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Identification of Isolutein (Lutein Epoxide) as *cis*-Antheraxanthin in Orange Juice

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The carotenoid profile of orange juice is very complex, a common characteristic for citrus products in general. This fact, along with the inherent acidity of the product, which promotes the isomerization of some carotenoids, makes the correct identification of some of these pigments quite difficult. Thus, one of the carotenoids occurring in orange juice has been traditionally identified as isolutein, a term used to refer to lutein epoxide, although enough evidence to support that identification has not been given. In this study, the carotenoid previously identified as isolutein/lutein epoxide in orange juice has been isolated and identified as a 9 or 9′-cis isomer of antheraxanthin as a result of different tests. To support this identification, a mixture of geometrical isomers of lutein epoxide isolated from petals of dandelions was analyzed under the same conditions used for orange juice carotenoids to check that neither their retention times nor their spectroscopic features matched with those of the orange juice carotenoid now identified as a cis isomer of antheraxanthin.

KEYWORDS: Antheraxanthin; C_{30}; carotenoids; isolutein; lutein epoxide; orange juice

INTRODUCTION

Orange juice is probably the most globally preferred fruit juice. The importance of carotenoids in orange juice color (*1*−*3*) along with the growing interest in these compounds owing to their likely health benefits (*4*−*6*) have stimulated the development of a wide variety of analytical methods for their determination in orange juice (*1*, *7*−*10*). Because there is a wide choice of methods for the analysis of orange juice carotenoids, research should focus on their accurate identification. The correct identification of these pigments in citrus fruits in general is more complicated, due to their complex carotenoid profile. On the other hand, their acidity is enough to promote rearrangements of 5,6-epoxides to 5,8-epoxides. In addition, the use of C_{30} columns, which are currently being used to study the carotenoid profile of different kinds of orange juices (*2*, *8*, *11*, *12*), leads to complex chromatograms, since different isomers of the same carotenoid can be separated (*13*−*15*). Thus, around 40 peaks corresponding to orange juice carotenoids have been obtained in some studies (*2*, *7*). Some of them remain unidentified, whereas many others have been identified in a tentative way by comparison of the spectroscopic data obtained with those reported in the literature. The aim of this study was to provide new data concerning the identity of one of these carotenoids, which had been previously identified as isolutein.

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MATERIALS AND METHODS

Pigment Extraction from Orange Juice and Saponification. The extract of carotenoids was obtained from 300 mL of ultrafrozen orange juice, kindly provided by Zumos Vitafresh (Almonte, Spain), which was thawed at room temperature. Extraction was carried out in a separatory funnel, using 500 mL of a mixture of ethanol:hexane (1:1). After the mixture was filtered, the colored extract was saponified overnight under an atmosphere of nitrogen. For that purpose, 500 mL of 10% ethanolic KOH was used. The saponified extract was washed four times with water (4 × 300 mL) to remove any trace of alkali and subsequently taken to dryness in a rotary evaporator at a temperature below 35 °C.

Thin-Layer Chromatography (TLC). For a preliminary study of the carotenoid profile of orange juice, an aliquot of the extract was chromatographed on silica gel TLC aluminum sheets (Merck, Darmstadt, Germany) using diethyl ether as the mobile phase. Lutein standard, obtained from spinach leaves according to standard procedures (*16*), was cochromatographed to determine the location of the band corresponding to dihydroxycarotenoids. After 30 min of development, it was seen that there were three bands below the one corresponding to dihydroxycarotenoids. The TLC aluminum sheet was left to dry exposed to air, and a few minutes later, it was clearly observed that two of those bands turned greenish, which revealed the presence of monoepoxycarotenoids, whereas the band at the bottom turned blueish, which revealed the presence of diepoxycarotenoids (*17*, *18*).

The rest of the extract was chromatographed on laboratory-made $60GF_{254}$ (Merck) silica gel plates (20 cm × 20 cm, 0.5 mm thickness) using the same solvent system. To determine the location of the dihydroxycarotenoid fraction, a small amount of lutein standard was cochromatographed on every plate. After 1 h, the bands located between the ones corresponding to dihydroxycarotenoids and hydroxycarotenoids

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with two epoxy groups (the band at the bottom) were scraped from the plates. The recovered bands were rechromatographed on laboratory-made aluminum G type E (Merck) plates (20 cm × 20 cm, 0.5 mm thick) using a mixture of acetone:petroleum ether (40−60 °C) (3:7) as the solvent system. After 1 h, the main band was scraped from the plate. The extract was kept dry at −18 °C and under a nitrogen atmosphere until analysis.

Antheraxanthin Standard. The antheraxanthin standard was obtained by treating 1 mg of zeaxanthin with 3-chloroperoxybenzoic acid as described in the literature (*19*, *20*). Antheraxanthin was purified by TLC on silica gel 60 F_{254} plates (20 cm × 20 cm, 0.7 mm thick) (Merck), using the mixture petroleum ether (65−95 °C)−acetone−diethylamine (10:4:1) (*18*). The high-performance liquid chromatography (HPLC) analysis of the extract under the conditions detailed below revealed that two optical isomers showing identical absorption maxima (444 and 472 nm, r_t = 19.07 and 20.29 min), namely, (3*S*,5*R*,6*S*,3′*R*)- and (3*S*,5*S*,6*R*,3′*R*)-antheraxanthin (*21*), were formed.

Cis/Trans Isomerization of the Antheraxanthin Standard. An ethanolic solution of the antheraxanthin standard under an atmosphere of nitrogen was heated in a boiling water bath for 30 min and subsequently illuminated for 12 h by means of an incandescent lamp (300 W).

Isolation of Lutein Epoxide. Lutein epoxide was isolated from a saponified extract of petals of dandelion (*Taraxacum officinale* Weber) according to standard procedures (*18*, *22*) as described previously (*23*).

Identification. Identification of antheraxanthin was carried out by routine procedures. The chromatographic behavior and the color on silica gel plates were taken into account, as well as the test for detecting 5,6-epoxy groups by chemical derivatization (*24*) and its UV/vis and mass spectra. In addition, the extract was cochromatographed with antheraxanthin standard on silica gel TLC aluminum sheets (Merck) using diethyl ether as mobile phase. The extract was also cochromatographed with a mixture of geometrical isomers of antheraxanthin in the HPLC system.

The electron impact mass spectra (EI-MS) were recorded on a Micromass AutoSpec instrument (Micromass, Manchester, United Kingdom) at an ionizing voltage of 70 eV. The temperature of the ion source chamber was 230−240 °C. Before the analysis, the extracts were purified through alumina minicolumns (Brockmann activity grade III) as recommended (*25*, *26*) and concentrated to dryness.

HPLC. An Agilent 1100 system, equipped with a quaternary pump, a photodiode array detector, and a column temperature control module (Agilent, Palo Alto, CA), was used for HPLC analyses. The column was kept at 17 °C, the flow rate was kept at 1 mL/min, and the diode array detector was set at 430, 450, and 486 nm. A 20 μL loop and a 250 mm × 4.6 mm i.d., 5 μm, C_{30} column (YMC, Wilmington, NC) were used. Three solvents, methanol (MeOH), methyl-*tert*-butyl ether (MTBE), and water, were used in the mobile phase. The gradient elution was the same as described previously (*8*, *27*): 0 min, 90% MeOH + 5% MTBE + 5% water; 12 min, 95% MeOH + 5% MTBE; 25 min, 89% MeOH + 11% MTBE; 40 min, 75% MeOH + 25% MTBE; 60 min, 50% MeOH + 50% MTBE; 62 min, 90% MeOH + 5% MTBE + 5% water. The reequilibration time between consecutive injections was 12 min. Both MeOH and MTBE contained small proportions of butylated hydroxytoluene (BHT) and triethylamine (0.1 and 0.05%, respectively) to protect the carotenoids during the chromatographic analysis (*28*).

RESULTS AND DISCUSSION

Identification of the Antheraxanthin Standard. The chromatogram of the extract of antheraxanthin obtained from zeaxanthin is shown in **Figure 1**.

As a consequence of the treatment of an aliquot of the extract with ethanolic HCl (0.1 M), the two epimers of antheraxanthin disappeared, whereas three isomers of the 5,8-epoxyderivative of antheraxanthin, mutatoxanthin (5,8-epoxy-5,8-dihydro-β,β-carotene-3,3′-diol) (chemical structures in **Figure 2**), were obtained (r_t = 19.24, 22.62, and 24.60 min, absorption maxima at 426 and 452 nm), as it can be observed in **Figure 3**. As a result of the 5,6-epoxide to 5,8-epoxide rearrangement due to the acidic treatment, a hypsochromic shift of 18 nm was observed in the absorption maxima of the isomers of mutatox-

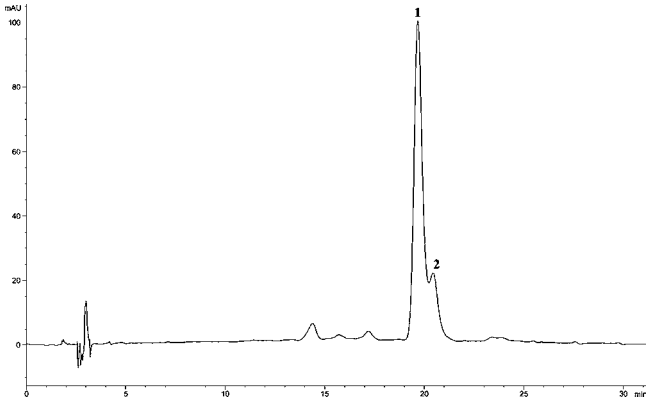

Figure 1. Chromatogram at 430 nm of the standard of antheraxanthin obtained from zeaxanthin.

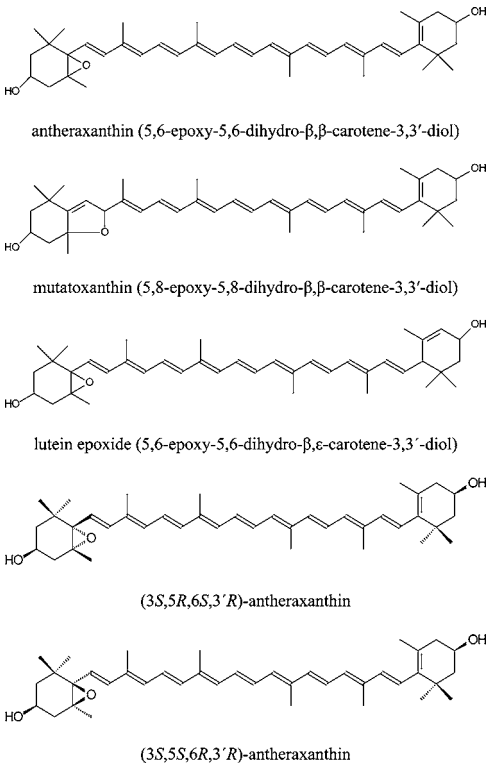

Figure 2. Chemical structures of antheraxanthin, mutatoxanthin, and lutein epoxide.

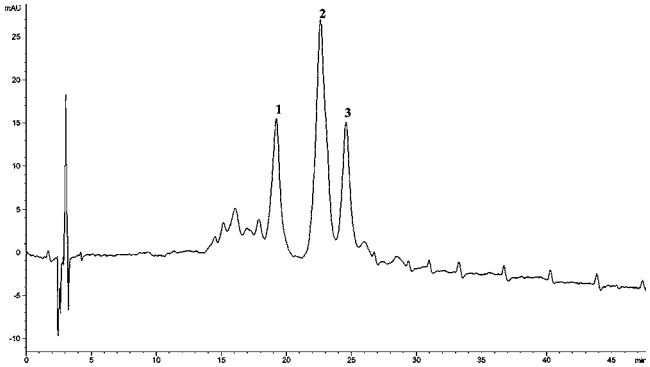

Figure 3. Chromatogram at 430 nm of the mixture of mutatoxanthin isomers obtained by treating the antheraxanthin standard with 0.1 M ethanolic HCl.

Table 1. Chromatographic and Spectroscopic Features of the Mixture of Isomers of Antheraxanthin

peak	r_t[a] (min)	isomer of antheraxanthin[b]	absorption maxima (nm)			D_B/D_{II}
1	16.78	mixture (13,13′,15-*cis*)	330	442	465	0.421
2	19.51	(3*S*,5*R*,6*S*,3′*R*) or (3*S*,5*S*,6*R*,3′*R*)		444	472	
3	20.26	(3*S*,5*R*,6*S*,3′*R*) or (3*S*,5*S*,6*R*,3′*R*)		444	472	
4	25.30	9 or 9′-*cis*	332	440	466	0.077
6	30.00	9 or 9′-*cis*	332	440	468	0.093
7	31.13	9 or 9′-*cis*	332	440	468	0.086

[a] Retention time. [b] Tentative identification.

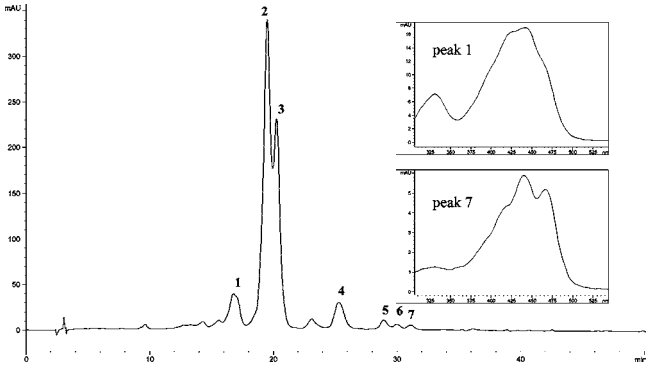

Figure 4. Chromatogram at 430 nm of the mixture of geometrical isomers of antheraxanthin (tentative peak identification and espectroscopic features in **Table 1**).

anthin. The spectra of all of the isomers formed showed little fine structure in comparison with those of other epoxycarotenoids, which is typical of mutatoxanthin (*2*).

The EI-MS of the antheraxanthin standard showed a molecular ion at 584 *m*/*z*, consistent with the formula $C_{40}H_{56}O_3$. The fragment at *m*/*z* 504 $[M - 80]^+$ revealed the presence of an epoxy group in the molecule, whereas those at *m*/*z* 352, 221, and 181 indicated that the epoxy group was in a ring with a hydroxy group (*29*).

Identification of the Geometrical Isomers of Antheraxanthin. The different geometrical isomers of antheraxanthin were tentatively identified on the basis of their spectroscopic properties in the mobile phase (**Table 1**). Thus, the hypsochromic shift of the absorption maxima of the cis isomers of carotenoids with respect to those of the all-trans isomer, around 2−6 nm in the case of mono-cis isomers and around 10−12 nm in the case of di-cis isomers (*30*, *31*), and the intensities of the cis peaks in the spectra were considered. To evaluate the intensities of a cis peak, which appears in the ultraviolet region, the ratio of its absorbance to that of the second absorption band in the visible region, known as Q ratio or D_B/D_{II} (*13*, *32*, *33*), was calculated. This parameter is of great utility, since it is well-known that the intensity of the cis peak is greater as the cis double bond is nearer the center of the molecule (*31*).

The chromatogram of the mixture of geometrical isomers of antheraxanthin is shown in **Figure 4** and **Table 1**. Both the shape of peak 1 and its spectrum (**Figure 4**) revealed the coelution of different isomers. The intense cis peak (D_B/D_{II} = 0.421) may indicate the coelution of the isomers 13, 13′, or 15-cis. Peaks 2 and 3 corresponded to the two isomers of all-*trans*-antheraxanthin obtained by treating zeaxanthin with 3-chloroperoxybenzoic acid, although they were not well-resolved in the chromatographic system used. Peaks 4, 6, and 7 showed smooth cis peaks (D_B/D_{II} < 0.1) and virtually identical

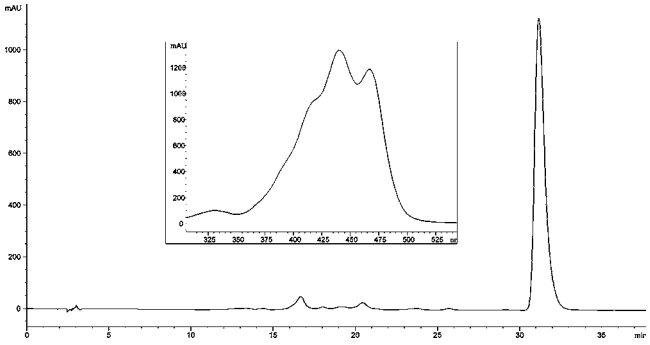

Figure 5. Chromatogram at 430 nm and spectra in the mobile phase of the *cis*-antheraxanthin isomer isolated from orange juice.

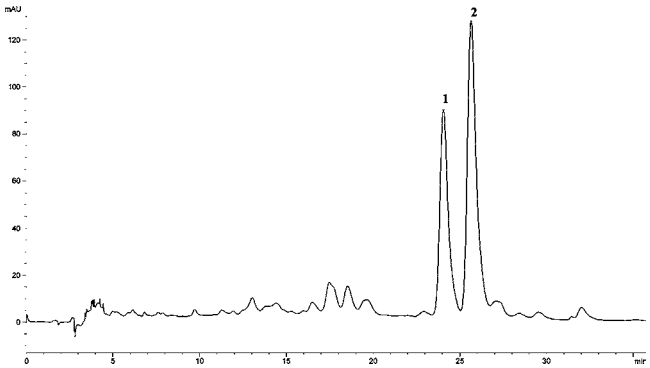

Figure 6. Chromatogram at 430 nm of the band isolated from orange juice after the acidic treatment with 0.1 M ethanolic HCl.

absorption maxima, so they were identified as 9 or 9′-cis isomers of the epimers of all-*trans*-antheraxanthin. Peak 5 was a rest of the zeaxanthin standard. Its retention time, 28.93 min, was lower than those of some isomers of antheraxanthin (peaks 6 and 7, 30.00 and 31.13 min, respectively), despite the fact that the latter carotenoid is more polar, which is a good example of the efficiency of C_{30} columns in the separation of carotenoids.

Identification of Antheraxanthin in Orange Juice. The color of the band isolated from orange juice was orange-yellowish on the TLC plates, although it turned greenish after a few minutes of exposure to air, which revealed the presence of a 5,6-epoxy group in the molecule (*18*). The chromatographic behavior of the extract and the antheraxanthin standard on silica was the same, which was checked on TLC aluminum sheets using diethyl ether as the mobile phase.

The chromatogram of the extract isolated from orange juice revealed the presence of only one compound. Both its retention time (31.17 min) and spectrum in the mobile phase (**Figure 5**), with absorption maxima at 332, 440, and 468 nm and a smooth cis peak, matched with those of the peak 7 of the mixture of isomers of antheraxanthin (**Figure 4**), identified as 9 or 9′-antheraxanthin.

As a consequence of the treatment of the extract with 0.1 M ethanolic HCl, the peak at 31.17 min disappeared completely, whereas two new compounds with identical spectra (r_t = 23.85 and 25.35 min, absorption maxima at 426 and 452 nm) were formed (**Figure 6**). A hypsochromic shift of 14 nm was observed in the absorption maxima of the isomers formed as a result of the acidic treatment, which revealed the presence of one 5,6-epoxy group in the carotenoid isolated from orange juice. The chromatographic and spectroscopic features of these 5,8-epoxy derivatives match with those of two of the three isomers of mutatoxanthin obtained from the antheraxanthin standard (peaks 2 and 3). In addition, their spectra showed little fine structure

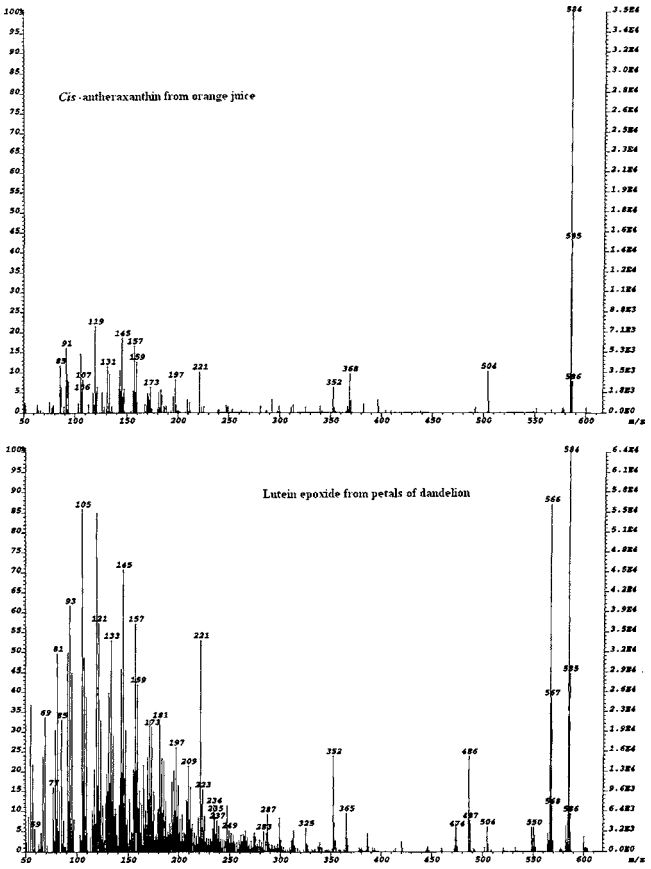

Figure 7. Electron impact mass spectra of *cis*-antheraxanthin from orange juice and lutein epoxide from petals of dandelion.

in comparison with those of other epoxycarotenoids, which is typical of mutatoxanthin. The hypsochromic shift was lower than the one observed when the antheraxanthin standard was subjected to acidic treatment (18 nm), which supported the identification of the compound isolated from orange juice as a *cis*-antheraxanthin isomer, probably 9- or 9′-*cis*-antheraxanthin. The occurrence of mutatoxanthin and other 5,8-epoxycarotenoids not only in orange juice but in other foods is related to their inherent acidity (*34*−*36*), since, as it is well-known, traces of acid are enough to bring about the isomerization of carotenoids 5,6-epoxide to their corresponding 5,8-epoxyderivatives.

The mass spectrum of the compound isolated from orange juice (**Figure 7**) was consistent with that of the antheraxanthin standard. It showed a molecular ion at 584 *m*/*z*, consistent with the formula $C_{40}H_{56}O_3$, and fragments at *m*/*z* 504 $[M - 80]^+$, 352, 221, and 181.

The EI-MS allowed the molecular weight of the carotenoid to be determined and confirmed the preliminary identification of antheraxanthin made on the basis of its chromatographic behavior on silica plates, visible absorption spectra, and the result of the test for detecting 5,6-epoxy groups. The minimum criteria for identification of carotenoids (cochromatography with standard, UV/vis spectrum, and mass spectrum) (*37*) were therefore fulfilled.

Identification of Isolutein as *cis*-Antheraxanthin in Orange Juice. The term isolutein was used to denote lutein epoxide (**Figure 2**) (*38*, *39*), although it is not currently being used (*21*). That term has appeared lately in recent papers to tentatively identify an orange carotenoid that, depending on the chromatographic conditions used, eluted a little before or later than zeaxanthin (*7*, *11*, *12*, *40*, *41*). This carotenoid had been recently identified as a cis isomer of antheraxanthin in a tentative way, mainly on the basis of its spectroscopic characteristics in the mobile phase (*2*). The assays carried out in the present paper confirm that this carotenoid is in fact a cis isomer of antheraxanthin, probably the 9- or 9′-cis isomer. In relation to this, the analysis of the mixture of geometrical isomers of lutein epoxide obtained from petals of dandelion under the same HPLC conditions used for orange juice carotenoids revealed that neither their retention times nor their spectroscopic features matched with those of the carotenoid now identified as 9- or 9′-*cis*-antheraxanthin. Furthermore, some differences can also be found in the EI-MS of lutein epoxide in comparison to that of *cis*-antheraxanthin isolated from orange juice, as it can be observed in **Figure 7**. Both spectra showed strong molecular ions at *m*/*z* 584, consistent with the formula $C_{40}H_{56}O_3$, as well as fragments at *m*/*z* 352, 221, and 181, typical of epoxycarotenoids. However, fragments at *m*/*z* 566 $[M - 18]^+$ and 548 $[M - 18-18]^+$ that indicated losses of two molecules of water due to the presence of two hydroxy groups in the molecule, among others, were found in the spectrum of lutein epoxide and not in the one corresponding to *cis*-antheraxanthin.

In conclusion, it can be said that the new data supplied in this study may confirm that the orange juice carotenoid previously identified as isolutein/lutein epoxide in a tentative way is actually a cis isomer of antheraxanthin.

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