

## Does the carotenoid neoxanthin occur in orange juice?

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### Abstract

Research on the biosynthesis of carotenoid pigments, from diverse points of view, has undergone a considerable boost in recent years due to the large body of studies that highlights their beneficial effects in humans. To obtain valuable information from such surveys, it is of paramount importance to reduce the tentativeness with which some carotenoids are still identified, above all, when they are key compounds for the synthesis of other important metabolites. This is the case for neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- $\beta,\beta$ -carotene-3,5,3'-triol), an epoxycarotenoid which can be found in the light harvesting apparatus of plants and whose cleavage leads to the formation of xanthoxin and then of the plant hormone abscisic acid, among other compounds. Hence, this paper presents pieces of evidence that may put in doubt the presence of such a carotenoid in orange juice, as a consequence of which we propose that the compound formerly tentatively identified as neoxanthin should be referred to as “unidentified” prior to its conclusive characterization and identification.

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### 1. Introduction

The assessment of the carotenoid content of carotenoid-rich products is a research area of growing interest owing to their beneficial effects for human health. Thus, although the vitamin A activity exhibited by some of them has long been known, many studies now focus on the assessment of their antioxidant activity and ultimately on their likely beneficial effects in relation to the prevention or alleviation of serious human disorders, namely several forms of cancer, eye disorders, cardiovascular disease, and light-induced erythema (Olson, 1999). Largely due to all these facts, research on the biosynthesis of these pigments, from various points of view and with diverse goals, has experienced a considerable boost (Fester et al., 2002; Hornero-Méndez & Britton, 2002; Hornero-Méndez, Gómez-Ladrón de Guevara, & Mínguez-Mosquera, 2000). To obtain mean-

ingful information from such biosynthetic studies, it is essential to reduce to a minimum the tentativeness with which some carotenoids are still identified, not only because of the importance of these pigments by themselves but also due to the fact that they are metabolically related to tocopherols, gibberellins, quinones, abscisic acid (ABA) and other important metabolites (Bouvier, Rahier, & Camara, 2005; Fraser, Pinto, Holloway, & Bramley, 2000; Fray et al., 1995). This fact is especially important in the case of orange juice, since its carotenoid pattern is reportedly one of the most complex among fruits (Gross, 1987) and therefore worth studying, not only from a biosynthetic point of view, but also from many other angles. In this sense, the target of this paper is to illuminate the occurrence, or not, in orange juices, of neoxanthin (5',6'-epoxy-6,7-didehydro-5,6,5',6'-tetrahydro- $\beta,\beta$ -carotene-3,5,3'-triol), an epoxycarotenoid that forms part of the light-harvesting apparatus of plants (Croce, Remelli, Varotto, Breton, & Bassi, 1999) and whose cleavage by the enzyme 9-*cis*-epoxycarotenoid dioxygenase (enzyme

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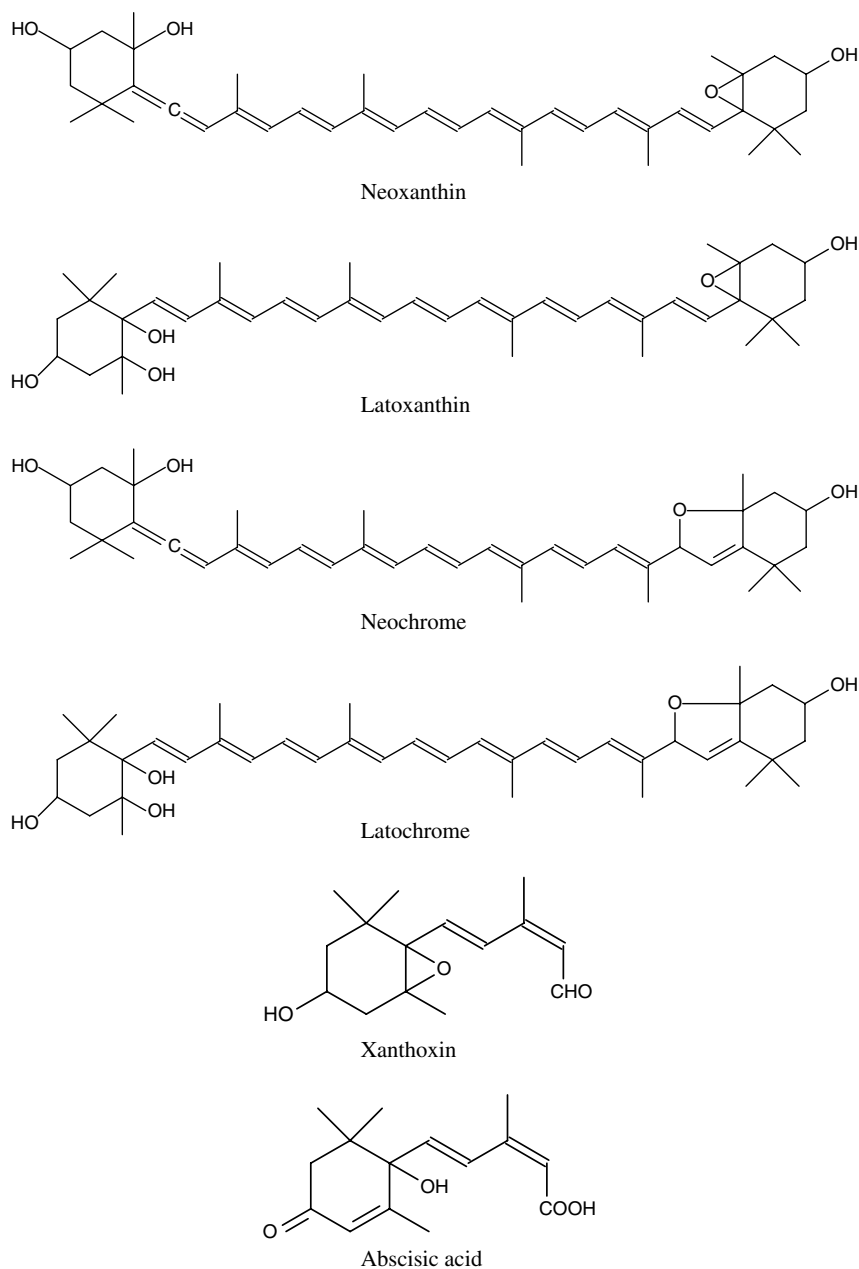


Fig. 1. Chemical structures of neoxanthin, latoxanthin, neochrome, latochrome, xanthoxin and abscisic acid.

unable to cleave the *trans*-isomer) leads to the formation of xanthoxin and then of abscisic acid (ABA) (Fig. 1), a plant hormone which plays important roles in the maturation of seeds, responses to water and other stresses (Qin & Zeevaert, 1999), and reportedly in the formation of the potent odorant  $\beta$ -damascenone (Bezman et al., 2005).

## 2. Materials and methods

### 2.1. Isolation and stereomutation of the neoxanthin standard

The (9'*Z*)-isomer of neoxanthin was isolated in accordance with standard practices (Britton, Liaen-Jensen, &

Pfander, 1995) from a saponified extract of spinach leaves (*Spinacia oleracea* L.) on silicagel TLC plates using diethyl ether as mobile phase. The stereomutation of the (9'*Z*)-neoxanthin standard was achieved through heating and heavy illumination, as explained elsewhere (Meléndez-Martínez, Vicario, & Heredia, 2007). The arrays of geometrical isomers of neoxanthin, so obtained (Table 1), were tentatively identified on the basis of their spectral characteristics by considering the displacements of the absorption maxima and the relative intensity of the “*cis*-peaks” appearing in the UV region of their spectra relative to that of the maximum in the visible region (Zechmeister, 1962), the latter parameter expressed as  $D_B/D_{II}$ .

Table 1  
Chromatographic and spectroscopic data of the neoxanthin isomers obtained after stereomutation

| Peak | $r_t$ (min) | Neoxanthin isomers        | Absorption maxima <sup>a</sup> (nm) |     |     |     | $D_B/D_{II}$ |
|------|-------------|---------------------------|-------------------------------------|-----|-----|-----|--------------|
| 1    | 10.54       | (15Z)-Neoxanthin          | 328                                 | 416 | 438 | 464 | 0.542        |
| 2    | 10.91       | (13Z) + (13'Z)-Neoxanthin | 328                                 | 412 | 434 | 460 | 0.389        |
| 3    | 11.84       | (Di-Z)-neoxanthin         | 328                                 | 408 | 430 | 456 | 0.233        |
| 4    | 13.09       | (All-E)-neoxanthin        |                                     | 418 | 440 | 468 |              |
| 5    | 15.55       | (9Z)-Neoxanthin           | 326                                 | 414 | 436 | 464 | 0.081        |
| 6    | 16.21       | (9'Z)-Neoxanthin          | 328                                 | 414 | 436 | 464 | 0.068        |

<sup>a</sup> Absorption maxima in the mobile phase.

## 2.2. HPLC analysis, UV/Vis spectroscopy and mass spectrometry

The orange juice carotenoids were separated on a C<sub>30</sub> column by the gradient method routinely used in our laboratory (Mouly, Gaydou, Lapierre, & Corsetti, 1999; Meléndez-Martínez, Britton, Vicario, & Heredia, 2005b). The UV/Vis spectra of the neoxanthin standard was recorded on a Hewlett Packard UV/Visible diode-array spectrophotometer, model HP8452, using a 10 mm pathlength glass cuvette. The electron impact mass spectra (EI-MS) were obtained with a Micromass AutoSpec instrument by using a direct insertion probe at an ionizing voltage of 70 eV and ion source temperature of 240 °C. Before the mass spectrometry analyses, the solutions were passed through alumina microcolumns (Brockmann activity grade III) to remove interfering material and subsequently concentrated to dryness.

## 2.3. Orange juice samples

Over 70 samples of orange juice analyzed for the realization of an extensive study on the carotenoids and colour of orange juices (Meléndez-Martínez, 2005) were surveyed. Such samples belonged to the different types of orange juices retailed in Spain, namely orange juices from concentrate (OJFC), ultrafrozen orange juices (UFOJ), orange juices from squeezed oranges (OJFSO) and orange juices from the ecological agriculture (OJFEA). Sample preparation was carried out as explained elsewhere (Meléndez-Martínez, Britton, Vicario, & Heredia, 2005c).

## 3. Results and discussion

### 3.1. Confirmation of the identity of the (9'Z)-neoxanthin standard

The absorption maxima of the (9'Z)-neoxanthin in acetone (416, 438 and 466 nm) were coherent with those broadly reported in the literature (Britton, Liaaen-Jensen, & Pfander, 2004). After adding a few drops of 0.1 M ethanolic HCl, a hypsochromic displacement of 14 nm was observed in the absorption maxima, as a result of the 5,6-epoxy to 5,8-epoxy re-arrangement to give neochrome (5',8'-epoxy-6,7-didehydro-5,6,5',8'-tetrahydro- $\beta,\beta$ -carotene-3,5,3'-triol) (Fig. 1). The electron impact mass spectrum

(EI-MS) showed a molecular ion at  $m/z$  600, which agreed well with the formula C<sub>40</sub>H<sub>56</sub>O<sub>4</sub>. The fragments at  $m/z$  582 [M-18]<sup>+</sup>, 564 [M-18-18]<sup>+</sup> and 546 [M-18-18-18]<sup>+</sup>, corresponding to the losses of 1–3 molecules of water, respectively, revealed the presence of 3 hydroxy groups in the molecule. The fragments at  $m/z$  520 [M-80]<sup>+</sup> and 502 [M-18-80]<sup>+</sup>, revealed the presence of an epoxy group, whereas those at  $m/z$  352, 221 and 181 suggested that the epoxy group was in a ring with a hydroxy group. The interpretation of the mass spectra of the neoxanthin standard was in accordance with that of the same pigment accomplished by Mercadante, Rodríguez-Amaya, and Britton (1997).

### 3.2. Identification of the isomers obtained upon stereomutation of the (9'Z)-neoxanthin standard

A chromatogram of the extract of (9'Z)-neoxanthin upon stereomutation is depicted in Fig. 2, the chromatographic and spectroscopic features of the various isomers obtained being summarized in Table 1. Peak 1 was readily identified as (15Z)-neoxanthin, due to it showing the most intense “cis-peak” ( $D_B/D_{II}$  = 0.542). The shape of peak 2 indicated that there was coelution of isomers, even though the spectra obtained throughout the chromatographic peak were virtually identical, as a result of which it was identified as a mixture of (13Z)- and (13'Z)-neoxanthin. The spectrum of peak 4 lacked the “cis-peak” and exhibited

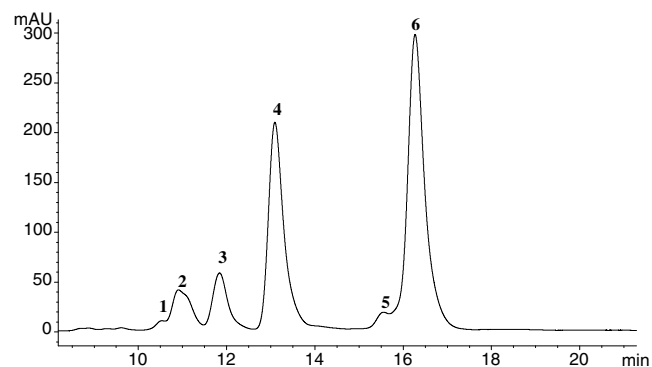


Fig. 2. Chromatogram, at 430 nm, of the extract of geometrical isomers of neoxanthin obtained upon stereomutation of the (9Z)-neoxanthin standard: (15Z)-neoxanthin (peak 1); (13Z) + (13'Z)-neoxanthin (peak 2); (di-Z)-neoxanthin (peak 3); (all-E)-neoxanthin (peak 4); (9Z)-neoxanthin (peak 5); (9'Z)-neoxanthin (peak 6).

a finer structure than did those of the other isomers. This fact, together with the location of its absorption maxima at the longest wavelength (440 nm) when compared with all the other isomers, led to its identification as all-*E*-neoxanthin. Peak 3 was identified as a di-*Z*-isomer of neoxanthin on the basis of the conspicuous hypsochromic shift of its absorption maxima in the mobile phase (430 nm) relative to that of the isomer identified as all-*E*-neoxanthin. The spectra corresponding to peaks 5 and 6 showed virtually identical absorption maxima and intensities of their “*cis*-peaks”. In relation to peak 5, the fact that the magnitude of its chromatographic peak was negligible in comparison to that of peak 6 and that its fine structure was also lower (data not shown), led us to identify it as the (9*Z*)-isomer, in which the nearness of the *Z* double bond to the allenic group brings about a higher steric hindrance than in the (9'*Z*)-stereoisomer. Anyhow, both the chromatographic and the spectroscopic features of peak 6 matched those of the (9'*Z*)-neoxanthin standard isolated from spinach leaves. The identification of the different geometrical isomers detected accorded with that reported by Strand, Kvernberg, Karlsen, and Liaaen-Jensen (2000).

### 3.3. Is neoxanthin present in orange juice?

The carotenoid neoxanthin has been tentatively identified in orange juices by our group and by other authors in a number of fairly recent studies (Cortés, Torregrosa, Esteve, & Frígola, 2006; Fanciullino et al., 2006; Lee & Castle, 2001; Meléndez-Martínez, Britton, Vicario, & Heredia, 2005a; Meléndez-Martínez, Vicario, & Heredia, 2003; Mouly et al., 1999). As a result of new sets of analyses performed in our laboratory, we have gathered a number of analytical observations that seem to indicate that such an important carotenoid may have been misidentified. In this sense, it has been checked in UFOJ that the reten-

tion time of that carotenoid ( $t_r = 8.19$  min, Fig. 3), thereafter named “unidentified 1”, did not match any of those corresponding to the different geometrical isomers of neoxanthin obtained by stereomutation of the (9'*Z*)-standard (Tables 1 and 2), despite the shape of its spectrum and the location of its absorption maxima in the mobile phase (418, 440 and 468 nm). This may have led to erroneously identifying it as all-*E*-neoxanthin, a pigment that clearly elutes later ( $t_r = 13.09$  min). In this sense, it seems that the identification of neoxanthin that we carried out in a previous study by using a proper neoxanthin standard (Meléndez-Martínez et al., 2003) was erroneous, despite the fact that both the retention time and the spectroscopic features of the compound matched those of the standard, which was due to the limitations of the isocratic chromatographic method and the C<sub>18</sub> column used in the separation of isomers, the co-elution in such a system of lutein and zeaxanthin being a good illustration of it.

The fact that the acidification of the carotenoid extract of UFOJ yields the disappearance of “unidentified 1”, revealing the presence in its molecule of a 5,6-epoxy group (Meléndez-Martínez et al., 2005a), contributed to the misidentification, even though neither of the two resulting 5,8-epoxyderivatives (thereafter referred to as “unidentified 2” and “unidentified 3”, Fig. 3) matched the chromatographic features of the two epimers of neochrome (5',8'-epoxy-6,7-didehydro-5,6,5',8'-tetrahydro- $\beta$ , $\beta$ -carotene-3,5,3'-triol) obtained upon acidification of the neoxanthin standard (Table 2). In relation to this, the occurrence of the carotenoids termed unidentified 2 and 3, but not of unidentified 1 was observed in most of the samples of the remaining types of orange juice (OJFC, OJFSO and OJFEA), which is in agreement with the observation that the main qualitative difference between such types of orange juices and UFOJ is the predominance in the former of 5,8-epoxycarotenoids over their 5,6-epoxy counterparts, which in many cases disappear completely (Meléndez-Martínez, 2005; Meléndez-Martínez, Britton, Vicario, & Heredia, 2006).

Besides these analytical observations, there are other pieces of evidence that point towards the non-occurrence of relevant levels of neoxanthin in orange juice. Thus, while (9'*Z*)-neoxanthin is well-known to be a major carotenoid in all green leaves and other photosynthetic tissues and in many algae, and (all-*E*)-neoxanthin occurs in some flowers

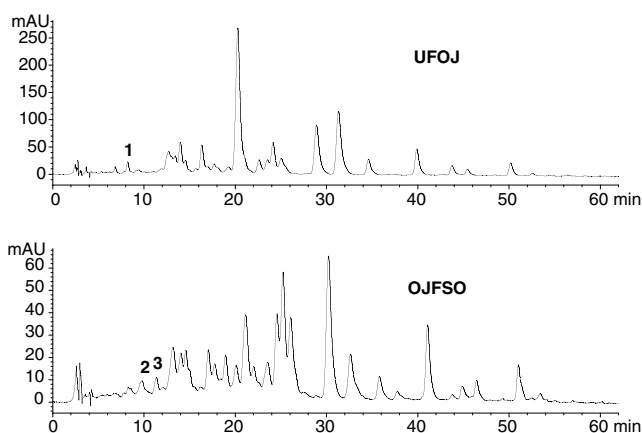


Fig. 3. Chromatograms, at 430 nm, of the carotenoid extracts of an ultrafrozen orange juice (UFOJ) and an orange juice from squeezed oranges (OJFSO), indicating the presence of the compounds referred to as “unidentified 1” (peak 1), “unidentified 2” (peak 2) and “unidentified 3” (peak 3).

Table 2

Chromatographic and spectroscopic features of the unidentified compounds and the neochrome isomers obtained from the neoxanthin standard

| $t_r$ (min) | Carotenoid         | Absorption maxima <sup>a</sup> (nm) |     |     |
|-------------|--------------------|-------------------------------------|-----|-----|
| 8.19        | Unidentified 1     | 418                                 | 440 | 468 |
| 9.41        | Unidentified 2     | 400                                 | 422 | 448 |
| 11.22       | Unidentified 3     | 400                                 | 422 | 448 |
| 14.27       | Neochrome isomer 1 | 400                                 | 422 | 448 |
| 15.79       | Neochrome isomer 2 | 400                                 | 422 | 448 |

<sup>a</sup> Absorption maxima in the mobile phase.

(Rodríguez-Amaya, 2001; Britton et al., 2004), the presence of whatever isomeric form of this carotenoid or its 5,8-epoxy counterpart, neochrome, in ripe fruits is not very common, although they have been identified in olives and mango (Mercadante et al., 1997; Gandul-Rojas, Cepero, & Mínguez-Mosquera, 1999). More importantly, as far as oranges and citrus in general are concerned, it has been observed in flavedo tissue that neoxanthin, which is a major carotenoid in green stages, progressively disappears to the extent that it is already not detectable in early chromoplast-containing tissue (Rodrigo, Marcos, & Zacarías, 2004; Alós, Cercós, Rodrigo, Zacarías, & Talón, 2006). In relation to this, it has just been hypothesized for oranges that (9*Z*)-violaxanthin may be the major substrate for 9-*cis*-epoxycarotenoid dioxygenase, (9'*Z*)-neoxanthin playing that role only in photosynthetic tissue, as it is virtually absent in full coloured tissue (Rodrigo et al., 2004).

In conclusion, it can be said that there is no sound evidence concerning the presence of detectable levels of neoxanthin in orange juice, at least in the wide range of orange juice brands marketed in Spain. Rather, the analytical data supplied in this study suggest that the carotenoid so tentatively identified may be in fact another one. Thus, it may well also be the tetrahydroxycarotenoid lutoxanthin (5',6'-epoxy-5,6,5',6'-tetrahydro- $\beta,\beta$ -carotene-3,5,6,3'-tetrol) (Fig. 1), whose spectroscopic features are similar to those of neoxanthin but which is more polar than the latter. Likewise, the 5,8-epoxy derivatives of such unidentified compounds, found in other samples of orange juice, may also be isomers of the 5,8-epoxy derivative of lutoxanthin, that is, lutochrome (5',8'-epoxy-5,6,5',8'-tetrahydro- $\beta,\beta$ -carotene-3,5,6,3'-tetrol) (Fig. 1), which has already been reported in orange juice (Gross, Carmon, Lifshitz, & Sklarz, 1975). In any case, and so as not to hinder the dissemination of valuable data on orange juice carotenoids, the compounds formerly tentatively identified as neoxanthin and neochrome should be referred to as “unidentified” until they are isolated and conclusively characterized and identified.

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