PhD THESIS

Zeaxanthin Food Sources and Strategies to Enhance its Bioaccessibility

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(SUMMARY OF THE DOCTORAL THESIS)

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SUMMARY

Over the last decades, carotenoid pigments have been the centre of attention of many researchers mainly due to the provitamin A activity of several compounds, but also because of their overall positive impact upon human health. The consumption of fruits and vegetables rich in carotenoids has been associated with a lower risk of many chronic diseases such as cardiovascular and eye disorders and certain types of cancer. As humans are not able to synthesize carotenoids *de novo*, the intake of these bioactive compounds is dependent on the diet.

Structurally, carotenoids are divided into two classes: carotenes (hydrocarbons, unoxygenated carotenoids) and xanthophylls (oxygenated derivatives of hydrocarbons). Belonging to the latter category, lutein and zeaxanthin are the only carotenoids able to accumulate in the macular region of the human retina. There, the two xanthophylls offer protection by filtrating high-energy blue light (known to be damaging to photoreceptors and to the retinal pigment epithelium) and by acting as antioxidants, limiting the oxidative stress (quenching excited triplet state sensitizers and singlet oxygen, scavenging reactive oxygen species). Moreover, a large number of publications establish an association between lutein and zeaxanthin intake and the prevention and treatment of several eye diseases such as age-related macular degeneration (AMD) and cataract.

In general, lutein has been the focus of considerably more research studies than its isomer zeaxanthin mostly due to the fact that lutein is more abundant in nature. Although both of these dihydroxycarotenoids along with *meso*-zeaxanthin (a non-dietary xanthophyll that is formed in the retina from lutein) are equally found in the human macula, the human dietary intake is more in favour of lutein than zeaxanthin. The alarming dietary ratio of lutein to zeaxanthin of approximately 5:1 clearly indicates the importance of finding food sources with a high zeaxanthin concentration.

Analysing the amount of carotenoids in different foods is now performable in every research unit having carotenoids as their research interest. High-performance liquid chromatography (HPLC) has proven to be a good method for separating, identifying and quantifying carotenoids in foods and is used in most of the published studies available in the literature. However, apart from the quantitative analysis,

another aspect of critical importance in investigating food sources with an elevated carotenoid content is represented by the gastrointestinal fate of carotenoids following the ingestion of the food matrices. The fraction of the ingested carotenoid that is transferred into mixed micelles during digestion is commonly known as the bioaccessible fraction. Subsequently, the amount of the carotenoid absorbed through the gastrointestinal tract that is able to reach appropriate tissues in order to exert its biological effects represents the bioavailable fraction.

In the literature, human studies are considered the "golden standard" in terms of carotenoid digestion and absorption evaluation. However, *in vitro* models that mimic physiological conditions *in vivo* have been used recently by scientists as an alternative for animal and human models because they are simple, fast, reproducible, inexpensive and have no ethical restrictions. *In vitro* digestion models typically include three phases (oral, gastric and small intestinal phase) and involve the use of simulated digestion fluids as solvents to which corresponding enzyme solutions and bile are added. More recently, a consortium of scientists proposed a standardized static *in vitro* digestion model (INFOGEST®) with the aim of obtaining a better comparability between studies of different research groups using gastrointestinal simulation.

The main objectives of the PhD thesis were:

The aim of the PhD thesis was to investigate natural food sources with a high zeaxanthin concentration and, more importantly, with an elevated zeaxanthin bioaccessibility. Therefore, after a rigorous screening of the existing literature on the subject, several zeaxanthin-rich food sources were selected. As a zeaxanthin source of plant origin, cold-pressed sea buckthorn oil was employed in the first part of the original research. To the best of our knowledge, this is the first study investigating carotenoid bioaccessibility from this food matrix. Sea buckthorn oil is characterized by a high concentration of zeaxanthin, mainly in its esterified form with one or two fatty acids. Considering this deposition form of zeaxanthin, the *in vitro* digestion method was amended and the use of cholesterol esterase was included so as to maximize the cleavage of the ester forms during digestion. Furthermore, an oil-in-water emulsion was prepared and subjected through the simulated digestion. Statistical analysis was performed between the oil and the oil-in-water emulsion as a means to evaluate the potential use of emulsions as delivery systems for lipophilic pigments such as carotenoids.

Considering that there are few animal sources of zeaxanthin, in the second part of the study egg yolk was selected for further investigation. The superior bioaccessibility of zeaxanthin from boiled yolks of commercially available eggs was in agreement with previous publications. The lower amount of macular xanthophylls in egg yolk was somewhat counterbalanced by their particularly high bioaccessibility owed to the high lipid composition of egg yolks.

There are only a few publications regarding the gastrointestinal fate of carotenoids from microalgal supplements. In the final part of the present study the organic dried biomass of *Spirulina* was investigated as a zeaxanthin-rich source of microalgal origin. Seeing that various bile extracts have been used in the literature, both porcine and bovine bile extracts were tested during the simulated digestion model to evaluate the influence on carotenoid bioaccessibility. In order to enhance carotenoid bioaccessibility from *Spirulina*, an additional dietary fat (organic cold-pressed coconut oil) was included in the protocol.

In order to achieve the research aim of the thesis, several objectives have been defined:

O1. Providing an exhaustive review of natural food sources of zeaxanthin and selecting food sources with a potential high bioaccessibility

O2. Employing and optimizing the standardized static *in vitro* digestion protocol for the assessment of zeaxanthin bioaccessibility from the selected food sources

O3. Investigating new strategies to enhance zeaxanthin bioaccessibility from the selected food sources

The results of the present thesis were published in one review article (in *Molecules*, ISI indexed Journal - IF 3.26) and two original articles (one in *Nutrients*, ISI indexed Journal - IF 4.54 and one in *Food Science & Nutrition*, ISI indexed Journal - IF 1.79). Currently, there is one more original manuscript submitted (under review, minor revisions) to an ISI indexed Journal.

All experiments presented in the current thesis were performed at the Department of Chemistry and Biochemistry of The University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, under the supervision of Prof. Dr. Adela Pintea.

The PhD thesis is structured in two parts: state of the art containing one literature review article and original research comprising the working hypothesis/objectives **(Chapter 1)**, general methodologies **(Chapter 2)**, followed by three original research articles **(Chapters 3-5)**, general conclusions and recommendations **(Chapter 6)** and innovative contributions of the thesis **(Chapter 7)**.

For the literature review presented in state of the art, publications that reported dietary sources, bioaccessibility assessment and health benefits of zeaxanthin were reviewed by using the online databases of Web of Science, Scopus and ScienceDirect. Following the literature review, food sources of different origin with a potential high concentration of zeaxanthin were selected and zeaxanthin bioaccessibility was investigated through a simulated digestion model. Cold-pressed sea buckthorn oil, organic egg yolks and organic dried powder of *Spirulina* purchased either from supermarkets or local health food stores were chosen as representatives for plant-based, animal-based and microalgal-based zeaxanthin food sources. These particular food sources were selected based on their affordable prices and availability to the population.

Carotenoid identification and quantification was performed using HPLC-DAD and the fatty acid profile by GC-MS. The international standardized *in vitro* digestion protocol (INFOGEST®) was used and optimized for each of the investigated food sources. For the food sources of plant and microalgal origin, suitable strategies were employed in order to increase zeaxanthin bioaccessibility. In the case of sea buckthorn oil, an oil-in-water emulsion was prepared and subjected to the simulated gastrointestinal digestion, while coconut oil was co-digested with the organic biomass of *Spirulina* to enhance zeaxanthin bioaccessibility. All analyses were performed in triplicate and the results are presented as mean ± standard deviation (SD).

Statistical analysis was performed using Graph Pad Prism, Version 6.0 (Graph Pad Software Inc.) and SPSS programme (SPSS Inc., Chicago, IL, USA). Samples were analysed using unpaired t test with Welch's correction, one-way ANOVA post hoc tests and pairwise multiple comparisons were conducted using Tukey's test. p values < .05 were considered statistically significant.

Chapter 3 aimed at validating sea buckthorn oil as a valuable source of bioaccessible xanthophylls. This study investigated the carotenoid and fatty acid profiles of cold-pressed sea buckthorn oil and examined the *in vitro* bioaccessibility of carotenoids from this food matrix as against an oil-in-water emulsion prepared using the selected oil.

Chapter 4 provides insight into the quality of some commercially available eggs in terms of carotenoid content and profile and into the *in vitro* bioaccessibility of the macular xanthophylls from organic boiled egg yolks.

In **Chapter 5** the carotenoid and fatty acid profiles of commercial microalgal supplements in powder form were investigated, along with their respective carotenoid bioaccessibility. This study points out the potential use of the edible biomass of microalgae as powerful carotenoid food sources and it emphasizes the positive influence of an additional dietary fat during the gastrointestinal digestion on carotenoid bioaccessibility.

The general conclusions were:

1. Cold-pressed sea buckthorn oil represents a strikingly rich natural source of carotenoids, in particular of zeaxanthin.
2. The addition of cholesterol esterase in the *in vitro* digestion model represents the best approach for the simulated digestion of food samples that contain a high amount of carotenoid esters.
3. Providing a lipid source during the gastrointestinal digestion facilitates the transfer of carotenoids into mixed micelles, thus enhancing their bioaccessibility.
4. Using oil-in-water emulsions as delivery systems for carotenoids improves the bioaccessibility of these naturally occurring bioactive compounds.
5. Due to the high lipid matrix, egg yolk represents an affordable source of highly bioaccessible macular pigments in human nutrition.
6. Organic egg production yields a higher total carotenoid content in egg yolks, along with a carotenoid profile composed mostly of lutein and zeaxanthin and the absence of synthetic compounds.
7. The dried biomass of microalgae represents a natural good source of carotenoids, especially of xanthophylls.
8. The use of bovine bile extract in the *in vitro* digestion model is recommended as against porcine bile extract due to its similarity in bile acid composition to human duodenal contents.
9. The addition of coconut oil, a lipid source rich in medium-chain saturated fatty acids, led to the formation of smaller mixed micelles and thus, increased zeaxanthin bioaccessibility from *Spirulina*.

Originality and personal contributions

The current PhD thesis presents natural bioaccessible food sources of zeaxanthin and the results obtained can be considered useful in the food industry and in biotechnology and nutrition research fields. The research performed throughout this thesis was carried out in such a way that the bioaccessibility of zeaxanthin from different food matrices could be assessed, compared and enhanced through various approaches.

The published literature review providing a comprehensive study on the existing literature on natural dietary sources of zeaxanthin facilitates the development of functional foods with an enhanced zeaxanthin bioaccessibility and provides a small-scale database which allows end-consumers and nutritionists to choose or recommend better dietary sources of zeaxanthin for populations vulnerable or affected by ophthalmic diseases such as age-related macular degeneration.

In general, research on zeaxanthin has been focused more on its concentration in various dietary sources rather than its liberation and gastrointestinal fate after ingestion. To the best of our knowledge, this is the first publication on carotenoid bioaccessibility from sea buckthorn oil. In a similar manner, the well-known dried biomass of *Spirulina* has gained increasing attention but there is only one available publication regarding zeaxanthin bioaccessibility from this food matrix. However, in the respective conference abstract, the *in vitro* digestion conditions were not entirely provided and the investigated *Spirulina* strain was not mentioned, while in the current thesis an international standardized *in vitro* digestion was applied.

By using the INFOGEST® method to evaluate carotenoid bioaccessibility from the plant-, animal- and microalgal-based food matrices, a direct comparison between the results presented and other results obtained by different research groups all over the world was enabled.

As the majority of the zeaxanthin-rich food sources are exotic fruits unavailable everywhere in the world, in the current thesis three food matrices affordable and available to the general population were researched and validated as dietary sources with an elevated zeaxanthin bioaccessibility.

Finally, the successful enhancement of zeaxanthin bioaccessibility by formulating a sea buckthorn oil-in-water emulsion and by including a fat source comprised mainly of saturated fatty acids (coconut oil) in the digestion of *Spirulina* constitute highly useful information for food developers in designing food products for targeted end-consumers.

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