DOCTORAL (PH.D.) THESIS

Qualitative Analysis of Natural and Semisynthetic Carotenoids by Liquid Chromatography

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I. Introduction

The carotenoids have not only aesthetic role, but have also antioxidant property, therefore they play a serious part in prevention of some diseases.

The most consumed foodstuff and food matrices contain some kind of carotenoids, among these the most important is the ß-carotene, as a vitamin-A precursor. In the retina zeaxanthin and lutein accumulate and they have role also in the prevention of the age related macular degeneration. Other carotenoid molecules have got general antioxidant properties.

They are absorbed with fats, carried with lipoproteins, stored in the liver and in adipose tissue. Main dietary carotenoids are the ß-carotene, α-carotene, lycopene, ß-cryptoxanthin, lutein and zeaxanthin. Through the foodstuff preparation techniques these structurally sensitive compounds may suffer degradation (oxidation, isomerization, decomposition). With the modern analytical methods these changes can be followed. At the Department of Biochemistry and Medical Chemistry of the University of Pécs there is a tradition on the carotenoid analysis and chromatography of different plant parts. At the end of the 1920s, the founder of our department, László Zechmeister and his research group developed already a column chromatography method for the analysis of plant extracts. With the improvement of the science and technology, we have now modern analytical devices as well.

Since the analysis of carotenoids are important as well in the field of nutrition science and also in health care, I proposed in my PhD. study to compare the diverse analytical methods in carotenoid analysis and to find the most appropriate analytical method for certain problems.

II. Aims of the study

- The comparison of the HPLC separations of carotenoids by C_{18} and C_{30} columns, their efficiency in the analysis of different structures (analysis of hydrocarbons, polar functional group containing molecules, structures with κ, ε and ß end groups, stereoisomers, epoxy carotenoids, geometrical isomers).

- To find the most appropriate qualitative analytical method for complex natural plant extracts containing a large number of carotenoids with diverse structures. To identify not only the main components of complex extracts, but also the minor carotenoids.

- Separation of configurational isomers of carotenoid 5,6-epoxides and, with application of chiral stationary phase, the determination of the absolute configuration. The separation of stereoisomers of the non-hydroxylated end-group compounds bearing epoxide.

III. Materials and methods

Reagents and standards

HPLC grade solvents were bought from Scharlab Ltd (methanol, acetone, *terc*-butyl-methyl-ether). Some part of the carotenoid samples were isolated or semisynthetically produced. The isolation of the so called 'rare' substances have been made earlier by our research group, the structural identifications were performed for each substances according to their MS, NMR and CD spectras. The isolations and synthetical modifications were carried out by our research group according to standard methods. Some special substances were supported by CaroteNature Gmbh.

Preparation of semisynthetic carotenoids

The semisynthetic carotenoid 5,6-epoxides were prepared from the corresponding precursor with epoxidation of the double bonds in 5,6-position by monoperphtalic acid. Carotenoid 5,8-epoxides were produced by treatment of the appropriate 5,6-epoxide compound with hydrochloric acid. The oxo carotenoids were prepared by the oxidation of the hydroxy derivatives. Geometrical isomers were obtained in iodine catalyzed photoisomerisation. If it was necessary, compounds prepared by derivatization were purified by classic $CaCO_3$ column chromatography.

Extraction of mamey

The pulp of red mamey fruit from Panama market (Panama City, Panama) was extracted according to standard procedures.

Devices

UV/Vis spectras were measured with Jasco-530 spectrophotometer in benzene.

The ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectras were performed by a Varian UNITY INOVA 400-WB spectrometer and a Bruker DRX Avance II (500/125 MHz for $^{1}H/^{13}C$) spectrometer.

High performance liquid chromatography (C_{18}, C_{30})

The HPLC analysis were carried out by Dionex P680 analytical pump, Dionex PDA 100 *UV/Vis* detector (Thermo Fisher Scientific, Inc. Waltham, MA), Dionex column thermostate and evaluated by Chromeleon 6.8 softver. The exact HPLC method can be found in the following chart.

HPLC conditions

I. Method	250 x 4.6 mm column, LiChrospher 100 RP 18e (Merck KGaA) 5 µm endcapped	flow: 1.25 ml/min eluents: A: H_2O/MeOH = 12/88 v/v% B: MeOH C: Acetone/MeOH = 50/50 v/v% 22 °C λ=450 nm	0-2 min 100 % A, 2-10 min to 80% A/20%B, 10-18 min to 50 % A/50% B, 18-25 min to 100 % B, 25-27 min to 100 % B, 27-33 min to 100 % C, 33-38 min 100 % C, 38-40 min to 100 % B (in linear steps)
II. Method	250 x 4.6 mm column, YMC C_{30} (YMC Europe GmbH) 3 µm endcapped	flow: 1.00 ml/perc eluents: A: MeOH/TBME/H_2O = 81/15/4 v/v% B: MeOH/TBME/H_2O = 6/90/4 v/v% 22 °C λ=450 nm	0-90 min 100% A/0% B to 100% B (in linear steps)

HPLC-MS analysis

HPLC-MS measurements were performed by a Dionex P580 NDG pump, on a 250 × 4.6 mm i.d. YMC C30, 3 µm particle size column. MS spectras were measured with the help of a Finnigan AQA single quadrupole mass spectrometer. The ion source was APCI, in positive mode, the adjustments of the device were: 4 kV corona voltage, 400 °C temperature, full scan mode, data processing 0.2 scan/s.

Chiral HPLC-DAD analysis

The analysis were carried out on a Dionex system (Thermo Fisher Scientific, Inc., Waltham, MA), P680 gradient pump, column termostate and PDA-100 detector, data were processed with Chromeleon 6.7 softver. For chiral separation 250 x 4,6 mm, 3µm Chiralcel OD (Daicel, Chemical Industries, Ltd.) column was used.

Eluents-**1**: (A) MeOH/EtOH (1:1); (B) MeCN/EtOH (1:1). Linear gradient from 100% A eluent to 100% B, in 30 minutes with 1 ml/min flow, on 28 °C.

Eluents-**2**: n-hexane/EtOH (1:1) isocratic elution with 0.5 ml/perc flow, on 28 °C.

HPLC-CD analysis

The analysis were performed on Jasco HPLC system, HPLC-UV and -OR chromatograms were measured with a Jasco-910 UV and OR-2090Plus chiral detectors. The HPLC-ECD curves were recorded with a Jasco-810 spectropolarimeter.

IV. Results

4.1. Comparison of C_{18} and C_{30} stationary phases

In the case of C_{18} stationary phase we used a formerly developed method, which is well known for paprika carotenoids (Method I.).

The elution order of carotenoids on C_{18} column follows the polarity order: the carotenes, keto- and epoxicarotenes absorb more stronger than the hydroxy-compounds. These relative polarity order is determined by the partition between methanol-petrol ether system.

The C_{30} stationary phase method (Method II.) was improved from a described system. However, the polarity of the eluent also lessen here, the elution order of the analyte molecules does not follow the polarity order on this stationary phase.

Minor differences in the molecular structure result in significant distinctions in the retention times for both stationary phases.

4.1.1. Separation of hydrocarbons

While on C_{18} phase the baseline separation could not be evolved, on C_{30} stationary phase the differences in the retention times of hydrocarbons are minutes.

The stronger interactions between the triacontyl groups of the stationary phase and the carotenoid molecules seems to be verified in the case of nonpolar carotenes. Very little disparity in the cyclohexene ring, such as the position of a double bond, causes important change in the retention times: Carotenoids with ε end group give shorter retention times than that with ß end group. The open chain molecules can take extended conformations and are able to interact with the stationary phase on a larger surface. The length of the conjugated polyene system also correlates with the retention parameters: increase in the number of conjugated double bonds results in longer retention times on C_{30} stationary phase. The extended conjugation of π bonds represents a high electron density, which is able to participate in stronger non-bonding interactions with the triacontyl chains of the stationary phase.

4.1.2. Separation of polar carotenoids

The difference in the retention times of oxo- and hydroxy-carotenoids is small on C_{18} phase, but on C_{30} this difference is significant. In the case of carotenoids having exclusively hydroxyl substituents the retention times decrease by the increasing number of hydroxyl groups on both stationary phases. **On a C_{30} stationary phase, however, the elution order does not follow the polarity of the carotenoids bearing epoxy group.** Epoxides and mainly diepoxides show shorter retention times on a C_{30} phase than it can be expected from their polarity. It suggest that the polar and bulky epoxide ring restrains the diffusion of the molecule in between the non-polar triacontyl chains, so these molecules are partially crowded out from the stationary phase. Because of the non-bonding interactions on the smaller surface area formed, these molecules eluted earlier than the monoepoxides and non-epoxides. Oxo-carotenoids elute later than hydroxy-carotenoids on both phases. The hydrogen bond acceptor oxo-group containing carotenoids spent longer time on the stationary phases than carotenoids with hydroxyl group being both acceptor and donor for hydrogen bonding.

4.1.3. Separation of κ-carotenoids

Carotenoids with kappa end group contain five-membered rings which can bear hydroxyl or oxo functions. On a C_{18} stationary phase, increase the number of polar functional (oxo and hydroxyl) groups results in a decrease of the retention times of κ carotenoids as well. On a C_{30} phase the epoxy κ-carotenoids show again shorter retention times than it is expected from their polarity. **Both phases give rather good separation for κ-carotenoids, however, C_{18} phase seems to be more suitable for the separation of complex natural extracts containing both capsorubin (75) and cryptocapsin-5,6-epoxide (69).**

4.1.4. Separation of carotenoids with ε and ß end groups

The ε and ß end groups bearing carotenoids differ in the position of one double bond in the cyclic end group. The α- and ß-carotene (**2**,**1**) or α- and ß-cryptoxanthin (**11**, **10**) could not be eluted to baseline separation on C_{18} column, but they separate well on C_{30}. Lutein (**14**) and zeaxanthin (**16**) - the macular pigments - showed absolutely identical retention times on octadecyl bonded silica, however, they could be separated excellently on C_{30} phase, and lutein (**14**) is eluted before zeaxanthin (**16**). As we mentioned before, **carotenoids with ε end group give shorter retention time than the ß end group bearing ones on C_{30} column. Both non-**

polar and polar carotenoids proved that the length of the conjugated polyene system influence the retention properties of carotenoids on triacontyl bonded silica: the more π-electrons conjugated, the longer the retention time is.

4.1.5. Separation of stereoisomers

Although C_{18} and C_{30} stationary phases are not chiral, separation of several stereoisomers can be achieved by them. **The steric position of the hydroxyl groups means a greater driving force for the separation on C_{18} column but this mission can hardly be performed on a C_{30} column.**

Processing raw plant materials can cause epimerization of the 3' hydroxyl group of lutein (**14**) and this way 3'-epilutein can be formed (**15**). These diastereomers can be nicely separated both on C_{18} and C_{30} stationary phases, though their elution order is different on the two silica phases. 3,5,6-trihydroxy-ß end-group theoretically has four stereoisomers, three of which occur in Nature. The absolute configuration of this end-group also reveals the mode of formation. Separation of these isomers is important and give information for their biosynthesis. The three natural stereoisomers of karpoxanthin (karpoxanthin (**19**), 6-epikarpoxanthin (**20**) and 5,6-diepikarpoxanthin (**21**)) do not really separate on C_{30} stationary phase but they do on C_{18}. 4-Hydroxy-carotenoid stereoisomers (e.g. 4-hydroxy-ß-carotene (**12**), 4,4'-dihydroxy-ß-carotene (**17**)), obtained by the reduction of the oxo function of carotenoids with six-membered end-groups (echinenone (**59**), canthaxanthin (**63**)), cannot be separated either on C_{18} or C_{30} bonded silica, but only on chiral stationary phases. Reduced hydroxy derivatives of 6-oxo-κ-carotenoids differ only in the steric position of the hydroxyl group, still they can be separated on both stationary phases. On C_{18} they elute 2 min far from each other, on C_{30} column this difference is only 0.8 min. Similar retention properties can be observed in the cases of other reduced κ-carotenoids.

4.1.6. Separation of epoxy-carotenoids

Carotenoid-5,6-epoxides occur together with 5,8-epoxides (furanoids) in petals and pollens of various flowers, as well as in fruits and in all green leaves. Some of them are bioprecursors of plant hormone, abscisic acid, others are involved in the xanthophyll cycle, which has a protective role against excessive illumination.

In nature the stereochemistry of the epoxide ring is *anti* compared to the steric position of the hydroxyl group on the cyclohexyl end group. Epoxidation of carotenoids with

percarboxylic acids **yields *anti* and *syn* epoxides, which can be separated on C_{18} stationary phases. The retention time of the *syn* isomers proved to be shorter than that of the *anti* compounds.**

Furanoid formation is catalyzed *in vivo* by acids which are present in plants and/or enzymatic effects, but during food processing the amount of furanoids also increases, as the cells are destroyed and their acid content is liberated. **5,8-epoxides with at least two hydroxyl groups separate excellently from their parent 5,6-epoxides on octadecyl bonded silica. However, epoxides with or without only one hydroxyl group cannot be separated on C_{18} phase. Triacontyl bonded silica at the same time gives very good separation for these less polar regioisomers.** On C_{30} stationary phase the more extended conjugation of double bonds was proved to give longer retention time in general. As mentioned earlier, in the case of 5,6-epoxides the bulkiness and polar character of the rigid epoxide ring may hinder the diffusion of the molecule in between the non-polar triacontyl chains of the stationary phase, which leads to shorter retention as expected from polarity. Here, the epoxide group seems to have more influence on the retention than the length of the conjugated polyene system: although 5,8-epoxides contain less conjugated double bonds than 5,6-epoxides, furanoids elute later than their regioisomer 5,6-epoxides. **Separation of 5,8-epoxide stereoisomers can be achieved only on C_{18} phase, if both six-membered cyclic end groups bear hydroxyl group.** Furanoid molecules containing only one hydroxyl group, or without hydroxyl group on the six membered end group cannot be separated on these stationary phases. The separation of these compounds can be performed on chiral stationary phases. In spite of the very similar properties, 5,6-epoxide and 3,6-epoxide regioisomers separate well on C_{18} column, but they do not on C_{30}.

4.1.7. Separation of geometrical (*Z*/*E*) isomers

Food processing techniques, especially heating, can lead to cis-trans (*Z*/*E*) isomerization of double bonds of carotenoids. It is important to follow the ratio of (*Z*/*E*) isomers and its changes in food samples.

On C_{18} phase the resolution of geometrical (*Z*/*E*) isomers of carotenoids is rather limited, mainly in the case of nonpolar carotenes, only polar compounds can be separated and the elution order succeeds as all-*E* < 9*Z* < 13*Z* < 15*Z*. For polar carotenoids the all-*E* isomer separates well from the *Z* isomers, but the separation of *Z* isomers from each other is poorer. **C_{30} stationary phase seems to be ideal for the separation of less polar carotenoids (hydrocarbons, monohydroxy compounds) and their *cis-trans* isomers. The elution order of geometrical isomers is 15*Z* < 13*Z* < all-*E* < 9*Z* on triacontyl bonded silica**. On C_{30}

stationary phase the retention times seem to be effected by the length of the carotenoid molecule: 15*Z* and 13*Z* isomers have 'bent' shape, which makes the molecule more sphere-like, and they look shorter than the all-*E* or 9*Z*-izomers.

4.2. Applications

Our research group isolates and analyses carotenoids from very diverse natural sources. In this study the usage of the above discussed stationary phases are introduced through some examples, the application of these methods are demonstrated on the analysis of several plants.

With the cooperation of University of Pécs Faculty of Pharmacy, Pharmacognosy Department the carotenoid content of two herbs, the Canadian goldenrod (*Solidago canadensis L.*) inflorescence and the greater celandine (*Chelidonium majus L.*) flower were analysed. The analysis of Canadian goldenrod achieved on C_{18} phase, which showed (*9Z,9'Z*)-lutein, namely neolutein C, and (9*Z*)- or (9'*Z*)-lutein as main components. After the partition of the total extract, only ß-carotene (**1**) as apolar carotene could be detected in the hexane epiphase. However in the methanolic hypophase, besides the *cis*-compounds we found lutein 5,6- (**43**) and 5,8-epoxides (**44**/**45**). With the help of $CaCO_3$ column chromatography of the hypophase, the minor components (neoxanthin (**56**), neochroms (**104**, **105**), violaxanthin (**47**), luteoxanthin (**50, 51**), (9*Z*)-anteraxanthin, zeaxanthin (**16**)) could also be identified. The main carotenoid in greater celandine was lutein 5,6-epoxide (**43**), in addition 5,8-epoxide (**44**/**45**), violaxanthin (**47**) and *cis* isomers of lutein-5,6-epoxid (**37**) were found. Apolar carotene could not be found in the extract.

In Hungary, there is a tradition of the pumpkin consumption, and many baby foods contain pumpkin as well. During our work the carotenoid content of fresh and cooked pumpkin samples were analysed on C_{18}- and C_{30} stationary phases. These pumpkin species were (Nagydobosi (*Cucurbita maxima*); Halloween, a fodder species (*Cucurbita pepo*); orange, or Canadian pumpkin (*Cucurbita moschata*) and the Japan Hokkaido (*Cucurbita maxima* Duchesne *ssp.*) species) raised or distributed in our country. It can be concluded according to our examinations, that the more and more popular orange species contains a minimal quantity of α-cryptoxanthin (**11**) and some α- (**2**) and β-carotene (**1**). The other three species contain higher amount of lutein (**14**) and other polar components, e.g. cucurbitaxanthin (**46**) as well. Because of the isomer formation, the effect of heat treating procedures (frying, cooking) can be examined on C_{30} stationary phase, especially in case of the orange pumpkin.

The well-known source of κ end group bearing carotenoids (capsanthin (**71**), capsorubin (**75**) etc.) is the red paprika, which has been investigated for almost 90 years in our department.

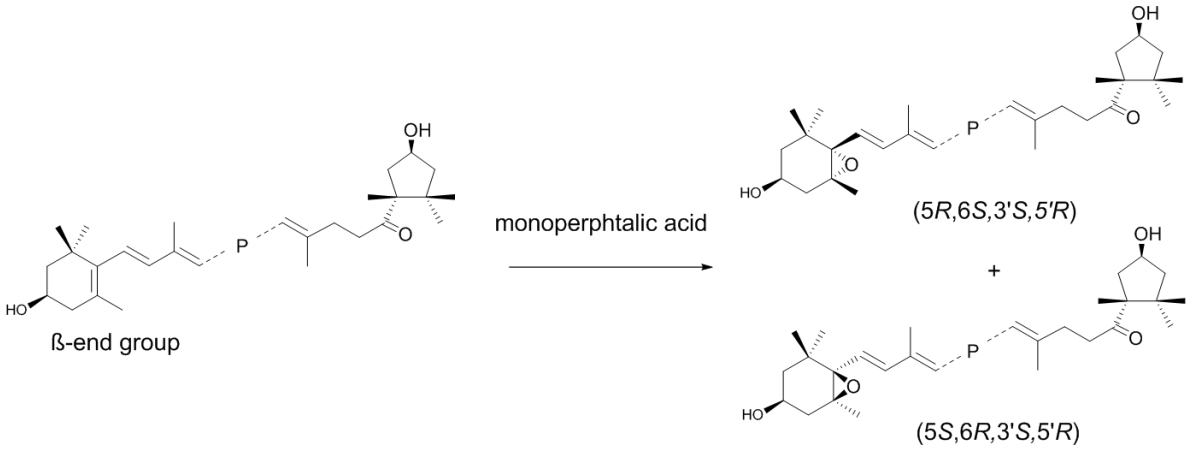
Numerous red paprika species were analysed on C_{18} and C_{30} stationary phases. Consequently the polar components in red paprika elute close to each other on C_{30} column, but they give better resolution on C_{18} phase.

The situation is reverse in the case of a Central American fruit, the red mamey (*Pouteria sapota*), which is an extremely rich κ-carotene source. The carotenoid content of this fruit does not seem to be complex on C_{18} stationary phase, until the analysis on C_{30} column shows a very rich chromatogram. However many components can be identified with the analysis of the total extract (29), numerous smaller peaks, which were mainly a mixture, stayed unidentified. To found these, the total extract were chromatographied on $CaCO_3$ column and gave seven fractions. The HPLC-DAD-MS analysis of these fractions were also executed on C_{30} column. The binding of carotenoids to $CaCO_3$ adsorbent are different than to silica gel, this way the components which covered by each other can be separated and identified as well.

4.3. The analysis of non-hydroxylated end group bearing carotenoid

The chiral separation and structural identification of carotenoid epoxides from different plant sources were also carried out. If the hydroxyl- and epoxide groups are on the same end group in the carotenoid, the two stereoisomer (*syn* and *anti*) would separate from each other on C_{18} stationary phase. In turn the diastereomer pairs which do not contain hydroxyl group can be separated only on chiral HPLC column.

To facilitate the identification of them, we prepared the semisynthetic compounds with monoperphtalic acidic treatment of the appropriate carotenoid. During the epoxidation a diastereomer pair formed, the reaction is demonstrated on the example of capsanthin:



When the steric position of the epoxide- and hydroxyl groups are the same is called *syn* (5*S*,6*R*,3'*S*,5'*R*), otherwise we are talking about *anti* (5*R*,6*S*,3'*S*,5'*R*) compound.

Cryptocapsin 5,6-epoxides (**69**, **119**), cryptocapsin 5,8-epoxides (**120**-**123**), ß-cryptoxanthin 5',6'-epoxides (**33**, **127**), ß-cryptoxanthin 5,6,5',6'-diepoxides (**36**, **124**-**126**), as well as ß-carotene 5,6-epoxides (**128**, **129**) and the sapotexanthin 5,6-epoxides (**130**, **131**) do not contain hydroxyl group on the epoxide containing ß-ring. These compounds were isolated from the reaction mixture, analysed with chiral chromatography method and structurally identified.

V. Summary

During my work, the behavior of 100 different carotenoid was investigated on C_{18} and C_{30} stationary phases, with two, practically used eluent system.

Octadecyl bonded silica phase gives good separation for the whole polarity range of carotenoids. The elution order on this stationary phase follows the polarity order of carotenoids. C_{18} phase enabled for the separation among very polar carotenoids and the non-polar carotenes. This phase could separate better hydroxy- and keto-carotenoids than carotenes, and did not allow good separation between the regioisomers of carotenoids (such as the pairs zeaxanthin (**16**) and lutein (**14**), or antheraxanthin (**37**) and lutein-5,6-epoxide (**43**)). The resolution of geometrical (*Z*/*E*) isomers is rather limited. In case of polar carotenoids the 9*Z*- and 13*Z*-isomers separate from each other (in the case of asymmetrical compounds 9/9' and 13/13' pairs are not separated), apolar compounds, especially carotenes do not separate from the *trans* structures. In spite of these, it is an excellent method for the separation stereoisomers of polar carotenoid (karpoxanthin epimers (**19**-**21**), capsanthol epimers (**78**-**79**), *syn* and *anti* epoxide pairs, etc.). Consequently the C_{18} reversed phase liquid chromatography seems to be a very suitable method for the conditional, retention time based identification of carotenoids in complex systems.

Fine analysis can be obtained by separation of natural extracts on a C_{30} stationary phase as well. The C_{30} stationary phase seems to be ideal for separation of apolar carotenes (hydrocarbon and monohydroxy-carotenoids) and their *cis*-*trans* isomers. This phase allows a very good separation between molecules with very similar structures (the position of one double bond, geometrical isomerism). However, the polar carotenoids are separated with poor resolution on C_{30}. The effect of hydroxy and epoxy groups for elution order is not clear, thus, the retention time of such compounds is not appropriate for the prediction of the structure of a carotenoid in a complex mixture.

The two chromatography methods were demonstrated on the example of analysis of extracts from natural sources. Thus, the usage of the C_{30} method for apolar and the C_{18} phase for polar carotenoids could be proved.

However, both columns are suitable for distinguish the *syn* and *anti* stereoisomers of hydroxylated 5,6-epoxide end group containing carotenoids, neither of them are able to separate the non hydroxylated epoxides. To solve this problem, we used chiral stationary phases. We prepared several semisynthetic diastereomer pairs (cryptocapsin 5,6-epoxide, ß-carotene 5,6-epoxide, ß-cryptoxanthin 5',6'-epoxide, ß-cryptoxanthin 5,6,5',6'-diepoxides) to facilitate the

identification of this type of carotenoids in mamey fruit. These components of mamey could be determined by our separation method using HPLC-CD technique.

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