

Figure 5. Structure of astaxanthin.

Astaxanthin can be found in various organisms but only plants, bacteria and fungi can synthesise it themselves. Animals such as fish, crustaceans or birds must take it up in the diet. The natural sources of astaxanthin are various algae, yeast, salmon, trout, krill, shrimp and crayfish. One of the best natural sources of astaxanthin, which is also used for human consumption and as source of pigment in the diet of salmon, trout and shrimp in aquacultures, is *Haematococcus pluvialis*. It is unicellular microalgae which accumulates high levels of astaxanthin under unfavourable conditions such as high salinity, nitrogen deficiency, high temperature and light. (Ambati et al., 2014). The production of astaxanthin consists of three main stages: cultivation in closed pond system then reddening in open pond system and extraction of dried *H. pluvialis* biomass. Astaxanthin can also be obtained by chemical synthesis (Fassett and Coombes, 2011).

1.2.1 Antioxidant effects

The red colour of astaxanthin is due to conjugated double bonds in the middle of the molecule. These types of bonds act as strong antioxidants because they can donate electrons and react with free radicals in order to convert them into more stable products, thus terminating the harmful free radical chain reaction in many organisms (Guerin et al., 2003). Astaxanthin has even proven to have better antioxidative properties, thus being more useful for various organisms, than other carotenoids. One of the reasons is that astaxanthin has both lipophilic and hydrophilic properties, therefore it can link within cell membranes. Cell membrane consists of double-layer of phospholipids which have polar heads heading out and non-polar tails heading in the membrane. Astaxanthins slightly polar terminal rings can be linked with polar heads of phospholipids whereas non-polar polyene chain of astaxanthin lays in the

middle of the membrane among non-polar phospholipid tails (Ambati et al., 2014; Kidd, 2011). The terminal rings can therefore scavenge radicals both inside and outside of the cell whereas the polyene chain can trap them inside of the cell membrane (Goto et al., 2001; Kidd, 2011). The other quality that makes astaxanthin better antioxidant than other carotenoids is the presence of both hydroxyl and keto group on each ring (Hussein et al., 2006).

Oxidative damage in organisms is caused by earlier mentioned singlet oxygen and ROS. These molecules have a very high reactivity and they are produced by regular aerobic metabolism. Oxidative molecules which are in excess may react with DNA, lipids or proteins resulting in damage of these molecules which contribute to aging, degenerative diseases and it can also lead to other disorders (Ambati et al., 2014). Astaxanthin can work as an anti-inflammation agent, it can prevent cardiovascular disease, it has anti-cancer activity and it can also enhance immune response, therefore is it already commercially used as a dietary supplement (Ambati et al., 2014; Dose et al., 2016; Fassett and Coombes, 2011; Kidd, 2011; Kishimoto et al., 2016). The problem with using astaxanthin as an antioxidant in diet is its low solubility in aqueous environment. Astaxanthin is a lipophilic molecule therefore it would require a chemical transport system in order to get to the targeted tissue. Based on the quality of astaxanthin to form complexes with various molecules, Polyakov et al. (2013) was able to complex various xanthophylls including astaxanthin with natural complexants creating a perspective transport system providing better stability and solubility of xanthophyll carotenoids.

1.2.2 Complexation

As mentioned above astaxanthin is very useful antioxidant. It can rapidly react with ROS preventing the damage caused by them. Another reason why astaxanthin is considered better antioxidant than other carotenoids can be his ability to form chelate complexes with metals. Astaxanthin has two neighbouring oxygen atoms on each terminal ring, one within hydroxy group and the other one within keto group. This structure is similar to the structure of many biologically active compounds such as α-hydroxy-ketones and hydroxy quinones. It is known that these compounds are able to form stable chelate complexes with metal ions, therefore astaxanthin should be able to form them as well (Polyakov et al., 2010). This metal complexing ability may play an important role in some organisms especially in those accumulating high levels of astaxanthin under unfavourable conditions. In *Haematococcus*

pluvialis accumulation of astaxanthin is thought to be a survival strategy. Under stress it accumulates 1% of cell mass as carotenoids of what 70% are monoesters of astaxanthin, 10% diesters and 5% free astaxanthin, the remaining 5% are mainly β-carotene, canthaxanthin and lutein. *H. pluvialis* also accumulates metals: 1% of the biomass of iron, 1% magnesium and 2% calcium (Lorenz, 1999).

Polyakov et al. (2010) found out that astaxanthin can form stable chelate complexes with Ca^{2+}, Zn^{2+} and Fe^{2+} which make significant changes in absorption spectra. The absorption maximum, in the presence of listed metals, shifts to the red. In this experiment also Cu^{2+} was used, but instead of complexation a blue shift and formation of cis-isomers was observed (Polyakov et al., 2010). Cis/trans isomerisation of astaxanthin in the presence of copper was also observed by Zhao et al. (2005). All-trans astaxanthin was readily converting to mainly 9-cis-astaxanthin and 13-cis-astaxanthin. There was another study conducted by Chen et al. (2007) focused on behaviour of astaxanthin in the presence of calcium ions. It was found that both complexation and cis/trans isomerisation occurs in the presence of Ca^{2+}.

The mechanism of astaxanthin-metal complex formation was also described by Polyakov et al. (2010). It was found that at low salt concentrations, less than 0,2 mM, in ethanol, there is a complex with only one metal on one of the ionone rings formed whereas at high salt concentration there is one metal on each ring (see Figure 6).

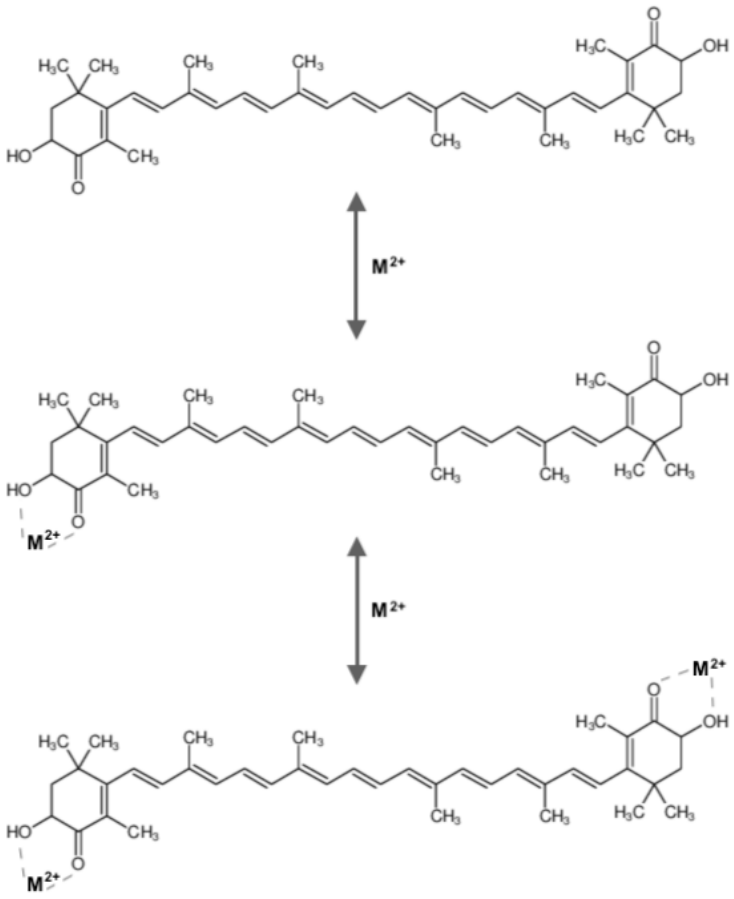

Figure 6. Complexation of astaxanthin with metals. Created based on Polyakov et al. (2010).

These results are also supported by a theoretical study by Hernández-Marin et al. (2012). It is stated that astaxanthin may form complexes with metal ions such as Ca^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+}, Cd^{2+} and Hg^{2+}. To gain most stable complexes metals are thought to be coordinated with the two oxygen atoms located on the terminal rings. The presence of these metals changes the absorption spectra by shifting the absorption maximum towards the red. The results are rather overrated though. For example, for zinc ion, the theoretical shift in ethanol should be from 471 nm (pure astaxanthin) to 531 nm whereas the observed shift in the study by Polyakov et al. (2010) was from 480 nm to 492 nm. Moreover, it was calculated that the metal complexes are slightly better electron donors and acceptors than pure astaxanthin thus having better antiradical capacity (Hernández-Marin et al., 2012).

2 Objectives

- To form complexes of astaxanthin with various metals.
- To characterize the acquired complexes using basic spectroscopic methods.
- To compare the results with data available in the literature.

3 Materials and methods

Chemicals used for preparation of samples are listed in Table 1. Chemicals which did not contain any water were used directly as obtained from manufacturer. Those which contained water, $Ca(ClO)_2 \cdot 4H_2O$ and $Zn(ClO_4)_2 \cdot 6H_2O$ were dried first. The drying took place on a petri dish placed on a hot plate, set to the temperature of 150 °C (zinc) or 300 °C (calcium). The process of drying was terminated when traces of water were no longer observed.

Table 1. Chemicals used for preparation of the samples.

Substance	Chemical formula	Manufacturer	CAS number
Astaxanthin > 97%	$C_{40}H_{52}O_4$	Sigma-Aldrich	472-61-7
Copper sulphate	$CuSO_4$	Sigma-Aldrich	7758-98-7
Calcium perchlorate tetrahydrate	$Ca(ClO_4)_2 \cdot 4H_2O$	Sigma-Aldrich	15627-86-8
Zinc perchlorate hexahydrate	$Zn(ClO_4)_2 \cdot 6H_2O$	Alfa Aesar	10025-64-6
Iron chloride	$FeCl_2$	Alfa Aesar	7758-94-3
Cadmium chloride	$CdCl_2$	Sigma-Aldrich	10108-64-2
Methanol for HPLC gradient grade	CH_3OH	VWR chemicals	67-56-1

3.1 Sample preparation

The protocol for sample preparation was optimised under various conditions. The main factors were temperature, light and solvent. In the end two protocols were used, one led to preparation of complexes, the one led to isomerisation of astaxanthin.

The first protocol looks as follows. Saturated stock solution of astaxanthin in methanol was prepared and required volumes, according to Table 2, were transferred to glass vials. The amounts of salts needed to make solutions of required concentrations were calculated using the equation number 4, where *m* stands for mass, *MW* for molecular weight, *V* for volume and *c* for molar concentration.

$$m = \frac{MW \cdot V}{c} \qquad (4)$$

The mass was measured using analytical balance (BOECO, BBI-31) and the measured values are stated in Table 2. The required amounts of salts were transferred directly into the glass vials containing saturated solution of astaxanthin. The samples were mixed thoroughly

by pipetting and left in the dark at room temperature. Because of the limited range of analytical balance, the least concentrated samples were made by diluting the 1 mM sample, in 1:1 ratio, by astaxanthin stock solution (2,5 ml of 1 mM + 2,5 ml of AST stock solution).

Table 2. The amounts of chemicals used for sample preparation.

	Molecular weight (g/mol)	Molar concentration (mmol/l)	Volume of AST solution (ml)	Mass of salt (mg)
$CuSO_4$	159,61	10	5	8
		1	10	1,6
		0,5	2,5	-
$Ca(ClO_4)_2$	238,97	10	5	12
		1	10	2,4
		0,5	2,5	-
$Zn(ClO_4)_2$	264,28	10	5	13,2
		1	10	2,6
		0,5	2,5	-
$FeCl_2$	126,75	10	5	6,4
		1	10	1,3
		0,5	2,5	-
$CdCl_2$	183,32	10	5	9,2
		1	10	1,8
		0,5	2,5	-

The other protocol involved heating. Saturated solution of astaxanthin in methanol was mixed, in 1:1 ratio, with 10 mM solution of salt in the same solvent. The sample was warmed up to 45 °C either directly in the spectrophotometer (in temperature controlled cell holder) or in the water bath.

The samples were first characterized by steady state absorption spectroscopy. Their absorption spectra were obtained using UV/Vis spectrophotometer (Shimadzu UV-2600) and compared with the absorption spectrum of astaxanthin in methanol. Absorption spectra were measured in 10 mm quartz cuvettes. For analysis of the data SigmaPlot 10.0 was used.

Further analysis, regarding composition of selected samples, was performed by HPLC (High Performance Liquid Chromatography) using Waters Alliance e2695 HPLC system with 2998 PDA detector. Two methods were used, one for separation of complexes and another one for separation of isomers. The first method was performed on Waters Nova-Pak C18 4μm

3x300 mm column. As mobile phase methanol was used for first 10 minutes followed by a gradient of acetonitrile for another 10 minutes. The other method was performed on YMC Carotenoid C30 3 μm 4,6x150 mm column. In this case, mobile phase was made according to Zhao et al. (2005). It was a mixture of dichloromethane, methanol, acetonitrile and water in the ratio 5:85:5:5. All of the used solvents were of HPLC quality. For both methods the detection wavelength was 480 nm, flowrate was 1 ml/min and temperature was 30 °C.

Selected sample was also analysed with transient absorption. Transient absorption spectrum was measured in a 2 mm quartz cuvette. Sample was excited at 520 nm and its transient absorption spectrum was later compared with a transient absorption spectrum of pure astaxanthin excited at 500 nm.

3.2 Principles of used methods

3.2.1 Steady state absorption spectroscopy

Measuring the absorption spectra is a simple method readily used for basic characterization of carotenoids. It can reveal whether a given substance is present in the sample or not and its concentration can be quantified. It is done by measuring the amount of light absorbed by the sample at different wavelengths. Plotting the absorbance against wavelength gives us the absorption spectrum which is unique for each molecule. The absorption of light of particular wavelengths is caused by interaction of electromagnetic field with charged particles in the sample, more specifically, it is due to electronic transitions between the ground state and higher excited states for which the transition is allowed (Hammes, 2005).

The amount of light absorbed by a sample is described by transmittance (T) it is a ratio of the intensity of transmitted light (I) and incident light (I_0), see equation 5. More widely used quantity for description of the phenomenon is absorbance which is a negative logarithm of transmittance, see equation 6. Absorbance is often expressed in a form of Beer-Lambert law which says that it is equal to the product of extinction coefficient (ε), concentration (c) and path length (l), see equation 6.

$$T = \frac{I}{I_0} \tag{5}$$

$$A = -\log T = \log \frac{I_0}{I} = \varepsilon c l \tag{6}$$

Using the Beer-Lambert law, concentration of a given sample can be computed if extinction coefficient, unique for every compound at a given wavelength, is known (Hammes, 2005; Prosser, 1989).

3.2.2 HPLC

High performance liquid chromatography (HPLC) is analytical technique used for separation of classes or groups of molecules. As in all of the chromatographic techniques, the separation takes place between two phases, a stationary phase, which is often bound to the solid, and a mobile phase, which is in this case liquid. In HPLC elevated pressure is needed to force the liquid through the stationary phase giving better resolution and shorter analysis time than chromatographic techniques using gravity to pull the liquid around the particles of stationary phase. HPLC is used to detect and quantify substances of interest in a mixture and also allows their isolation and purification. One of the most widely used techniques is the reversed-phase chromatography where compounds are separated according to their polarity between stationary phase which is nonpolar and mobile phase which is very polar (Neue, 1997). An HPLC system consists of a solvent reservoir, a pump, an injector, the analytical column, and a detector. Solvent reservoir contains the mobile phase which is delivered into the system on the column by a pump while the sample is delivered by the injector. The column can be filled with various types of stationary phase and it is eluted by both sample and mobile phase. On the outlet of the column there is a detector which detects individual fractions of the sample.

3.2.3 Transient absorption spectroscopy

To study extremely fast processes, happening on the timescale of few picoseconds or less, time resolved methods must be applied. Femtosecond spectroscopy, more specifically transient absorption, is one of the most widely used methods to study such systems. In principle, a laser beam which consists of short pulses (<100 fs) is divided into two beams; pump and probe. Pump is the excitation beam while the probe is the beam which is probing the sample in various time points after excitation. This method is also called the pump-probe technique. After splitting, pump and probe are sent to the sample through different paths. Pump of desired wavelength goes through the chopper which blocks every other pulse enabling comparison between excited and non-excited state of the sample. Probe is a white

light continuum and before reaching the sample it goes through a delay line, to achieve the resolution in time, and it is split into two beams; reference and probe. Probe beam overlaps with the pump beam while reference beam does not. After the beams have passed through the sample they are directed to the detector where the transient absorption spectra can be generated. Transient absorption is calculated as a difference between absorbance of excited sample (pumped) and absorbance of non-excited sample (unpumped), see equation 7 where I_{ref} stands for intensity of reference beam, I_{pp} for pumped probe beam and I_{up} for unpumped probe.

$$TA = \log\frac{I_{ref}}{I_{pp}} - \log\frac{I_{ref}}{I_{up}} \quad (7)$$

Transient absorption carries the information about dynamics of the investigated sample. Both negative and positive signal can be observed. The negative signal is a result of ground state bleaching. After stimulation of sample with pump pulse, some molecules are excited into higher states and therefore the amount of molecules in the ground state decreases in comparison with non-excited sample. Positive signal appears as a result of excited state absorption because excited molecules can absorb photons of another wavelengths than molecules in the ground state (Chábera, 2010; Keşan, 2016).

4 Results

4.1 Complex formation

Following the first protocol of sample preparation mentioned in the previous chapter, altogether 15 samples with 5 different salts were prepared. Their spectra were measured at various time points. Four of the samples, namely those using $CuSO_4$, $Ca(ClO_4)_2$ and $Zn(ClO_4)_2$ and $FeCl_2$, showed significant changes in their absorption spectra and will be shown in detail below. Samples containing cadmium chloride did not show any changes in any salt concentration therefore will not be mentioned any further.

4.1.1 Copper

Samples with three different concentrations of copper sulphate were prepared according to Table 2. The first measurement of each concentration was taken on the same day as the sample was prepared, but it did not show any changes. First changes were observed on the next day when the absorption maximum, for all of the samples, was shifted to the red compared to pure astaxanthin. Sample with the highest salt concentration (10 mM) was shifted by 6 nm while the less concentrated samples (1 mM and 0,5 mM) were shifted by 8 nm. Three days later both colour and absorption spectrum of the most concentrated sample changed considerably leading to the conclusion that the astaxanthin was most likely degraded with such a high amount of copper. Both less concentrated samples showed even more pronounced red shifts: 1mM by 16 nm and 0,5 mM by 11 nm. These changes remain stable for a long time (months) if kept in a fridge. Since the absorption maximum of the sample with 1mM concentration was shifted the most, only this one was further analysed. The changes of absorption spectra of 1 mM sample are shown in Figure 7.

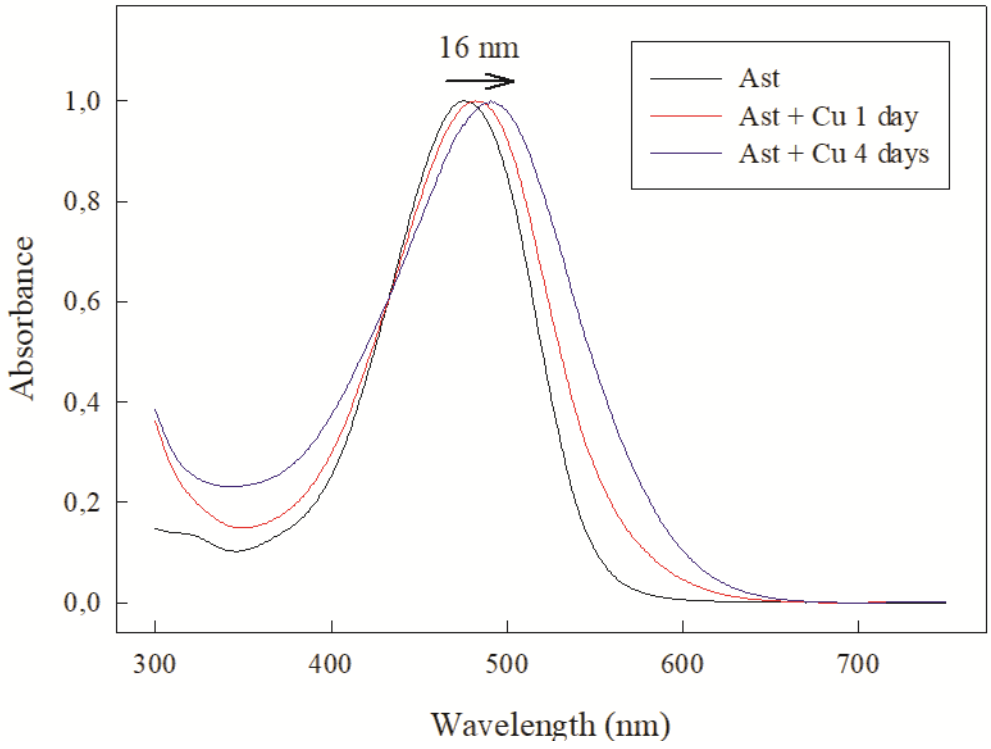

Figure 7. Absorption spectra of the sample with astaxanthin and copper (1 mM) measured at different times after preparation. Spectra are normalized.

4.1.2 Calcium

Samples with three different concentrations of calcium perchlorate were prepared according to Table 2. After three days absorption spectra were measured for each sample with the following results. The only sample, where a significant red shift was observed, was the most concentrated one (10 mM). Its absorption maximum was shifted by 6 nm compared to astaxanthin and it has not shifted any further over time. Both less concentrated samples did not show any changes, neither in 3 days nor in 4 weeks after the sample was prepared. The changes in absorption spectra of 10 mM sample are shown in Figure 8.

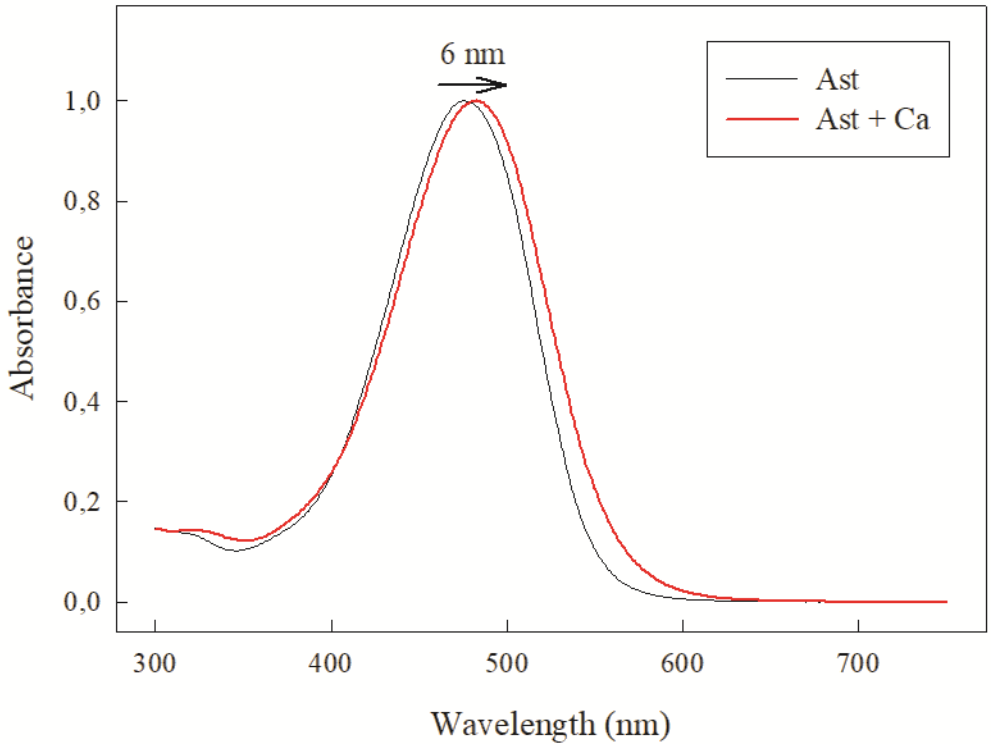

Figure 8. Absorption spectra of the sample with astaxanthin and calcium (10 mM) measured at different times after preparation. Spectra are normalized.

4.1.3 Zinc

Samples with three different concentrations of zinc perchlorate were prepared according to Table 2. After three days absorption spectra of each sample were measured and it has been found that absorption maxima of all three of them were shifted to the red. The absorption maximum of the most concentrated sample (10 mM) was shifted the most, by 11 nm. The sample with 1 mM concentration was shifted by 5 nm and the least concentrated sample (0,5 mM) was shifted only by 2 nm. The absorption maxima of the samples did not stay the same, instead they kept shifting further to the red. Finally, in three weeks both 10 mM and 1mM samples were shifted by 16 nm while the 0,5 mM sample was shifted only by 4 nm. The absorption spectra of the most concentrated sample are shown in Figure 9. In all the samples containing zinc, unlike in the others, red flakes of unknown composition appeared.

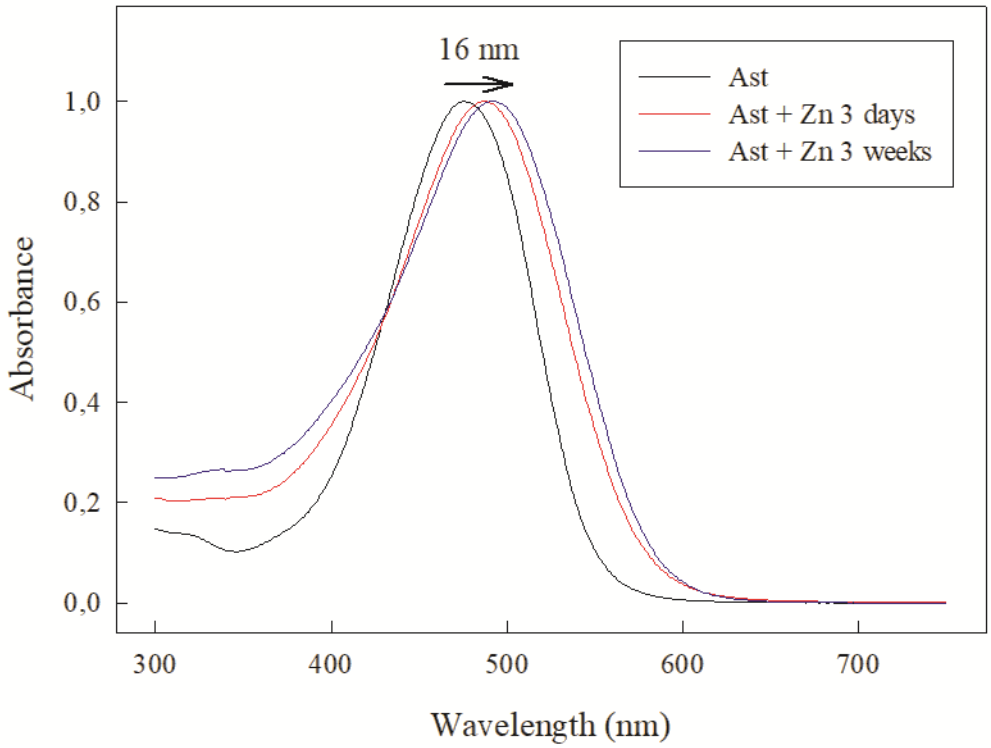

Figure 9. Absorption spectra of the sample with astaxanthin and zinc (10 mM) measured at different times after preparation. Spectra are normalized.

4.1.4 Iron

Samples with three different concentrations of iron chloride were prepared according to Table 2. After three days absorption spectrum of each sample was measured with the

following results. The absorption spectrum of the most concentrated sample (10 mM) changed completely leading to the conclusion that such a high concentration of iron causes degradation of astaxanthin. Neither of the less concentrated samples showed any changes. In three weeks all of the samples seemed to be degraded changing their originally orange colour to yellow.

4.2 Isomerisation

Following the second protocol of sample preparation, mentioned in previous chapter, led to formation of cis isomers of astaxanthin in the presence of copper. The absorption spectrum was measured every hour after the sample was prepared and there were noticeable changes at every time point. The absorption maximum was shifting to the blue. The maximal shift, 20 nm, was observed after two days. In addition to the blue shift, a shoulder around 370-380 nm appeared and was gradually rising over time, see Figure 10. Together with the rising absorbance of the shoulder around 375 nm the absorbance of astaxanthin at 476 nm was decreasing leading to the conclusion that all-trans astaxanthin has been converted to cis astaxanthin. This phenomenon cannot be seen in Figure 10 because the data are normalized.

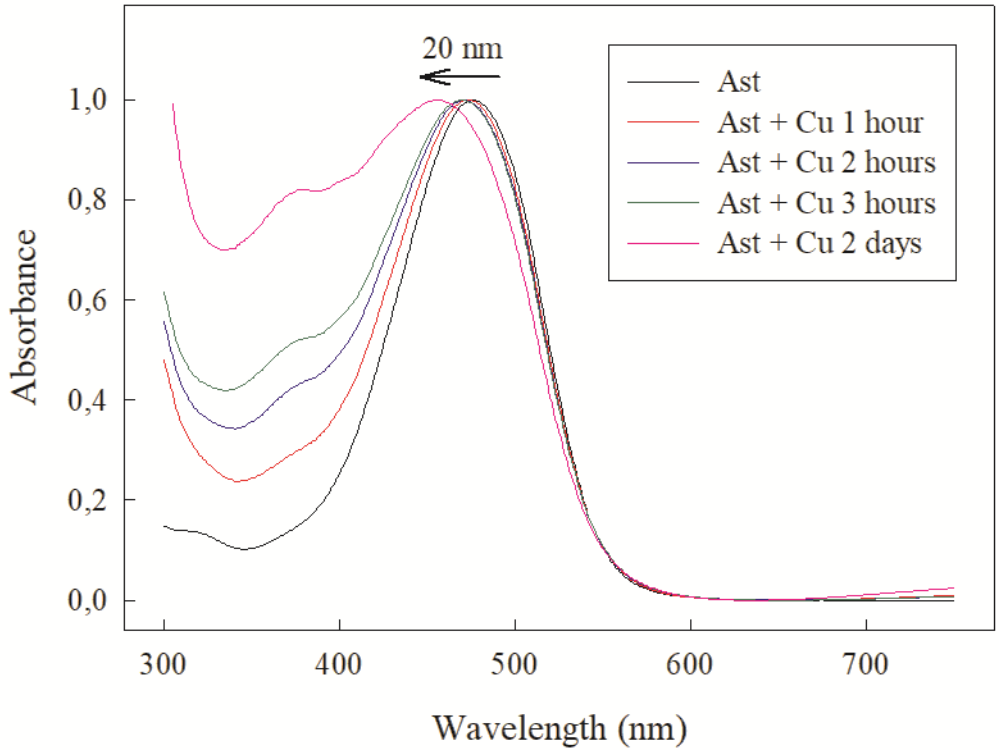

Figure 10. Formation of cis isomers of astaxanthin in the presence of copper measured at different times after preparation. Spectra are normalized.

4.3 HPLC analysis of isomers

The presence of cis isomers of astaxanthin was verified by HPLC with method by Zhao et al. (2005) and the final chromatogram is shown in Figure 11. The highest peak between fourth and sixth minute represents all-trans astaxanthin. Smaller peak which appeared at 6,3 min just after all-trans astaxanthin was identified as 9-cis astaxanthin and the last peak which appeared at 6,7 min was identified as 13-cis astaxanthin. The identification of the two products is based on previous work on astaxanthin (Yuan and Chen, 1997, 1999; Zhao et al., 2005).

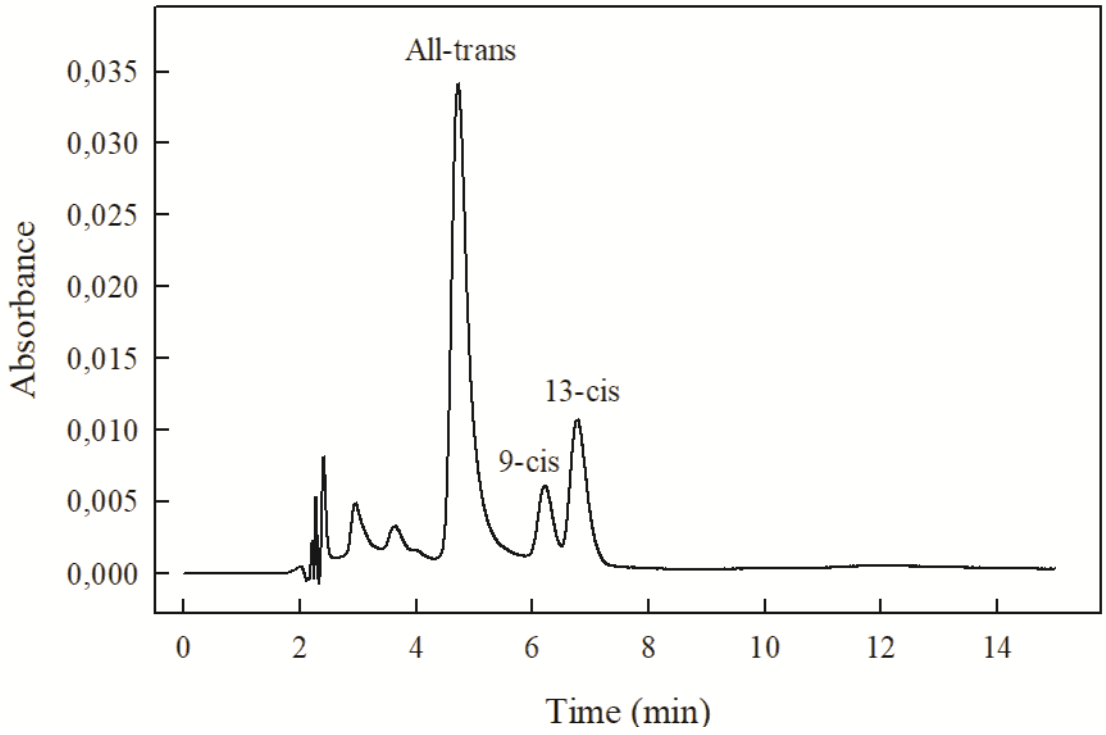

Figure 11. Chromatogram of the sample with isomers.

All-trans astaxanthin absorbs the most at 478 nm. For the 9-cis astaxanthin the absorption maximum is blue shifted by 9 nm compared to all-trans astaxanthin and there is a little bump around 370 nm. The 13-cis astaxanthin has two distinct peaks in the absorption spectrum. The higher one is blue shifted by 12 nm compared to all-trans astaxanthin and the smaller one, cis-peak, appears around 370 nm. The two peaks that appeared before all-trans astaxanthin, at 2,9 min and 3,6 min have their absorption spectra shifted way to the blue area, therefore it is assumed that they are degradation products of astaxanthin. Absorption spectra of individual isomers are shown in Figure 12.

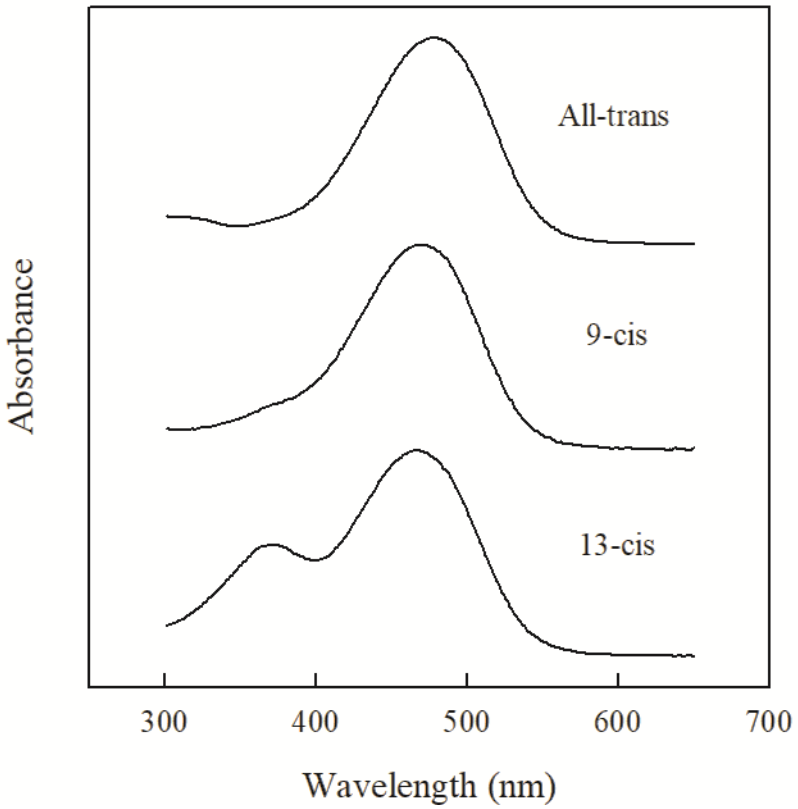

Figure 12. Absorption spectra of all-trans, 9-cis and 13-cis astaxanthin.

4.4 HPLC analysis of complexes

Samples prepared according to the protocol leading to formation of astaxanthin complexes with copper, calcium and zinc were analysed by HPLC. Without any obvious reasons, the analyses of samples with calcium and zinc, were not successful. For the sample with copper a chromatogram was acquired showing several products of complexation, see Figure 13.

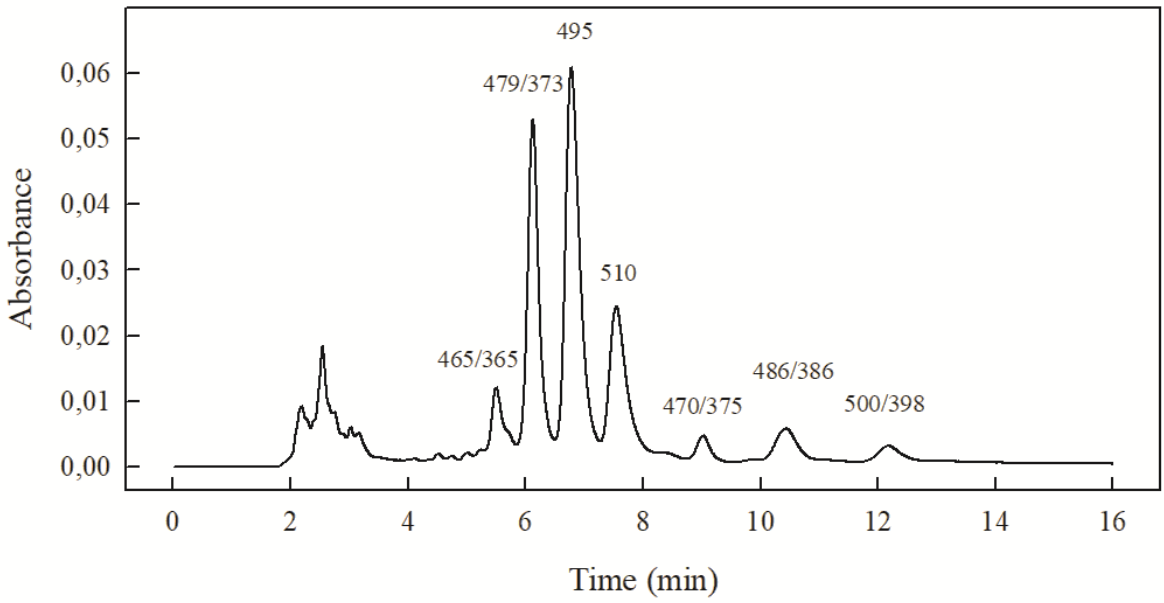

Figure 13. Chromatogram of the sample with astaxanthin and copper (1 mM). The numbers above each peak denote wavelength (nm) of main absorption peaks.

The most red shifted products without any shoulders in UV were the ones eluted at 6,8 and 7,5 min. Complex eluted at 6,8 min had the absorption maximum at 495 nm thus it was shifted by 18 nm compared with all-trans astaxanthin (the absorption maximum of all-trans astaxanthin detected by HPLC was at 477 nm). Complex eluted at 7,5 min was the most red shifted with the absorption maximum at 510 nm thus it was shifted by 33 nm compared to all-trans astaxanthin. The absorption spectra of these two complexes are shown in Figure 14.

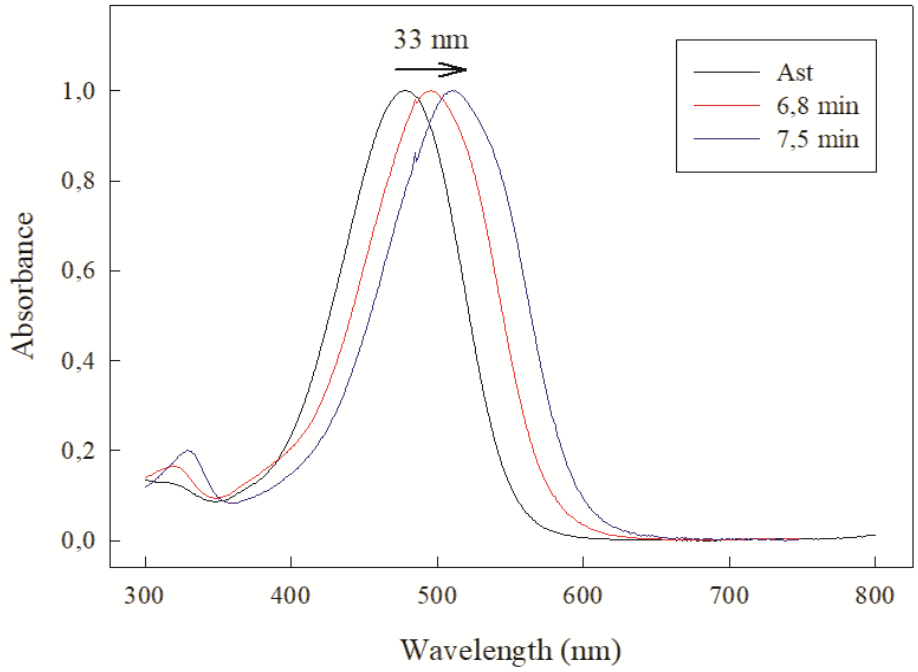

Figure 14. Absorption spectra of complexes eluted at 6,8 and 7,5 min and astaxanthin.

Among rest of the products a shoulder in UV appeared in their absorption spectra. The very first product eluted from the column had an absorption spectrum with two peaks one at 465 nm and another in the UV (around 365 nm). This spectrum is very similar to the spectrum of 13-cis astaxanthin obtained from isomerisation experiment. Another product, eluted at 9 min, had the absorption maximum at 470 nm and a shoulder around 375 nm and it is very similar to the spectrum of 9-cis astaxanthin from the isomerisation experiment. The absorption spectra of these products are shown in Figure 15.

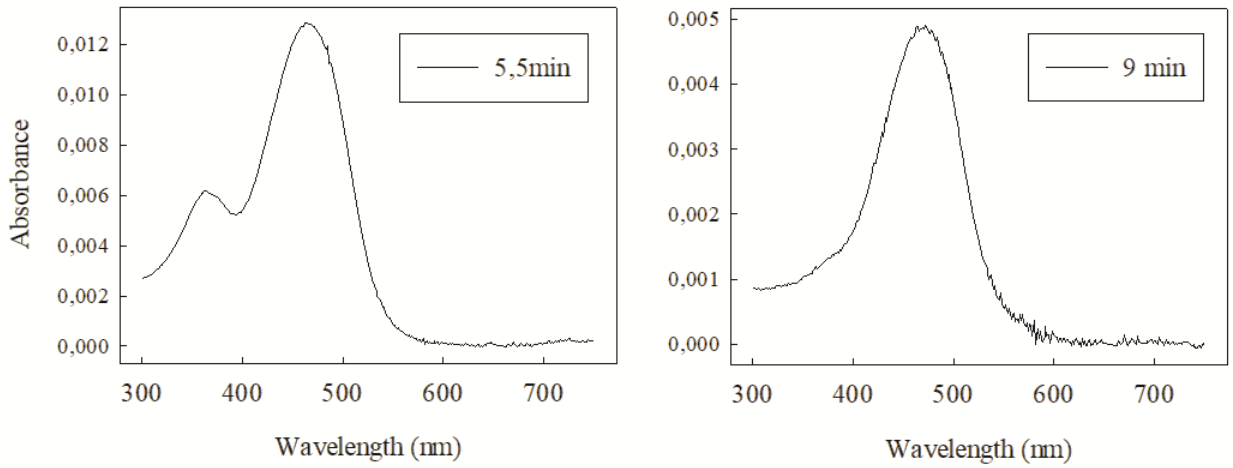

Figure 15. Absorption spectra of products eluted at 5,5 min and 9 min.

The absorption maxima of the rest of the products were shifted to the red in comparison with all-trans astaxanthin and there were shoulders present in UV. The product eluted at 6,1 min had the absorption maximum at 479 nm and the shoulder appeared at approximately 373 nm. The product eluted at 10,4 min had the absorption maximum at 486 nm and the shoulder appeared at 386 nm. The last product, eluted at 12,2 min, had the absorption maximum at 500 nm and a broad shoulder around 398 nm. Absorption spectra of these products are shown in Figure 16.

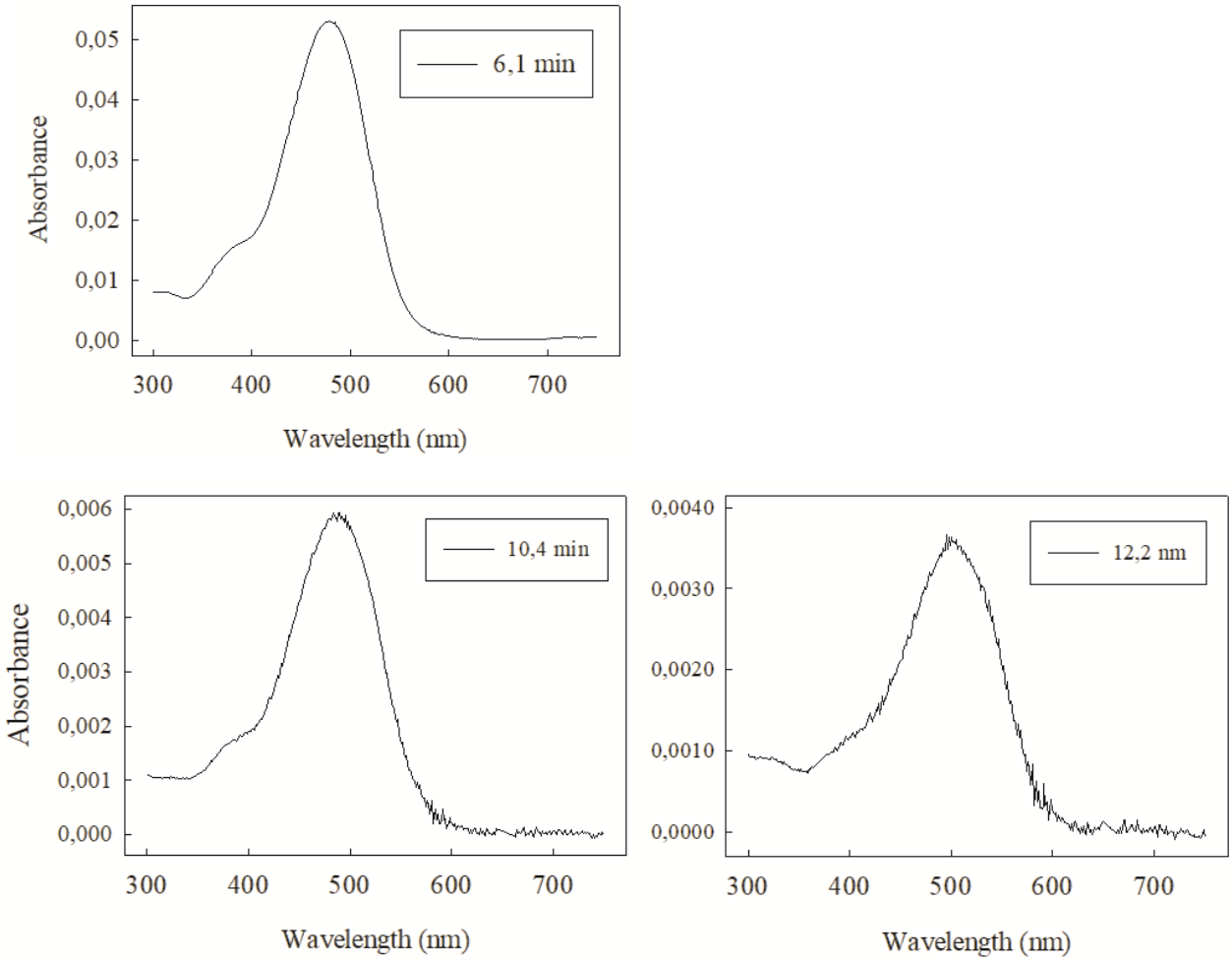

Figure 16. Absorption spectra of the products eluted at 6,1; 10,4 and 12,2 min.

4.5 Transient absorption spectra of astaxanthin-copper complexes

Femtosecond transient absorption was measured on the sample with astaxanthin and copper (1 mM) containing complexes as verified by HPLC. Spectra were measured at 1 ps after excitation at 520 nm in case of the complex and at 500 in case of pure astaxanthin. The S_1-S_n maximum of the complex peaks around 650 nm, thus is red-shifted by nearly 25 nm compared to pure astaxanthin in methanol. Moreover, in the transient absorption spectrum of the complex another band, around 800 nm, is observed (see Figure 17). This band is most likely due to an intramolecular charge transfer (ICT) state induced by binding of copper.

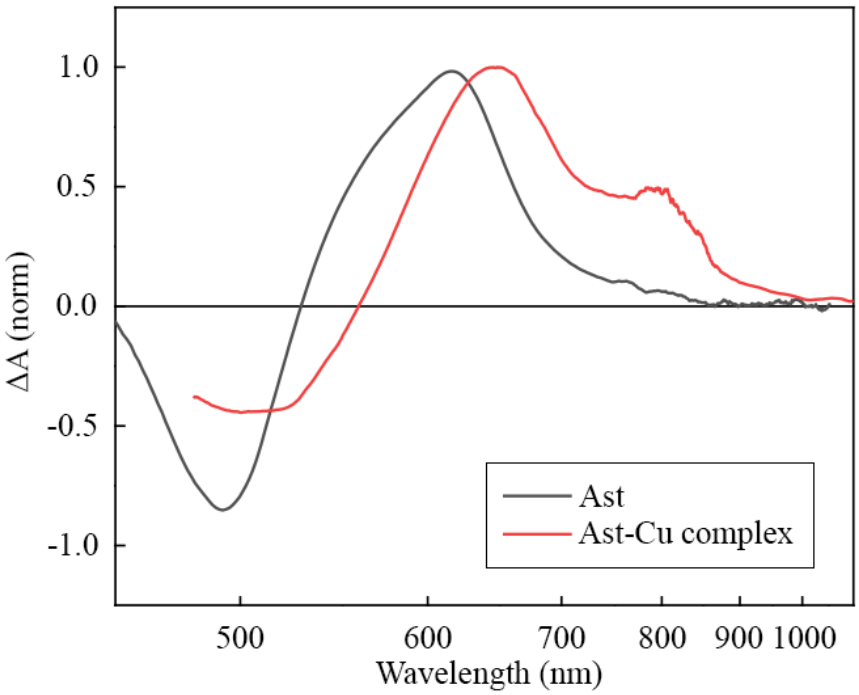

Figure 17. Transient absorption spectra of astaxanthin and its complex with copper. Spectra are normalized to maximum.

Another important outcome in femtosecond spectroscopy is the information about ultrafast dynamics corresponding to the excited-state dynamics. Kinetics, which describes decays of excited states in time, are shown in Figure 18 and correspond to a decay of the astaxanthin S_1 state. Pure astaxanthin, which has been excited at 500 nm, decays monoexponentially with 5 ps time constant whereas the life-time of the Ast-Cu complex is shorter due to presence of copper. The dominant decay component has a lifetime of 1,9 ps but there is also a minor component with 3,5 ps life-time.

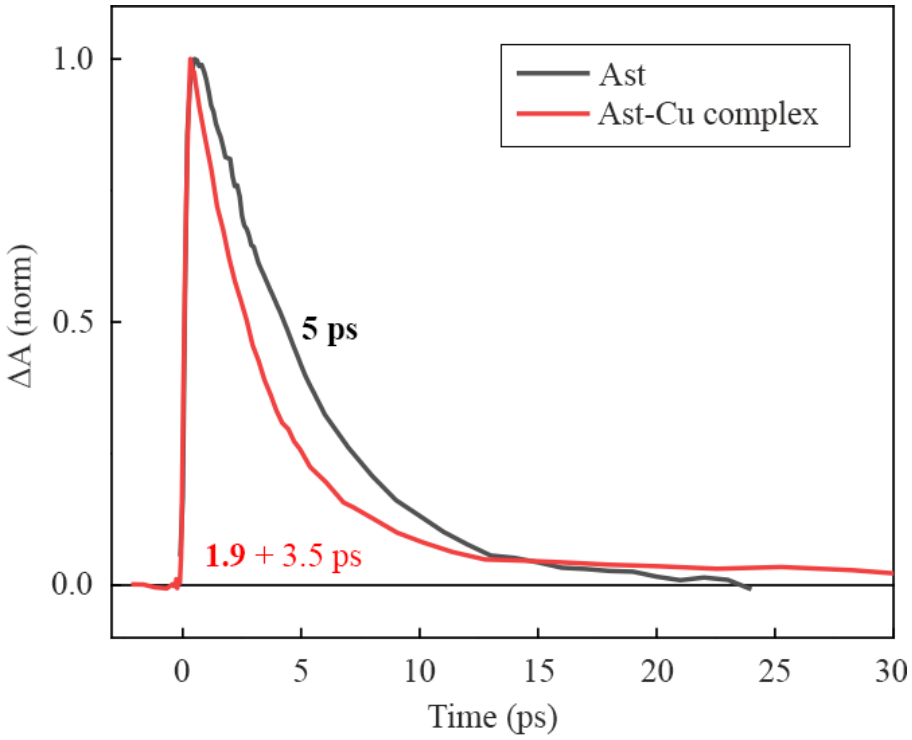

Figure 18. Decay of the S_1 state of pure astaxanthin and its complex with copper. Kinetics are normalized to maximum.

5 Discussion

Many scientists have been lately concerned with the idea of how does astaxanthin behave in the presence of metals. Some of them were using computational modelling (Hernández-Marin et al., 2012) whereas others were experimenting and characterizing the outcomes with various methods such as UV-Vis spectroscopy, HPLC, nuclear magnetic resonance spectrometry or mass spectrometry (Chen et al., 2007; Polyakov et al., 2010; Zhao et al., 2005). The objective of this thesis was to form complexes of astaxanthin with various metals. It turned out that the preparation of such complexes is not an easy task, because the preparation procedure is poorly described in the literature. In the beginning, protocol involving heating the samples to the temperature of approximately 45 °C was used. Although this protocol led to red shifts in some samples, in case of the sample containing copper it led to inconsistent results. First it showed a pronounced red shift, but when replicated it showed a blue shift with a shoulder around 375 nm. The blue shift in the presence of copper was also observed by Polyakov et al. (2010) and Zhao et al. (2005). Using this protocol it was not possible to obtain the red shift anymore, therefore various conditions were modified including light, temperature, solvent and salt concentration. Finally, using a different source of methanol and stored in the dark at room temperature, the red shift was observed again with copper and with both calcium and zinc as well. In the end, two protocols, as described in methods, were used each consistently leading to the same results, therefore the experiments are replicable.

5.1 Complexation

Formation of complexes of astaxanthin with copper, calcium and zinc was accomplished whereas the same attempt with iron and cadmium failed, although according to the theoretical study, the formation of complexes with these metals should be possible as well. When cadmium is present, it seems that astaxanthin does not react with it at all, whereas when iron is present, the absorption spectrum changes completely. The peak of astaxanthin disappears and it is replaced by a broad peak rising at 700 nm going all the way to the UV, peaking around 350 nm. The change can also be observed by eye, when the orange colour of astaxanthin in methanol changes to yellow. To the possible better outcome, the protocol for sample preparation could be further modified or metals in other than chloride form could be used.

5.1.1 Red shifts

The maximal shift of the samples containing calcium was by 6 nm, it was observed very soon, and it has not changed any further. Similar red shift in the presence of calcium, by 4 nm, was reported by Chen et al. (2007) while Polyakov et al. (2010) reported more pronounced red shift, around 12 nm. The theoretical study by Hernández-Marin et al. (2012) is predicting the Ast-Ca complex to shift by 43 nm (if there is just one calcium ion bound on one ring) or by 29 nm (if there are two calcium ions bound, one at each ring). These values seem to be very overrated, but it must be kept in mind that they are computed for individual complexes while what was measured is probably a mixture of various compounds that contains both pure astaxanthin and complexes. The composition of this sample was not managed to be analysed by HPLC to confirm the presence of complexes.

More red shifted were the samples with copper and zinc both equally shifted by 16 nm. The shifting of the sample containing zinc took longer than with calcium or copper. Moreover, little red flakes appeared on the bottom of the vials. Similar red shift, around 12 nm, was reported by Polyakov at al. (2010) and the theoretical study by Hernández-Marin et al. (2012) is predicting the Ast-Zn complex to shift by 76 nm (one zinc ion bound) or by 60 nm (two zinc ions bound). Observed red shift more or less corresponds with what was reported by Polyakov et al. (2010) but the results of the theoretical study seem to be overrated.

Finally, the sample with copper was shifted by 16 nm and the shifting was quicker than with zinc. This red shift was not observed yet. Instead a blue shift with formation of cis isomers of astaxanthin was reported by Polyakov et al. (2010) and Zhao et al. (2005). The formation of complexes was again predicted by Hernández-Marin et al. (2012). Ast-Cu complexes should have the highest red shifts of all the analysed compounds. Complex with one copper ion bound should be shifted by 112 nm and complex with two copper ions bound should be shifted by 380 nm. It seems, unlike with calcium and zinc, that complex with 2 copper ions has the absorption maximum shifted more than the complex with one copper ion. However, using another model, swapped values were reported (1 copper ion by 439 nm, 2 copper ions by 116). To sum up, theoretically computed values for shifting of the absorption maxima seem to be highly overrated and whether complex with one ion or two ions of copper is shifted more is not clear.

5.1.2 Concentration dependence

Experiments showed a high dependence of complex formation on concentration of salts in the samples. For each salt, the concentration needed to make the most shifted sample was different. The optimal concentration for calcium turned up to be 10 mM. Less concentrated samples did not produce any changes in the absorption spectra. In the future, even higher concentration of calcium in the sample could be used to find out whether higher concentration would produce more pronounced shifts. For zinc, the optimal concentration was not very strictly defined since both 10 mM and 1 mM produced the same shift. The absorption spectra of the least concentrated sample changed as well, but the shift was not as pronounced. The optimal concentration for copper was 1 mM. Higher concentration resulted in degradation of astaxanthin while lower concentration did not produce such a pronounced red shift.

5.1.3 Dependence on the source of the ions

The complex formation is most likely dependent on the type of compound from which the salt ions come from. In case of calcium both Chen et al. (2007) and Polyakov et al. (2010) used calcium perchlorate ($Ca(ClO_4)_2$) as a source of calcium ions. The same salt was used in this work and the results were similar. In case of zinc, Polyakov et al. (2010) used zinc perchlorate ($Zn(ClO_4)_2$), again, the same salt as in this work and similar results were observed. To produce copper complexes different compounds were used. Polyakov et al. (2010) and Zhao et al. (2005) used copper chloride ($CuCl_2$) as a source of copper ions and no red shifts were reported. Blue shift with formation of cis isomers was observed instead. In this work copper sulphate ($CuSO_4$) was used resulting in both red and blue shift under different conditions. In case of complexation of astaxanthin with iron, iron perchlorate ($Fe(ClO_4)_2$) was used by Polyakov et al. (2010) and a red shift was reported, whereas in this work iron chloride ($FeCl_2$) was used resulting in degradation of astaxanthin instead of red shift. Cadmium used in this work was also in a form of chloride and it did not produce any changes. Chlorides have not proven to be effective in complexation of astaxanthin with metals, therefore using iron and cadmium in a different form (e. g. perchlorate or sulphate) should be tested in the future.

5.2 Isomerization

In the presence of copper, at elevated temperature, all-trans astaxanthin was readily converting into its cis isoforms. This process is well described in the literature by Polyakov et al. (2010) and Zhao et al. (2005), therefore there is no doubt that isomerization is what was observed in this work. Data measured by UV-Vis spectroscopy are also supported with the data obtained from HPLC. What remains unclear is, where is the balance between isomerization and complexation, in the presence of copper. The temperature is most likely not the only factor because the isomerization observed by Zhao et al. (2005) was carried out at 4 °C. It is reported though that higher temperature promotes isomerization of astaxanthin (Yuan and Chen, 1997, 1999). Another factor might be the source of copper, copper chloride was used by Polyakov et al. (2010) and Zhao et al. (2005) while copper sulphate was used in this work. The most important factor may be the source of solvent. Methanol used for complexation was HPLC gradient grade whereas in the previous experiments, involving isomerization, methanol of lower quality was used. Therefore there was probably more water in the samples and it has been proven in our work, that water is breaking down the Ast-Cu complexes.

5.3 HPLC

The interpretation of chromatogram of Ast-Cu complexes was not an easy task because the complexation of astaxanthin with copper has not been reported yet. Compounds, relatively easy identified, were eluted at 5,5 and 9 min. They were based on their absorption spectra, identified as 13-cis astaxanthin and 9-cis astaxanthin, respectively. The succession of peaks (13-cis eluted first, 9-cis eluted later) is reversed in comparison with chromatogram of isomerisation products, but since a different column and eluents were used the results are not contradictive.

All the other compounds were red shifted in comparison with all-trans astaxanthin. The shape of the absorption spectra of the compounds eluted at 6,8 and 7,5 min was similar to all-trans astaxanthin, but the absorption maxima were red shifted. Therefore, they were identified as all-trans complexes of astaxanthin, one with one copper ion on one of the terminal rings, another one with one copper ion bound to each terminal ring.

Remaining three compounds exhibited a red shift and a shoulder in UV. The shape of the absorption spectra was very similar to the absorption spectrum of 9-cis astaxanthin, therefore

it is assumed that the compounds are cis isomers of produced complexes eluted at 6,8 and 7,5 min. The compound eluted at 6,1 min has the second highest absorption, therefore it is probably more stable than the other two cis isomers, and may be the 9-cis isomer of the all-trans compound eluted at 6,8 min which has the highest absorption. The compounds eluted at 10,4 and 12,5 min have way lower absorbance and may be the cis isomers of the compound eluted at 7,5 min which exhibits lower absorbance as well. Furthermore, compound eluted at 12,5 min has the absorbance maximum shifted further than the all-trans complex eluted at 6,8 min, therefore cannot be its cis isomer.

Based on this observation, compound eluted at 6,8 min was identified as all-trans Ast-Cu complex with one copper ion on each terminal ring and compound eluted at 6,1 min as its 9-cis isomer. Compound eluted at 7,5 min was identified as Ast-Cu complex with only one coper on one of the rings and compounds eluted at 10,4 and 12,2 min as its 9-cis isomers. There are two 9-cis isomers because 9-cis astaxanthin is 'bent' asymmetrically and copper ion can be bound either to the ring on the shorter or longer part of the molecule.

This identification of products is only preliminary and may not be entirely correct, but is the best possible based on the acquired data. For better description of individual compounds generated by the complexation process, their separation in HPLC would be essential. This has proven to be nearly impossible in this work. Although the absorbance of the sample containing complexes measured by UV-vis spectroscopy was high enough, the absorbance of individual compounds eluted from HPLC system was very low. Possible reason for this behaviour is that the complexes were breaking down in the column. To solve this problem, more concentrated sample was made and various methods and columns were used, however the improvement was not good enough. If the separation of the compounds was successful in the future, the individual compounds could be measured by femtosecond spectroscopy or mass spectrometry to find out more about their structure and behaviour.

5.4 Transient absorption

Transient absorption spectra were measured on the Ast-Cu complexes. This sample was chosen because it produced the most pronounced red shifts. Thanks to the chromatogram obtained from HPLC analysis it was known that the transient absorption spectra were measured on a mixture of compounds. Therefore the excitation wavelength (520 nm) was chosen to hit predominantly the red shifted compounds (complexes). The main purpose of this

experiment was to find out whether complexation of astaxanthin with metal can promote activation of the ICT state, which has never been observed in transient absorption spectra of pure astaxanthin. The transient absorption spectrum of Ast-Cu complex contains a new band occurring around 800 nm that can be associated with the presence of the ICT induced by the presence of the Cu ion. This conclusion is further supported by shortening of the excited-state lifetime of astaxanthin in Ast-Cu complex, because increased charge-transfer character is typically accompanied by shortening the lifetime of the lowest excited state (Polívka and Sundstrom, 2004).

6 Summary

Complexes of astaxanthin with calcium, zinc and iron were formed. All of these were measured by UV-Vis absorption spectrophotometry at various time points to acquire information about the evolution in time. Observed absorption spectra were compared with the data in the literature. Complexes of astaxanthin with calcium showed the least-shifted absorption spectra and the attempts to assess composition of the sample with HPLC failed. Complexes of astaxanthin with zinc exhibited more pronounced red shift of absorption spectra, but the HPLC analysis was not successful either. Complexes of astaxanthin with copper have proven to be the most successful, producing a pronounced red shift in the shortest time. Composition of this sample was determined by HPLC and the main compounds were identified. Furthermore, the transient absorption spectra were measured, therefore the information about ultrafast dynamics of the Ast-Cu complexes were acquired and ICT state was observed. Furthermore, presence of copper, under slightly modified conditions, induced isomerization of all-trans astaxanthin into its cis isoforms. This phenomenon was verified by HPLC.

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