Determination of astaxanthin and canthaxanthin in food products by HPLC method

Oznaczanie astaksantyny i kantaksantyny w produktach spożywczych metodą HPLC

Małgorzata Krępska[1*], Katarzyna Sieczyńska[1], Magdalena Lasoń-Rydel[1, 2]

[1] Łukasiewicz-Łódzki Instytut Technologiczny, [2] Politechnika Łódzka

Abstract

Astaxanthin and canthaxanthin are carotenoids produced mainly by algae, fungi and bacteria. These dyes give the characteristic pink-orange color to salmon, shrimp and flamingo feathers. In the case of farm animals, astaxanthin and canthaxanthin are added to the feed, and their amount is strictly established and should not exceed certain limits. The article presents a method of determination of astaxanthin and canthaxanthin by HPLC method. This analysis determines whether these dyes are detected in the product and what their content are. This makes it possible to assess compliance with the limits and often also to determine the origin of the product.

Abstrakt

Astaksantyna i kantaksantyna to karotenoidy wytwarzane głównie przez algi, grzyby i bakterie. Barwniki te nadają charakterystyczny różowo-pomarańczowy kolor piórom łososi, krewetek i flamingów. W przypadku zwierząt gospodarskich astaksantyna i kantaksantyna są dodawane do paszy, a ich ilość jest ściśle określona i nie powinna przekraczać określonych limitów. W artykule przedstawiono metodę oznaczania astaksantyny i kantaksantyny metodą HPLC. Ta analiza określa, czy barwniki te są wykrywane w produkcie i jaka jest ich zawartość. Pozwala to ocenić przestrzeganie limitów, a często także określić pochodzenie produktu.

Keywords: natural dyes, carotenoids, xantophylls, HPLC

Słowa kluczowe: barwniki naturalne, karotenoidy, ksantofile, HPLC

[*]autor korespondencyjny: Małgorzata Krępska: malgorzata.krepska@lit.lukasiewicz.gov.pl
DOI: 10.57636/67.2022.1.8

1. Introduction

Astaxanthin and canthaxanthin are popular carotenoids that give the characteristic pink-orange color to salmon, shrimp or flamingo feathers [1]. These dyes are not synthesized by animals, but they are consumed by them with food. Natural sources of astaxanthin and canthaxanthin for wild animals are algae and crustaceans, in the case of farm animals it is specially colored feed. These dyes are added to feeds for fish, poultry or ornamental birds, which changes the color of their feathers, meat and eggs. The amount of dyes that may be included in the feed used for various farm animals is strictly defined in the European Union Regulations [2, 3, 4]. Determination of the content of astaxanthin and canthaxanthin allows to determine whether the requirements of the Regulations are met. Knowledge about the natural food sources of certain animals and what pigments they contain, it is possible to assess the origin of meat or eggs. This allows to assess the quality of meat or eggs, which is very important in terms of food safety.

2. Structure, properties and application of astaxanthin and canthaxanthin

Carotenoids are yellow, orange and red natural dyes produced by plants, algae, bacteria and fungi. It is a large group of over 700 compounds with similar molecular structures containing only carbon, hydrogen and oxygen. Molecules containing only carbon and hydrogen belong to the group of carotenes, while their oxygen derivatives belong to the group of xanthophylls [5]. Both carotenes and xanthophylls participate in the photosynthesis process by absorbing light and protecting tissues against the harmful effects of photooxidation. The dyes astaxanthin ($C_{40}H_{52}O_4$) and canthaxanthin ($C_{40}H_{52}O_2$) belong to the group of xanthophylls, i.e. they contain oxygen in their molecules.

Astaxanthin (3,3′-dihydroxy-β, β-carotene-4,4′-dione) is a dye containing hydroxyl and ketone groups at both ends of the molecule [6]. The hydrophilic-

hydrophobic structure of this compound causes a strong antioxidant effect stronger than other carotenoids, i.e. beta-carotene, lycopene, lutein, as well as known antioxidants, such as vitamin C or vitamin E. Due to the antioxidant properties of astaxanthin, it has a very beneficial effect on human health, i.e anti-cancer, anti-inflammatory or immunomodulatory effects [7]. For this reason, astaxanthin has also been used as a dietary supplement. It is produced mainly by the algae: Haematococcus pluvialis, Chlorella zofingensis, and the yeast Phaffia rhodozyma and Xantophyllomyces dendrorhous, and synthetic astaxanthin is also produced [8]. The main sources of astaxanthin in the human diet are fish and seafood [9, 10]. The greatest amounts of astaxanthin are in lobsters 15 mg/kg, shrimps 3-15 mg/kg, crabs 12 mg/kg and salmon about 4,4 mg/kg. The acceptable daily intake of astaxanthin (ADI) is 0.2 mg/kg body weight/day [11, 12].

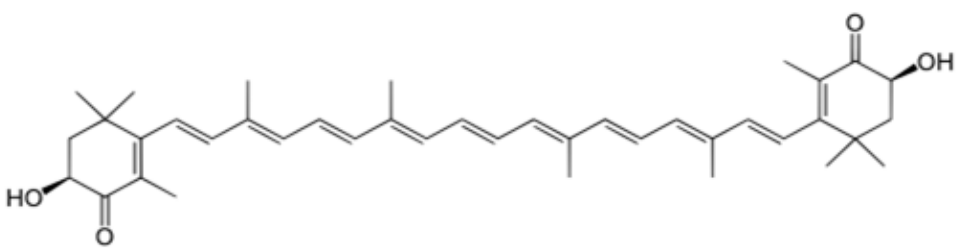


Fig. 1. Structure of the astaxanthin molecule

Canthaxanthin (β, β-carotene-4,4′-dione) is a dye containing two hydroxyl groups in the molecule. It is produced by the algae Botryococcus brauni, the fungi Aspergillus carbonarius and the bacteria Halophilic Archaeon (Haloferax alexandrines), and synthetic canthaxanthin is also produced for industrial purposes [13, 14]. Like astaxanthin, canthaxanthin has an antioxidant effect, but it is lower than astaxanthin. However, some studies have shown that too much canthaxanthin added, among others for sunbathing pills may cause changes in the macula of the eye and retinopathy [15, 16]. For this reason, the acceptable daily intake of

canthaxanthin ADI is 0.03 mg/kg body weight/day [17, 18]. Canthaxanthin, like astaxanthin, has found application in animal feed, while unlike astaxanthin, it is not used as a dietary supplement.

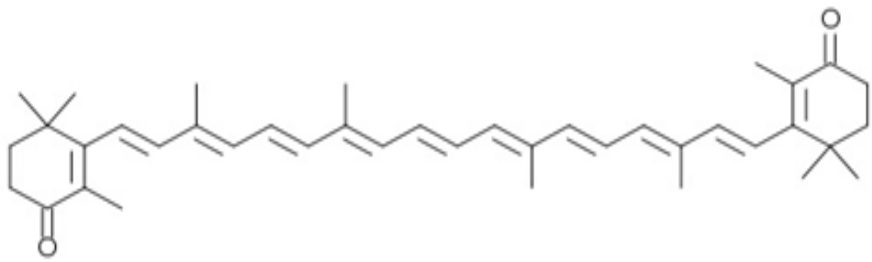


Fig. 2. Structure of the cantaxanthin molecule.

Permitted limits for astaxanthin and canthaxanthin in the feedingstuffs are strictly regulated and vary depending on the species of animal (Tab. 1).

Tab. 1. Permitted levels of astaxanthin and canthaxanthin in feed [12, 17, 18]

Species or category of animal	**Maximum conten t [mg/kg complete feedingstuffs]**	
	Astaxanthin	**Canthaxanthin**
Fish	100	25
Ornamental fish and ornamental birds	-*	100
Laying hens	-*	8
Chickens for fattening	-*	25

*-undefined

There are also limits on the content of astaxanthin and canthaxanthin as food additives or dietary supplement ingredients. Canthaxanthin as a food additive is allowed only for coloring Sousicces de Strasburg in the amount of up to 15 mg/kg [19]. The content of astaxanthin in dietary supplements has been determined at a maximum of 8 mg/day [20]. There is also an acceptable residue level for canthaxanthin in animal species [21].

Tab. 2. Maximum residual limits of canhaxanthin in animal species [21].

Species or category of animal	**Specific tissue**	**Canhaxanthin Maximum residual limits in the relevant foodstuffs of animal origin [mg/kg wet tissue]**
Poultry other than laying hens	Liver	15
	Skin/fat	2,5
Laying hens	Eggs yolk	30
Salmon	Muscle	10
Trout	Muscle	5

3. Experimental

3.1. Reagents and chemicals

Astaxanthin and canthaxanthin were purchased by Sigma Aldrich (Sr. Louis, MO, USA). Methanol HPLC grade, acetonitrile HPLC grade, hexane pure, ethanol 96% pure were supplied by the Chempur Company (Piekary Śląskie, Poland). Standards with a concentration of 10.0 µg/ml were dissolved in ethanol and stored in a fridge at 5°C for 3 months.

3.2. Sample preparation

Ecologically farmed salmon, chicken eggs yolk, animal feed and dietary supplement in capsules were examined. The meat was homogenized in a laboratory mill (IKAPOL, Poland), and then about 5 g of the sample was weighed with an accuracy of 0.0001 g using an analytical balance (PrSeries, OHAUS). The eggs were separated into yolk and white, and then about 5 g of yolk were weighed with an accuracy of 0.0001 g. The capsules were opened, and then about 1 g of the filling was weighed with an accuracy of 0.0001 g. The feed was weighed in the amount of about 1 g with an accuracy of 0.0001 g. The dyes in all matrices were extracted with

5 ml ethanol /2-propanol/ hexane (1:2:6 v/v/v) in an ultrasonic bath for 10 min[22,23].This procedure was repeated 3 times to obtain colorless supernatnant. Collected supernatants was filtered and purified by SPE column (Strata X, Phenomenex). The samples were transferred to a 25 ml volumetric flask and made up to the mark with ethanol.

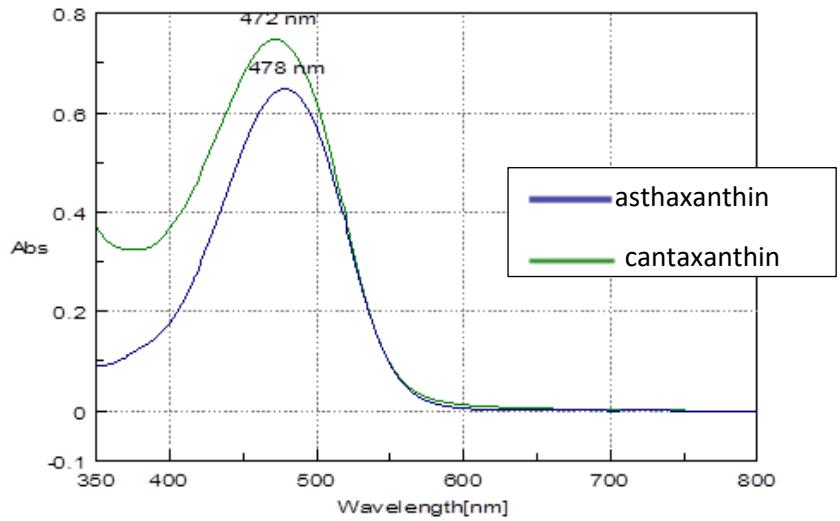
3.3. HPLC conditions

For HPLC separation a Prominence-i Shimadzu liquid chromatograph with a photo diode detector (PDA) was used. Chromatographic separations were obtained under isocratic conditions using a C_{18} Kinetex column (250 mm x 4,6 mm; 5 μm). The column was maintained at a temperature of 30°C. The mobile phase consisted acetonitrile and methanol [70 :30 v/v][24-27]. The wavelength was 475 nm, injection volume 15 μL. To determine maximum wavelength for both dyes spectrophotometer Jasco V-550 was used.

4. Results

The following results were obtained in the determinations:

1. Determination of the maximum wavelengths of dyes.
   The maximum wavelength for astaxanthin is 478 ± 2 nm and for canthaxanthin is respectively 472 ± 2 nm (Graph 1).



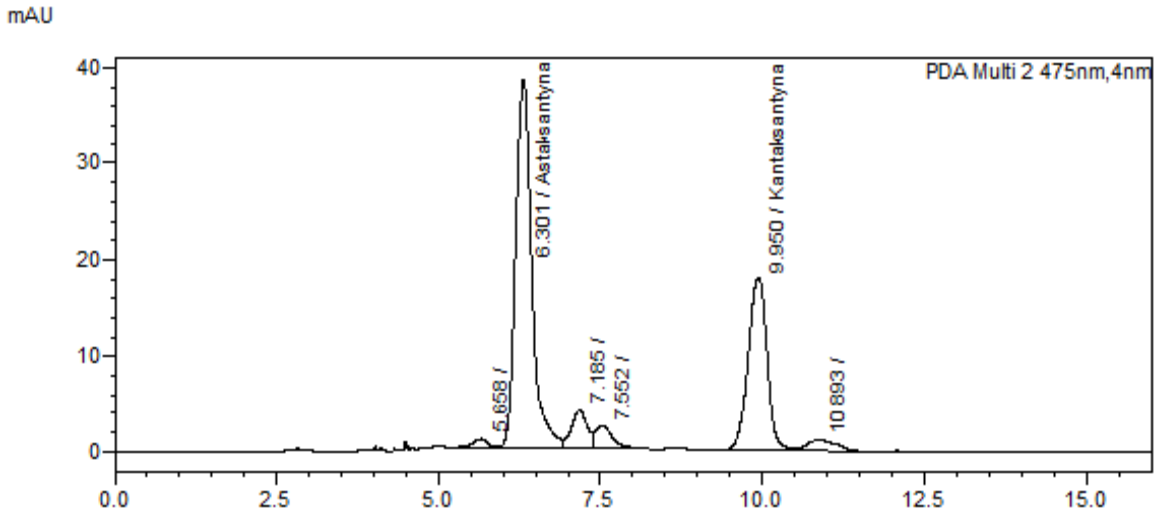
Graph 1. Absorbance of astaxanthin and canthaxanthin standard solutions

2. Determination of canthaxanthin and astaxanthin in food products by HPLC method

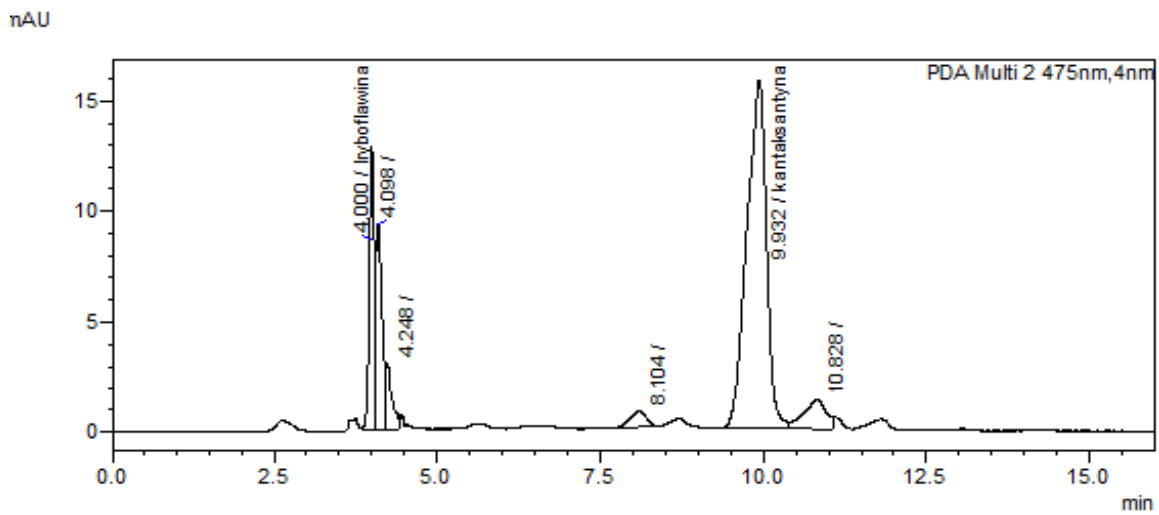
As a result of the performed determinations, the separation of canthaxanthin and astaxanthin dyes was obtained both in the case of standard solutions (Graph 2) and test sample solutions - animal feed (Graph 3). On this basis, the presence and content of dyes in the tested samples were determined (Tab. 3) . With regard to the values in Tab. 1 and Tab. 2, all tested products meet the requirements of the regulations.

Tab. 3. Results of determination of astaxanthin and canthaxanthin content in food products.

Tested product	**Astaxanthin (mg/kg of product)**	**Cantaxanthin (mg/kg of product)**
Ecologically farmed salmon	4,8 ± 0, 8	Not detected
Eggs yolks	Not detected	0,5 ± 0,1
Animal feed	Not detected	83,8 ± 12,5
Dietary supplements	103,5 ± 15,5 (6,0 mg/1capsule)	Not detected



Graph 2. The chromatograph of mix of standards: asthaxanthin and canthaxanthin



Graph 3. The chromatograph of analytes (animal feed)

5. Conclusions

Determination of astaxanthin and canthaxanthin in food samples, mainly in products of animal origin, is an important element in ensuring the quality of this

type of food. It allows to determine compliance with the limits of the regulations, and often to determine the origin of the tested product and whether it is fully organic. The dyes in the tested samples were determined by liquid chromatography (HPLC). The HPLC method in this case is optimal method. As a result, in a short time after extraction and cleaning of the test samples, it is possible to precisely determine both of dyes. This method can be successfully used in this type of determination.

Source of funding

Research carried out as part of the statutory work entitled: Rozdział i oznaczenie ilościowe dwóch barwników karotenoidowych: kantaksantyny i astaksantyny w produktach spożywczych metodą wysokosprawnej chromatografii cieczowej (HPLC). (nr : 00/LBŚ/01/00/1/4)

References

[1] Chitanaya Lakshmi G.: *Food coloring: The Natural Way*, Research Journal of Chemical Sciences, 4(2), 2014, str. 87-96.
[2] Commision Directive 2003/7/EC of 24 January 2003 amending the conditions for authorisation of canthaxanthin in feedingstuffs in accordance with Council Directive 70/524/EEC.
[3] Commission Implementing Regulatoin 2015/1486 of 2 sempember 2015 concerning of authorisation of canthaxanthin as feed additive for certain categories of poultry, ornamented fish and ornamented birds..
[4] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods (Text with EEA relevance).
[5] Delgado-Vargas F, Jiménez A. R, Paredes-López O.: *Natural Pigments: Carotenoids, Anthocyanins, and Betalains Characteristics, Biosynthesis, Processing, and Stability*, Critical Reviews in Food Science and Nutrition, **40:3**, 2000, str. 173-289.
[6] Higuera- Ciapara I., Felix-Valenzuela L., Goycoolea F. M.: *Astaxanthin: A reviev of its chemistry and applications.* Critical Revievs in Food Science and Nutrition, 46(2006), str. 185-196.
[7] Yousry M. A. N.: *Antioxidant activities of astaxanthin and related carotenoids*. Journal

of Agricultural and Food Chemistry, **48**, 2000, str. 1150-1154.
[8] Martinez-Camara S., Ibanez A., Rubio S., Barreiro C., Barredo J.: *Main Carotenoids Produced by Microorganisms.* Encyclopedia, **1**, 2021, str. 1223-1245.
[9] Pogorzelska E., Hamułka J., Wawrzyniak A.: *Astaksantyna-budowa,właściwości i możliwości zastosowania w żywności funkcjonalnej.* Żywność. Nauka. Technologia. Jakość, **1(104)**, 2016, str. 5-16.
[10] Brizio P., Benedetto A., Righetti M., Prearo M., Gasco L., Squadrone S., Abete M. C.: *Astaxanthin and Canthaxanthin (Xanthophyll) as Supplements in Rainbow trout Diet: In Vivo Assessment of residual Levels and Contributions to Human Health*. Agricultural and Food Chemistry, **6**, 2013, str. 10954-10959.
[11] Brendler T., Williamson E.M.: *Asthaxanthin. How much is too much? A safety reviev.* Phytothetapy Research, **33**, 2019, str. 3090-3111.
[12] EFSA Scientific Opinion. *Safety of astaxanthin for its use as a novel food in food supplements.* EFSA Journal,**18**, 2020, 5993.
[13] Esatbeyoglu T., Rimbach G.: *Canthaxanthin: From molecule to function*. Molecular Nutrition& Food Research, **61**, 2017, str. 1600469
[14] Baker T.M. Remi.: *Canthaxanthin in aquafeed applications: is there any risk?* Trends in Food Science & Technology, **12**, 2002, str. 240-243.
[15] Hueber A., Rosentreter A., Severin M.: *Cantaxanthin rethinopathy; long-term observations.* Opthalmic Res., **46**, 2011, str. 103-106.
[16] Arden Goeffrey B., Felix M. B.: *Canthaxanthin and the eye; a critical ocular toxicologic assesment.* Cutaneous Ocular Toxicology, **10**, 1991, str. 115-155.
[17] EFSA Scientific Opinion: *Scientific opinion on the safety and efficacy of canthaxanthin as a feed additive for poultry and for ornamental birds and ornamental fish,* EFSA Journal, **12**, 2014.
[18] EFSA Scientific Opinion: *Scientific Opinion on the re-evaluation of canthaxanthin(E161g) as a food additive*. EFSA Journal, **8(10)**, 2010.
[19] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives
[20] Commision Implementing regulation (EU) 2021/1377 of 19 August 2021 authorising the change of the conditions of use of the novel food astaxanthin-rich oleoresin from Haematococcus pluvialis algae under Regulation (EU) 2015/2283 of the European Parliament and of the Council and amending Commission Implementing Regulation (EU) 2017/2470
[21] Commission Regulation (EC) No 775/2008 of 4 August 2008 establishing maximum residue limits for the feed additive canthaxanthin in addition to the conditions provided for in Directive 2003/7/EC (Text with EEA relevance)
[22] Tzanova M., Argirova M., Atanasov V. : *HPLC Quantification of astaxanthin and canthaxanthin in Salmonidae eggs*. Biomedical Chromatography, **31**, 2017, str. 1-8.

[23] Tzanova M.: *Quantification of Astaxanthin and Canthaxanthin in Muscle Tissues of Rainbow Trout Oncorhynchus mykiss and Brook Trout Salvelinus fontinalis.* Turkish Journal of Fisheries and Aquatic Sciences, **18**, 2018, str. 1053-1061.
[24] Gonzalez M. , Gallego M., Valcarcel M.: *Liquid chromatographic determination of natural and synthetic colorants in lyophillizes foods using an automatic solid-phase extraction system*. Journal of agricultural and food chemistry, **51**, 2003, str. 2121-2129.
[25] Chen D., Wu M., Xie S., Li X., Tao Y., Wang X., Huang L., Pan Y., Peng D.: *Determination of tartrazine, lutein, capsanthin, canhaxanthin and beta-carotene in animal-derived foods and feeds by HPLC method*. Journal of chromatographic science, **57**, 2019, str. 462-468.
[26] Luterotti S., Markovic K. , Franco M. , Bicanic D., Madzgolf A., Kljak K.: *Comparison of spectrophotometric and HPLC methods for determination of carotenoids in foods*, Food chemistry, **140**, 2003, str. 390-397.
[27] Biehler, E., Mayer, F., Hoffmann, L., Krause, E., Bohn T.: *Comparison of 3 spectrophotometric methods of carotenoid determination in frequently consumed fruits and vegetables*, Journal of Food Science **75(1)**, 2010, str. 55-61.