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Review

Isolation and Analysis of Carotenoids in Hungary from Zechmeister until Today

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Abstract: László Zechmeister, one of the most important pioneers of carotenoid chemistry, died 50 years ago. He founded a carotenoid research group in Pécs (Hungary), which is the only place in the world where carotenoid research has been conducted continuously over the past 95 years. This review presents the life of Zechmeister and gives a summary about the evolution of the methods of analysis, isolation, and structure elucidation of carotenoids from the 1930s until today, based on the results of the research group founded by Zechmeister.

Keywords: carotenoids; column chromatography; HPLC; detection; isolation

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1. Introduction

Fifty years ago, on 28 February 1972, László Zechmeister (Figure 1), one of the most important pioneers of carotenoid chemistry and classical column chromatography, passed away. As members of the Hungarian carotenoid research group founded by Zechmeister, we consider it of great importance to commemorate him and his achievements. This paper deals with him as a dedicated scientist and his work on carotenoids. Further, we provide an overview of the progression of classical carotenoid research.

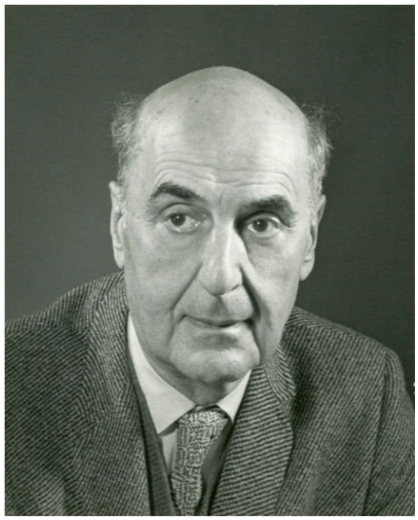

Figure 1. László Zechmeister, 14 May 1889 (Győr, Hungary)—28 February 1972 (Pasadena, CA, USA).

This review provides the possibility to show comprehensive details of the development of carotenoid isolation and analysis over the years. Despite the lack of modern analytical devices, Zechmeister's team achieved many novel findings. With the evolution

of instrumental techniques, the analytics evolved at a great pace, as well; however, some separation methods which were developed by Zechmeister and co-workers are still used in our laboratory in the same form as they were 90 years ago.

2. Historical Overview: The Life of László Zechmeister and His Research Group

Many scientists today know little about László Zechmeister or his work, yet his books [1–4] and scholarly publications were widely read and cited during the 1930s to the 1950s. He was one of the researchers who rediscovered chromatography and demonstrated its use for separating and isolating complex natural pigments.

László Zechmeister was born on 14 May 1889 in the city of Győr, a town in the northwest of Hungary. He received his primary education in Győr, and he finished high school in 1906. In 1907, Zechmeister commenced his studies in chemistry at the Swiss Federal Institute of Technology (ETH) in Zurich, where he followed lectures and enjoyed the guidance of the future Nobel Prize laureate Richard Willstätter. He received his degree as a chemical engineer in 1911. When Willstätter left for Berlin in 1912, Zechmeister followed him and worked as his assistant from 1912 to 1914. During this period, he wrote his doctorate thesis about cellulose and lignins [5].

It was Willstätter who sparked his interest in the investigations of natural products such as chlorophylls and carotenoids, a subject which he never gave up. Willstätter was the first to determine the correct molecular formulae of some plant carotenoids, thus placing a key in the hands of carotenoid researchers for further investigations. Zechmeister admired his professor (he kept a photo of him above his desk), and he often said, citing the work style of Willstätter, "to achieve the maximum possible result, we have to wish for the impossible".

His scientific career was interrupted when World War I began. Zechmeister was enlisted in the Hungarian army and had to fight on the front line, where he was injured twice. He was taken captive and sent to a Russian prison camp in Siberia for three years. At the end of the war, he left the prison and came home to Hungary. Due to post-war chaos, he could only find temporary positions, such as the work of a chief scientific officer at the Chinoin pharmaceutical factory in Hungary, where he conducted industrial chemical research. At the same time, he worked at a veterinary college with György Hevesy. He was offered a teaching appointment at the Royal Danish Agriculture and Veterinary Academy in Copenhagen, where he worked as an instructor and scientific assistant of Niels Bjerrum in 1922–1923.

In 1923, Zechmeister was offered a professorship and directorship of a chemistry laboratory at a medical school in the newly established Erzsébet University in Pécs. This was, of course, a great honor, as Zechmeister was only 33 years old, and there had never been such a young person holding a position such as that in Hungary before [6]. The university, as an institution, had operated in earlier years in Pozsony (today Bratislava). In respect to the equipment, everything stayed in Bratislava, and only the name was transferred to the new university. Zechmeister was mainly involved in organizational work, such as the building process of the premises and the laboratories and the hiring of scientific staff. In a short time, he developed his department into an effective teaching and research institute. He had the ability to collect brilliant colleagues around him, who played an important role in educational and research work, and who later became prominent in academic circles. His colleagues in the department worked in different fields of chemistry, but he had the greatest passion for carotenoid research. In the preface of his book, "Carotinoide" [1], Zechmeister wrote: "It would be an understatement to say that carotenoids are just a special class of natural colorants and put them into a system. On the one hand, the peculiar structure poses new tasks to the organic chemist and on the other hand, the vast distribution of these polyene pigments in a large number of different organisms gives a strong indication that these compounds are essential for life". Zechmeister soon became a respected teacher and a worldwide acknowledged authority in carotenoid research. By the end of the 1930s, he

had gained several awards and a well-deserved reputation in Europe, as well as in the US. He was often invited to teach at various universities.

As an authority in the field of chromatography and a well-known expert of research on pigments and stereoisomers, Zechmeister was also the founder of the book series *Progress in the Chemistry of Organic Natural Products* and was its editor until 1969 [7]. The first volume was inaugurated in 1938, and, remarkably, the series has not yet completed with the recent publication of the 118th issue. Although in former times the series was simply referred to as "Fortschritte", when German was still the language of science, it is now also known under the short form "Zechmeister", in memory of its founder, or simply as "Progress".

The prosperous years of scientific research were again interrupted by political events. The outbreak of the World War II in 1939 prompted Zechmeister to leave Hungary. He accepted an invitation by Linus Pauling to work at the Gates and Crellin Laboratories of Chemistry at the California Institute of Technology in Pasadena, where he continued his work on pigments and carotenoids. In 1959 László Zechmeister became professor emeritus at the Department of Organic Chemistry at CalTech. Until this time he had published 254 scientific papers with 87 co-authors. Nevertheless, he remained active even after his retirement. In 1962, he published a monograph on *cis* and *trans* isomeric carotenoids [4], which is very useful even today. Zechmeister received many honours during his life, including the Medaille Pasteur from the Biochemical Society (Paris), the Medaille Claude Bernard, the Grand Prize of the Hungarian Academy of Science, the Semmelweis Medal of the American-Hungarian Medical Association, and the American Chemical Society's Award on Chromatography and Electrophoresis. He was an honorary member of the Hungarian Academy of Science and that of the Austrian Chemical Society. He was a foreign member of the Royal Dutch Academy of Science, and in 1971 the University of Pécs Medical School conferred on him an honorary M.D. degree.

He continued teaching and researching until the summer of 1971 when he fell ill and passed away on 28 February 1972 in Pasadena. As he requested, his ashes were scattered into the ocean near Los Angeles.

After Zechmeister left, the carotenoid research group in Pécs was led by László Cholnoky, head of the Chemistry Department since 1946. Cholnoky was ten years younger than Zechmeister, and he was enrolled as a student of pharmacy at the University of Budapest and showed a great interest in analytical chemistry already during his studies. He obtained his Ph.D. in pharmacy in 1924, and then he moved to Pécs and started to work as Zechmeister's assistant. In 1940 he was promoted to a department professor. Prior to World War II he spent some months in Jena (Germany), where he studied the theoretical and practical questions of optical measurements, and later he stayed at Graz with F. Pregl to learn methods of organic microanalysis. Cholnoky also played a very active role in the university community; he was a vice dean, vice rector, and finally the rector of the university between 1964 and 1967. He was awarded the Kossuth Prize in 1959 and became a corresponding member of the board of the Hungarian Academy of Sciences in 1960. His sudden death on 12 June 1967 was a great shock for everybody.

After the death of Cholnoky, József Szabolcs became the head of the carotenoid research group and later also head of the department. He graduated as a chemist at the Loránd Eötvös University (Budapest) in 1951 and then started to work at the Department of Biophysics of the University of Pécs, and he joined the Department of Chemistry in 1954.

In 1990 József Szabolcs unexpectedly retired, and Gyula Tóth became the new head of the carotenoid research group. He completed his studies as a medical doctor at the University of Pécs in 1961. After his graduation he began to work with László Cholnoky, and, at his suggestion, the name of the department was changed in 1991 to the Department of Medical Chemistry. In 2002 the Department of Medical Chemistry was fused with the Department of Biochemistry to become the Department of Biochemistry and Medical Chemistry, hosting other groups for the research of analytical chemistry and biochemistry. In 2004 Gyula Tóth retired, and since then József Deli has been leading the carotenoid research group.

3. Advances in the Chromatographic Separation of Carotenoids

In this paper we give a summary on the evolution of the methods of analysis, isolation, and structure elucidation of carotenoids from the 1920s until today, based on the results of our research group. The field of analytical research of carotene pigments covers isolation and characterization. Determination of the carotenoid content of a biological sample requires first sampling and sample preparation, carotenoid extraction, separation, identification, quantification, and validation of the used methods. Today, for carotenoid analysis, high-performance liquid chromatography (HPLC) and spectrophotometry are the most frequently used methods.

To establish the identity of isolated or synthesized compounds and to characterize their structure (configuration and conformation), a versatile repertoire of techniques is available: UV/VIS spectrometry, infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR), mass spectrometry (MS), and X-ray crystallography.

It should be noted here that most of the research of Zechmeister was conducted without the help of modern methods. For structure elucidation he had to rely only on the absorption spectra of carotenoids and the changes of those on simple chemical transformations, such as reduction, acylation, and reactions with acids. Zechmeister and co-workers developed the hydrogenation of carotenoids, determined the number of double bonds in the molecules, and confirmed the structures by catalytic hydrogenation of some carotenes (1928–1933). They realized the polyene structure, which is characteristic for carotenoid pigments, and introduced the term "Polyene" [8–10].

3.1. Open-Column Chromatography

It is difficult to determine the exact time when Zechmeister started using the column chromatography technique, but he certainly played a major role in the expansion of its use from the 1930s and onwards. Already in 1934 in his book *Carotinoide* [1], Zechmeister dealt in detail with the principles and applications of chromatography. The researchers in Pécs were the first to realize the importance and possibilities of the application of this method in organic chemistry. It is highly remarkable that, although Willstätter himself did not consider chromatography an important tool, it was his student who brought back this forgotten method.

In the 1930s Zechmeister and his colleagues developed chromatography into a high-performance preparative separation method for organic compounds (Figure 2). They applied calcium carbonate powder as a stationary phase, and the coloured carotenoids could be well-seen on the column. The relation between their chromatographic behaviour and their structure could easily be recognized [11–13]. There is a lot of information about the retention properties of carotenoids, especially on normal phases such as calcium carbonate, which still comes from the era of Zechmeister and Cholnoky.

In 1937 Zechmeister and Cholnoky published the book *Die Chromatographische Adsorptionsmethode*, which was the very first handbook on chromatography [2]. As Leslie L. Ettre, the former editor of the journal *Chromatographia*, stated: "This was the right book published by the right people at the right time, and it became an instant best-seller. In fact, it was such a success that within one year a second, greatly enlarged edition was published" [6]. It was later translated into English and Russian and reprinted many times. With this method at hand, Zechmeister and Cholnoky had great success in the isolation and the determination of the constitution of carotenoid dyes and their properties.

Based on their own methods, they reported many novel findings: the isolation and structure elucidation of paprika dyes capsanthin and capsorubin [14–21], lycophyll, and lycoxanthin [22,23]; alkaline degradation of capsanthin to β-citraurin [24] (Figure S1); photometric and colorimetric determination of carotenoids [25]; and the investigation of different flowers and fruits (*Solanum dulcamara* [26], *Tamus communis* [27], *Lycium halimifolium* [28], *Calendula officinalis* [29]). They established the occurrence of zeaxanthin dipalmitate (physalien) in Physalien berries, and the occurrence of different capsanthin esters in paprika [18].

Figure 2. Column chromatography from around 1930 and from the present day.

After the leaving of Zechmeister in the 1940s, the intensity of publication slackened, and the next articles were published only in the 1950s [30]. However, these articles were the result of tedious work and presented important results: this was the first time when Cholnoky reported the physiological importance of carotenoids in plants and supported it with plant analytical data [30,31]. He emphasized again the importance of carotenoid epoxides as possible oxygen carriers in photosynthesis [32].

In the following years, using column chromatography Cholnoky and his co-workers discovered new carotenoids: α-cryptoxanthin (1958) [33,34] and cryptocapsin (1963) [35]. During those years the department had been lacking modern equipment. The determination of carotenoid spectra was carried out using a Löwe–Schumm grating spectroscope, and they measured the extinction rates of the different wavelengths one by one. They made a table of the results, and then they drew the spectrum on paper.

The fruitful organic microanalysis was based on this precise but tedious work, which led to the structural revision of some compounds. Cholnoky and his co-workers determined the structure of capsanthin and capsorubin in 1960 [36,37], at the same time as Paul Karrer (Zürich) [38] and Basil C. L. Weedon (London) [39]. These compounds possessed an interesting new feature among carotenoids, since instead of a trimethyl-cyclohexan ring they contain a trimethyl-cyclopentan ring (Figure S1). However, in the structure determination Karrer and Weedon enjoyed priority, as they already had IR equipment at their disposal.

The Hungarian research group was engaged in the Oppenauer oxidation in carotenoids, as well. They elaborated the $LiAlH_4$ reduction of carotenoid-epoxides and furanoids [40]. In a collaboration with Weedon's group, using the modern instruments of his department, they investigated the mass-spectrometry of carotenoid epoxides and furanoids [41]. In addition, they determined the structure of neoxanthin, carotenoids which can be found in higher plants containing allene bonds [42–44] (Figure S1).

In 1967 József Szabolcs led the carotenoid research group in Pécs. The group continued fruitful collaboration with a research group in London [45–48]. As a result of this important cooperation, a basic article about Optical Rotatory Dispersion of Carotenoids was published in 1969 [49], which allowed the determination of the absolute configuration of carotenoids. During this period of time the investigation of 5,6- and 5,8-epoxides, including their *cis* isomers, was continued [50–60]. While still using classical column chromatography, József Szabolcs, Gyula Tóth, and Péter Molnár isolated a huge number of naturally occurring carotenoid 5,6- and 5,8-epoxides (antheraxanthin, violaxanthin, lutein-5,6-epoxide, flavoxanthin, chrysanthemaxanthin, etc.), most of which derived from petals. They also studied the occurrence of *cis*-isomers in Nature. The main mono-*cis* isomers of some symmetric (zeaxanthin, capsorubin) and asymmetric (lutein, capsanthin, antheraxanthin, lutein

5,6-epoxide) molecules were prepared by thermal and iodine catalyzed photoisomerization for all-*trans*-carotenoids (Figure S1). For structure elucidation, they had ^{1}H- and ^{13}C-NMR- as well as CD-spectroscopies at their disposal. These results were presented as a plenary lecture on the 8th International Carotenoid Symposium in Boston by József Szabolcs [61].

In the 1990s the focus lay on the study on paprika carotenoids (see HPLC section) and on the further investigation of *cis-trans* isomeration [62–69]. Collaborations developed with the research group of Conrad H. Eugster in Zürich and later with the group of Hanspeter Pfander at the University of Bern.

As the many examples above demonstrate, chromatography on calcium carbonate is an efficient tool for the separation of diverse carotenoids, which is the reason that this type of open column chromatography has been used in our laboratory until today (Figure 2). Even very similar structures, such as configurational isomers, can be separated easily on calcium carbonate. The only disadvantage of this stationary phase is its very small capacity. Only 10–20 mg of carotenoid mixture can be separated on hundreds of grams of calcium carbonate, and a huge amount of eluent is needed, thus this method is of limited use for the preparative scale. To improve the capacity of column chromatography, silica gels can be an obvious choice as a stationary phase; however, their acidic character may induce some undesired transformations of sensitive samples.

A simple alkaline treatment of commercial silica gels can overcome this issue by converting silica to a basic form without lowering the capacity and without any change in retention properties. We demonstrated the affordability of such modified silica gels on acid-sensitive carotenoid epoxides [70]. The resolution of separation on silica is much less effective than on calcium carbonate, but it can be applied successfully for the separation of molecules of considerably different polarity. For instance, carotenoid glycosides from crude extracts of algae can be separated from other carotenoids and chlorophylls very efficiently, even in a gram-scale using modified silica (Figure 3) [71].

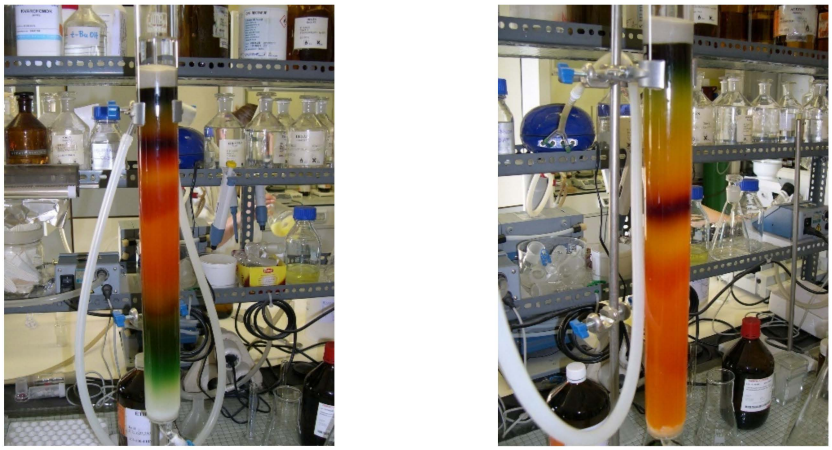

Figure 3. Column chromatography of an alga extract on modified silica gel.

Now, we use and propose the combination of the two methods: modified silica gel can be used for the bulk pre-purification of extracts, and the classical calcium carbonate stationary phase is excellent for further fine purification of the gained fractions.

3.2. Thin-Layer Chromatography

Thin-layer chromatography, as a form of planar liquid-solid phase chromatography, dates back to the late 1950s, when it began to replace paper chromatography. Although in biochemical and analytical laboratories there is now a tendency to forget TLC and to try to solve all problems of carotenoid isolation and identification by HPLC, this simple method remains very useful. Practical application now focuses more on a combination of both TLC and HPLC, rather than TLC alone. For preliminary tests of biological extracts as well as for monitoring reactions, TLC remains a cheap and indispensable alternative [72–74].

On a TLC plate without any sample preparation, some information can be obtained in a few minutes about the approximate composition of the sample. Additionally, during a chemical reaction, if it is to be monitored quickly, thin-layer chromatography can solve this task. HPLC requires sample preparation, and in the case of a carotene mixture—such as a raw extract—the elution time can be long as well. On the other hand, preparative TLC can be used for the separation of carotenoid extracts or reaction mixtures, as well [75]. Occasionally, only small amounts are available from a natural isolate or reaction mixture, and therefore the isolation or purification cannot be made with column chromatography. In this case preparative TLC offers a fast and inexpensive alternative. For a precise as well as for a qualitative analysis, HPLC-UV is necessary.

3.3. High Performance Liquid Chromatography

The HPLC method was introduced to the research of carotenoids in the 1970s. Since then, a large number of applications of HPLC methods have been published along with the evolution of stationary phases and the detection method. Both normal- and reversed-phase systems are used, either in isocratic or gradient elution modes. Reversed-phase systems are generally preferred over normal-phase HPLC having, e.g., lower column stability and poorer reproducibility of the retention times. In present times, in reversed-phase systems C_{18} and C_{30} chemically bonded phases are used [76–79]. The thirty-carbon group at the stationary phase contacts a high surface area of the carotene molecules, which causes more secondary interactions. As a result, the retention times for C_{30} phases are considerably higher than for C_{18} phases [80].

We investigated and compared the separation of many carotenoids for both types of stationary phases [80]. Our results demonstrate that the C_{30} stationary phase seems to be ideal for the separation of nonpolar carotenes (hydrocarbons, mono-hydroxy compounds) and their *cis-trans* isomers, whereas the separation of these compounds for C_{18} is very poor. Although the C_{18} column did not achieve baseline separation of hydrocarbons, the C_{30} column shows well-resolved separation with retention time differences of several minutes (Figures 4 and S1).

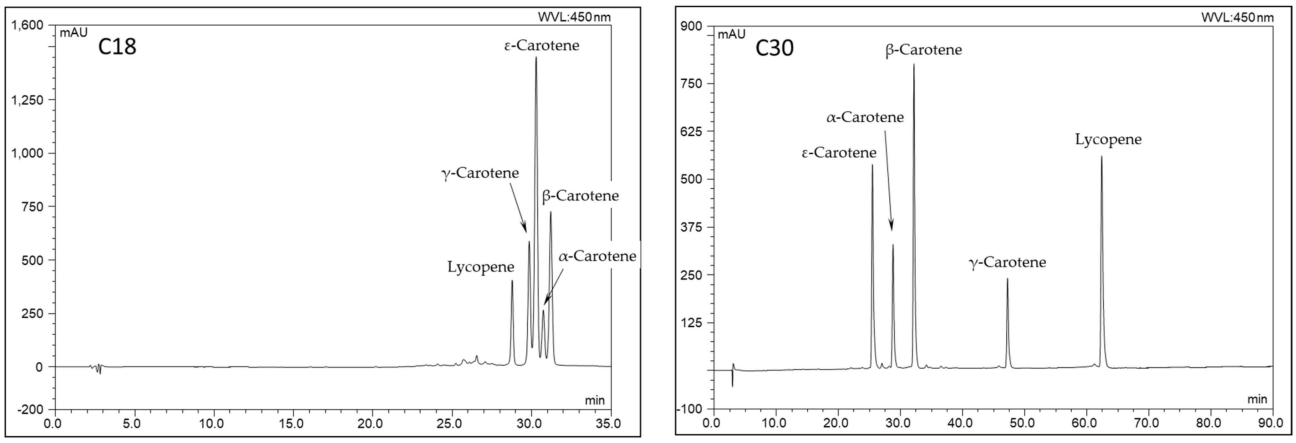

Figure 4. Separation of carotene mixtures for C_{18} and C_{30} reversed-phase columns. C_{18} Conditions: Technokroma C18 5 µm stat. phase (250 × 4.6 mm), 22 °C. Eluents: A: MeOH/H_2O = 12/88 *v*/*v*%, B: MeOH, C: Acetone/MeOH = 50/50 *v*/*v*%. Gradient program: 0′ 80% A, 20% B, 8′ 50% A, 50% B, 17′ 100% B, 15–17′ 100% B, 24′ 100% C, 24–31′ 100% C, 33′ 100% B, 33–34′ 100% B, 35′ 80% A, 20% B. Flow rate: 1.25 cm^3/min; λ = 450 nm; C_{30} condition: YMC C30 3 µm stat. phase (250 × 4.6 mm), 22 °C. Eluents: A: MeOH/TBME/H_2O = 81/15/4 *v*/*v*%, B: MeOH/TBME/H_2O = 6/90/4 *v*/*v*%. Gradient program: 0–90′ 100% B, 95′ 100% A. Flow rate: 1.00 cm^3/min; λ = 450 nm.

However, the C_{18} bonded silica gel phase is great at providing a total carotenoid profile of a natural isolate. The elution order for this stationary phase follows the polarity

order of carotenoids, whereas C_{30} is not appropriate for the prediction of the structure of a carotenoid in a complex mixture.

The C_{18} phase gives better separation in the case of mixtures which contain larger amounts of polar carotenoids, for example, paprika extract. From the middle of the 1980s, using C_{18} HPLC methods, we investigated the carotenoid composition of different kinds and differently coloured varieties of paprika during ripening [81–84]. Our studies showed three characteristic carotenoid profiles. In all unripe (green) fruits of different kinds of paprika, the typical chloroplast pigments, lutein (without esterification) and β-carotene, are the main carotenoids. The ripe yellow paprika whose colour never turns red contains violaxanthin, antheraxanthin, zeaxanthin, lutein, α- and β-cryptoxanthin, and α- and β-carotene as main carotenoids. The red colour of ripe red paprika is due to carotenoids with κ-end group(s). The main carotenoids are capsanthin, zeaxanthin, cucurbitaxanthin A, β-cryptoxanthin, and β-carotene. All hydroxy carotenoids are present as esters (Figure S1).

Our laboratory revealed novel pigments from red paprika using a C_{18} stationary phase. These new carotenoids contained a 3,6-epoxy end group, such as cycloviolaxanthin, cucurbitaxanthin A and B, cucurbitachrome epimers, and capsanthin 3,6-epoxide [85]. We also found novel paprika carotenoids bearing a 3,5,6-trihydroxy end group, such as 6-epikarpoxanthin, 5,6-diepikarpoxanthin, 5,6-diepicapsokarpoxanthin, and 5,6-diepilatoxanthin [86]. During the isolation of these compounds, two carotenoids with interesting 6-hydroxy γ-end groups, namely nigroxanthin [87] and prenigroxanthin [88], were isolated (Figure S1).

Although all isolated compounds were carefully characterized by modern spectroscopic methods (UV-VIS and mass spectrometry, NMR and circular dichroism spectroscopy), they did not give any indication for the configuration at C(6′). Thus, we explained the configuration by the biosynthetic pathway of paprika carotenoids, and the proposed structures with the 6′*S* configuration for nigroxanthin and prenigroxanthin were strongly supported [88].

We have investigated or reinvestigated other plants which contain κ-carotenoids, such as the buds of *Asparagus officinalis* [89], *Lilium tigrinum* [90], *Asparagus falcatus* [91], and flowers of different species of *Aesculus* (horse chestnut) [92]. In this way, two other minor carotenoids were also isolated and, based on their spectral data, identified, namely capsoneoxanthin [93] and (9*Z*)-capsanthin 5,6-epoxide [64]. Capsoneoxanthin contains another interesting allenic end group (Figure S1). Later, capsoneoxanthin was isolated also from red mamey [94].

The C_{30} phase is recommended if the extract contains larger amounts of nonpolar carotenoids, for example, in the case of red mamey (*Pouteria sapota*). Using a C_{18} column, only few carotenoid peaks are visible, whereas on a C_{30} phase 47 peaks can be detected [95]. Based on the HPLC analytical results, we isolated a few interesting carotenoids which contain a non-hydroxylated kappa end group, for instance, sapotexanthin [96], deoxy-capsorubin and dideoxy-capsorubin [97], cryptocapsin epoxides [98], and sapotexanthin 5,6-epoxide [99] (Figure S1).

3.4. Detection Methods for HPLC

The most common method used in the analysis of carotenoids is HPLC, employing various detection techniques. The success of the determination of a compound depends equally on the separation and the detection steps.

Simple UV-VIS detection is by far the most common because carotenoids absorb strongly in the visible region between 400 and 500 nm (all-*trans* isomers), whereas *cis* isomers also exhibit absorption near the UV region, around 320 nm. The detectors are simple and cheap, and photometric detection is readily compatible with gradient elution. Diode array detection (DAD) provides certain possibilities for analyte identification, which however, should not be overestimated. Diode array detection yields spectra sometimes with fine structures. Although the spectra of many carotenoids are very similar (e.g., those of β-carotene and zeaxanthin), the retention time and the spectrum of the peak all together are

informative. In the 1980s, we described a method for identification of aldehydes, ketones, 5,6-epoxides, and 5,8-epoxy groups in carotenoids using simple, known chemical reactions, such as the reduction of sodium borohydride or furanoid rearrangement on acid treatment. These transformations are followed by characteristic changes in the UV-VIS spectra, which are useful in the structure elucidation studies [100].

The main advantage of MS detection is that it enables not only the analyte quantification, but also the elucidation of its structure on the basis of molecular mass and fragmentation. The coupled diode array and mass detection with a known retention time may allow for complete structure elucidation in certain cases. However, there are exceptions, such as in the separation of antheraxanthin and cucurbitaxanthin A on C30. Both compounds have the same retention time, UV-VIS spectra, and molecular mass, thus they cannot be distinguished this way. In this case, a simple chemical derivatization, such as a reaction with acids, is very useful [100]. Antheraxanthin, being a 5,6-epoxide, rearranges with acids and mutatochromes to show a shift in retention time and spectra, whereas cucurbitaxanthin A does not (Figure 5).

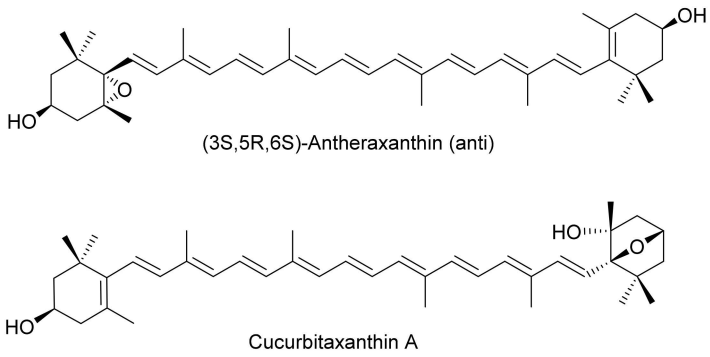

Figure 5. Structures of antheraxanthin and cucurbitaxanthin A.

In our laboratory, the detailed carotenoid analysis of red mamey (*Pouteria sapota*) was achieved by HPLC-DAD-MS, chemical tests, and co-chromatography with authentic samples. Altogether, 47 components were detected and 34 were identified from the total extract or after fractionation with classical column chromatography [95].

On-line *HPLC-CD* detection is a powerful tool which opens new perspectives in the identification of enantiomers or diastereomers of carotenoids. The separation of carotenoid epimers on chiral phases has been known for a long time, but CD (circular dichroism) detection is the newest technique.

During the investigation of mamey carotenoids, we isolated a new compound, cryptocapsin 5,6-epoxide (Figure 6) [98]. This carotenoid was characterized by its UV-Visible spectra, ^{1}H-NMR, mass, and circular dichroism (CD) spectra. As the enantiomeric non-hydroxylated (5*R*,6*S*)- and (5*S*,6*R*)-5,6-epoxy β-end groups cannot be distinguished by the ^{1}H-NMR spectra, the elucidation of the configuration at the cyclohexane ring was based on chiroptical data. To fully characterize the structure of the natural cryptocapsin 5,6-epoxide, it was semisynthetized from cryptocapsin by epoxidation with monoperoxyphthalic acid. The epoxidation reaction always yields two 5,6-epoxy diastereomers with (5*R*,6*S*, natural) and (5*S*,6*R*) absolute configurations. Generally, mainly in the case of an unsubstituted β-ring, the separation of such isomeric epoxides is not easy. In this case, the separation of the two stereoisomers is only possible on a chiral HPLC column. The chromatogram shows that the two epoxides form approximately in equal amounts during the reaction. The CD spectra of the natural (*anti*) and semisynthetic (*syn*) cryptocapsin 5,6-epoxide are opposite, demonstrating the different configuration of the 5,6-epoxy end groups [98].

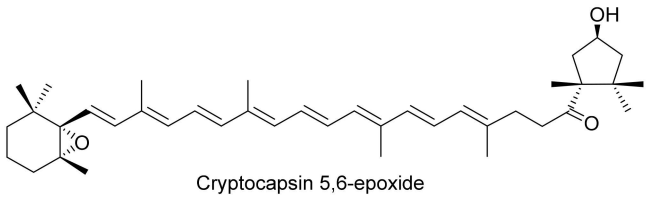

Figure 6. Structure of naturally occurring cryptocapsin 5,6-epoxide with a non-hydroxylated (5*R*,6*S*)-5,6-epoxy β-end group.

Twenty five years ago, β-cryptoxanthin was epoxidized with monoperoxyphthalic acid [101]. By classical column chromatography, three monoepoxides and two diepoxides could be separated, which were characterized by NMR and CD spectroscopy as well as mass spectrometry. These compounds were identified as *anti*- and *syn*-5,6-monoepoxides, *anti* 5′,6′-monoepoxide, as well as *anti*,*anti*- and *syn*,*syn*-diepoxides of β-cryptoxanthin. We have recently reinvestigated the 5′,6′-monoepoxide, which was previously identified as the *anti*-compound. On a chiral stationary phase, we obtained two peaks with the same UV spectra. As in the case of cryptocapsin epoxides, the non-hydroxylated *syn* and *anti* 5,6-epoxy carotenoids could not been separated, neither on calcium carbonate nor C_{18} or C_{30}; however, they could be separated on a chiral phase (Figure 7) [102].

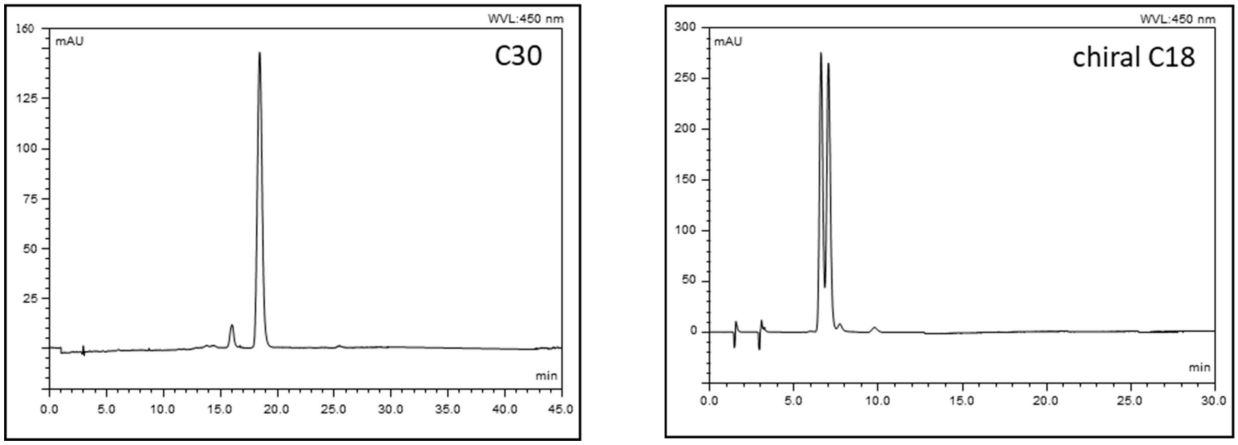

Figure 7. HPLC separation of β-cryptoxanthin 5′,6′-monoepoxides. C_{30} Conditions: see Figure 4. Chiral chromatography conditions: Chiralcel OD C18 3 µm stat. phase (250 × 4.6 mm), 22 °C. Eluents: A: MeOH/EtOH = 50/50 *v*/*v*%, B: MeCN/EtOH = 50/50 *v*/*v*%. Gradient program: from 0% B to 100% B eluent in 30 min. Flow rate: 1.00 cm^3/min; λ = 450 nm.

NMR detection is the best method for the identification of unknown stereoisomers. It can be used either off-line or on-line. Measurement of NMR spectra requires pure components in greater amounts than those of UV/Vis or MS detection modes. Klaus Albert and co-workers identified all lutein [103] and anhydrolutein I [104] stereoisomers using HPLC-NMR. In our laboratory, we investigated the carotenoid composition of cooked vegetables, for example, sorrel [105]. We found that lutein converts to 3′-epilutein and anhydrolutein I and II under acidic conditions, and we also observed the formation of *cis* isomers of these compounds [106,107]. For full characterization of these compounds, the semisynthetic *Z*-isomers were prepared by thermal and I_2-catalyzed photoisomerization [108]. By classical column chromatography, we could not separate the *Z*-isomers of anhydrolutein I, and thus for the separation and identification of these *cis*-isomers, combined HPLC-MS and HPLC-NMR on-line coupling techniques were used, in cooperation with Klaus Albert's group [104].

4. In the Footsteps of Zechmeister

In the second half of the 20th century, the main research field of the Hungarian carotenoid group shifted to carotenoid analysis of natural extracts, isolation, and identification of carotenoids with interesting structures. However, we kept using chemical reactions for structure elucidation studies. Péter Molnár in particular investigated and fully characterized the transformation of geometrical isomers of diverse carotenoids, revealing their kinetic and thermodynamic aspects [69]. After László Zechmeister left for the US, aside from carotenoid analysis, he continued his investigation on the in vitro chemical transformations of carotenoids and described many interesting findings [108]. In the last 15 years, we hearkened back to these works and applied Zechmeister's results in order to prepare novel derivatives of natural carotenoids. The synthesis with carotenoids became a new direction in our research aiming to create covalently modified derivatives with improved solubility and antioxidant properties [109]. Nevertheless, we continue fruitful carotenoid analysis to find new structures and to explore the biosynthetic pathways of carotenoids.

5. Conclusions

In the past 60–70, but mostly in the past 40 years, separation science and instrumental analysis underwent a huge development. As a result of this, isolation and structure elucidation of minor components became possible, helping the understanding of biosynthetic and metabolic pathways. The precision and the development of analytical tools make isolation and structure elucidation of new pigments possible. However, in some cases, old findings should also be revised with the help of new and powerful analytical methods.

Although most natural carotenoids have been discovered so far, tropical plants offer new sources for novel types of carotenoids, so there remains much to conduct in the field of isolation. Comprehensive chromatographic methods (such as supercritical chromatography) can help the scientific community to make the separation of natural products faster and more effective to be able to isolate previously unknown minor pigments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr10040707/s1, Figure S1: Structure of carotenoids.

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