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Identification of Zeinoxanthin in Orange Juices

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The monohydroxycarotenoid fraction of orange juice has been isolated by TLC and studied to determine whether the carotenoid accompanying β-cryptoxanthin was α-cryptoxanthin or zeinoxanthin. The provitamin A carotenoid α-cryptoxanthin has been widely reported in orange juice, although its identification has been carried out mainly on the basis of its spectral features, which are virtually identical with those of its non-provitamin A isomer, zeinoxanthin. As a result of a study of the UV−vis and mass spectra of the monohydroxycarotenoid fraction and of the methylation test, it was concluded that the carotenoid accompanying β-cryptoxanthin was the non-provitamin A carotenoid zeinoxanthin.

KEYWORDS: Carotenoids; α-cryptoxanthin; EI-MS; orange juice; provitamin A; vitamin A; zeinoxanthin

INTRODUCTION

Carotenoids are one of the main classes of natural pigments. Apart from being responsible for the color of a wide variety of foodstuffs and their likely protective role in several diseases, the provitamin A activity of some of them is well-known (*1*−*3*). So far, the existence of over 600 carotenoids has been reported, although only a few have provitamin A activity. The minimum structural requirement for this activity is an unsubstituted β-ring with an 11-carbon polyene chain, which is only satisfied by ∼60 carotenoids (*4*). The most important provitamin A in terms of both bioactivity and widespread occurrence is β-carotene. However, the importance of β-cryptoxanthin must also be highlighted, which, despite its lower bioactivity compared to β-carotene, is the main provitamin A carotenoid in many fruits, such as peach, nectarine, orange-fleshed papaya, and orange juice (*5*, *6*). Other examples of carotenoids satisfying the structural requirement mentioned above are α-carotene, γ-carotene, α-cryptoxanthin, β-zeacarotene, and β-carotene-5,6-epoxide, among others, all of which would have approximately half the bioactivity of β-carotene (*5*).

The assessment of the vitamin content of foodstuffs is of vital importance, not only due to the nutritional and biological relevance of these compounds but also in order to satisfy consumers' demands concerning the control of nutritional labels and the supply of accurate data for food databases and the establishment of dietary reference intakes. In this sense, carotenoids are becoming increasingly important (*7*, *8*).

Orange juice is probably the most globally accepted fruit juice, and it is worldwide recognized as a good source of provitamin A carotenoids (*9*). Although the occurrence of the provitamin A compounds α-carotene, β-carotene, and β-cryptoxanthin in orange juice has been demonstrated (*10*−*12*), identification of most orange juice carotenoids has been usually carried out in a tentative way. Due to these facts, this work is aimed at finding out if the monohydroxycarotenoid accompanying β-cryptoxanthin is the provitamin A carotenoid α-cryptoxanthin (β,ε-caroten-3′-ol) or zeinoxanthin (β,ε-caroten-3-ol), which lacks provitamin A activity.

MATERIALS AND METHODS

Isolation of Standards and of the Monohydroxycarotenoid Fraction of Orange Juice. The β-cryptoxanthin and lutein standards (structures in **Figure 1**) and the monohydroxycarotenoid fraction of orange juice were obtained according to standard procedures (*13*, *14*).

β-Cryptoxanthin was isolated from a saponified extract of red peppers (*Capsicum annuum* L.). TLC separation was performed on silica gel 60 GF_{254} plates (20 × 20 cm) (Merck, Darmstadt, Germany), using light petroleum ether (bp 65−95 °C)/acetone/diethylamine (10:4:1) as solvent system (*15*).

Lutein was isolated from a saponified extract of spinach leaves (*Spinacia oleracea* L.) on silica gel 60 GF_{254} plates (20 × 20 cm) (Merck) using diethyl ether as mobile phase (*16*).

The monohydroxycarotenoid fraction of orange juice was isolated from a saponified extract of ultrafrozen orange juice on silica gel 60 GF_{254} plates (20 × 20 cm) (Merck) using diethyl ether as mobile phase (*16*).

Identification. The identity of the β-cryptoxanthin and lutein standards was confirmed by their UV−vis and electron impact mass spectra (EI-MS).

Prior to HPLC analysis the UV−vis spectra of ethanolic solutions of the standard carotenoids were recorded on a Hewlett-Packard UV−visible diode array spectrophotometer, model HP8452, using a glass cuvette (10 mm path length).

The EI-MS spectra were obtained with a Micromass AutoSpec instrument using a direct insertion probe at an ionizing voltage of 70 eV and ion source temperature of 240 °C. Before mass spectrometry,

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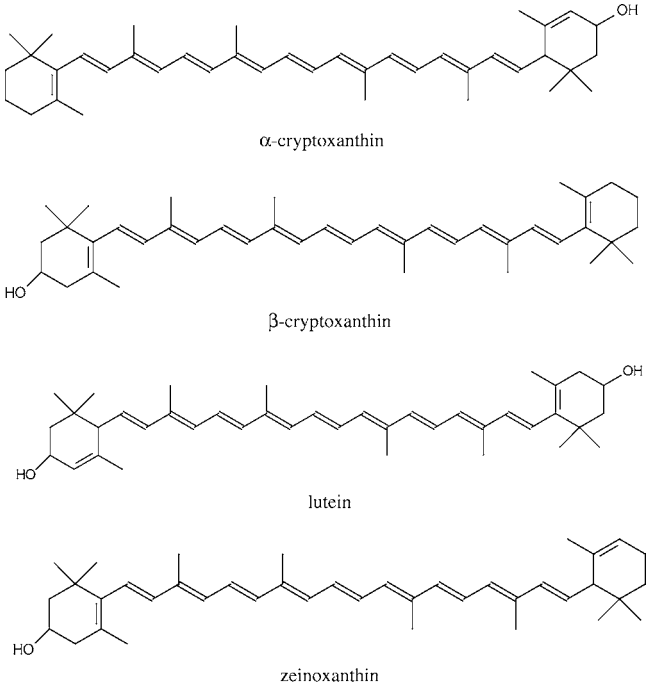

Figure 1. Chemical structures of α-cryptoxanthin, β-cryptoxanthin, lutein, and zeinoxanthin.

the solutions containing the carotenoids were purified through alumina microcolumns (Brockmann activity grade III), as recommended (*17*, *18*), and concentrated to dryness.

To help identify cis-isomers of β-cryptoxanthin that may occur in the monohydroxycarotenoid fraction of orange juice, an ethanolic solution of the standard was heated at 80 °C for 30 min in a water bath and subsequently illuminated overnight by means of a powerful lamp (300 W). The vial containing the ethanolic solution was gently blanketed with nitrogen to avoid oxidation reactions. Tentative identification of the different geometrical isomers obtained was carried out by considering their spectral properties in the mobile phase.

To find out if the monohydroxycarotenoid accompanying β-cryptoxanthin in orange juices was α-cryptoxanthin or zeinoxanthin (**Figure 1**), the methylation test with acidified methanol was carried out according to the recommended guidelines (*4*, *19*). For that purpose the whole monohydroxycarotenoid fraction of orange juice was concentrated to dryness, redissolved in methanol, and treated with a few drops of methanolic HCl (0.2 M) under nitrogen for 3 and 6 h.

High-Performance Liquid Chromatography (HPLC). HPLC analyses were carried out by means of a Hewlett-Packard 1100 system, consisting of a quaternary pump, a photodiode array detector, and a column temperature control module (Hewlett-Packard, Palo Alto, CA). A 20-μL loop and a C30 YMC column (5 mm, 250 × 4.6 mm) (Wilmington, NC) were used. The column was kept at 17 °C, and the flow rate was 1 mL/min. The diode array detector was set at 430, 450, and 486 nm. Methanol (MeOH), *tert*-butyl methyl ether (TBME), and water were used in the mobile phase. The gradient elution was the same as described elsewhere (*20*): 0 min, 90% MeOH + 5% TBME + 5% water (v/v/v); 12 min, 95% MeOH + 5% TBME (v/v/v); 25 min, 89% MeOH + 11% TBME (v/v/v); 40 min, 75% MeOH + 25% TBME (v/v/v); 60 min, 50% MeOH + 50% TBME (v/v/v); 62 min, 90% MeOH + 5% TBME + 5% water (v/v/v). MeOH and TBME contained a small proportion of butylated hydroxytoluene (BHT) (0.1% w/v) and triethylamine (0.05% v/v) to protect the carotenoids during the chromatographic analysis (*21*).

Description of Samples. Two samples of four different types of orange juice [orange juices from concentrate (OJFC), orange juices from squeezed oranges (OJFSO), orange juices from the ecological agriculture (OJFEA), and ultrafrozen orange juices (UFOJ)] were analyzed to check the occurrence of zeinoxanthin.

Sample Preparation. Ten-milliliter aliquots of UFOJ or 25-mL aliquots of the remaining types of orange juices (OJFC, OJFSO, and OJFEA) were gently mixed with 50 mL of the extracting solvent (methanol/acetone/hexane, 25:25:50, v/v/v, containing 0.1% BHT), and the resulting mixture was subsequently centrifuged for 10 min at 1250*g*, in accordance with other authors (*22*). The top hexane layer containing the carotenoid pigments was recovered and washed four times with ~25 mL of water to remove any trace of acetone. Saponification was carried out by adding 25 mL of ethanolic KOH (10% w/v). After 1 h, the reaction was stopped by adding ~100 mL of aqueous NaCl (10% w/v) to remove the alkali. The carotenoid extract was washed three more times. The hexane extract was concentrated to dryness in a rotary evaporator at a temperature below 35 °C, and the carotenoids were redissolved in 1 mL of a mixture of acetone/methanol (1:2, v/v, containing 0.1% BHT). Prior to injection in the HPLC system the extract was filtered through Millipore PVDF Millex filters (13 mm × 0.45 μm) (Bedford, MA).

Table 1. Chromatographic and Spectral Data in the Mobile Phase (Conditions Described in the Text) of the Different Geometrical Isomers of β-Cryptoxanthin Obtained

peak	t_r (min)	tentative identification	absorption maxima (nm)	D_B/D_{II}
1	31.19	15-*cis*-β-cryptoxanthin	338, 448, 474	0.471
2	33.46	13- or 13′-*cis*-β-cryptoxanthin	338, 444, 466	0.454
3	41.03	*all-trans*-β-cryptoxanthin	450, 476	
4	45.09	9- or 9′-*cis*-β-cryptoxanthin	342, 446, 470	0.090
5	46.14	9- or 9′-*cis*-β-cryptoxanthin	342, 446, 470	0.084

RESULTS AND DISCUSSION

Confirmation of the Identity of the β-Cryptoxanthin and Lutein Standards. The UV−vis spectra of the ethanolic solution of the lutein standard showed absorption maxima at 422, 445, and 475 nm, which was in agreement with the maxima tabulated (*4*). The EI-MS spectrum showed a molecular ion of 568 mass units, consistent with the formula $C_{40}H_{56}O_2$. Radical cations at *m*/*z* 550 $[M - 18]^+$ and 532 $[M - 18 - 18]^+$, corresponding to the loss of one and two molecules of water, respectively, revealed the presence of two hydroxy groups. The mass spectrum agreed with those reported by other authors (*23*).

The UV−vis spectra of the ethanolic solution of the β-cryptoxanthin standard showed absorption maxima at 450 and 478 nm, which agreed with the maxima reported elsewhere (*4*). In the EI-MS spectrum a molecular ion of 552 mass units was observed, which was coherent with the formula $C_{40}H_{56}O$. A fragment at *m*/*z* 534 $[M - 18]^+$ revealed the presence of one hydroxy group. The mass spectrum was in agreement with those reported by other authors (*18*, *24*).

Tentative Identification of the Geometrical Isomers of the β-Cryptoxanthin Standard. The spectral and chromatographic properties of the different isomers of β-cryptoxanthin (**Figure 2**) are summarized in **Table 1**. For their tentative identification on the basis of spectral features, two parameters were taken into account: on the one hand, the hypsochromic shift with respect to the all-trans-isomer (*4*) and, on the other hand, the intensity of the cis-peak in the spectrum. For this purpose, the ratio between its absorbance and the one corresponding to the absorption maxima in the visible region (*Q* ratio or D_B/D_{II}) was calculated (*25*−*27*).

Peaks 1 and 2 were tentatively identified as 15- and 13- or 13′-*cis*-β-cryptoxanthin, respectively. The intensities of their cis-peaks were similar, although their absorption maxima (**Table 1**) were clearly different. In the case of the peak identified as 15-*cis*-β-cryptoxanthin, the absorption maxima were located at higher wavelengths, which was consistent with the identification of other 15- and 13-cis-isomers of carotenoids carried out by other authors (*28*−*30*). As for peaks 4 and 5, they both showed

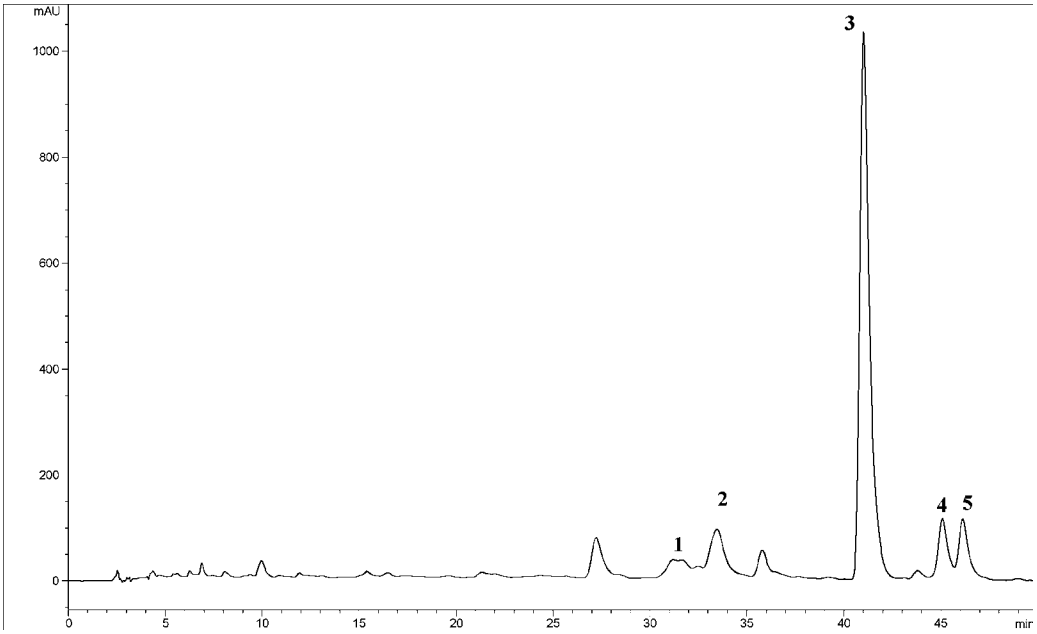

Figure 2. Chromatogram of the mixture of geometrical isomers of β-cryptoxanthin obtained (identification in **Table 1**).

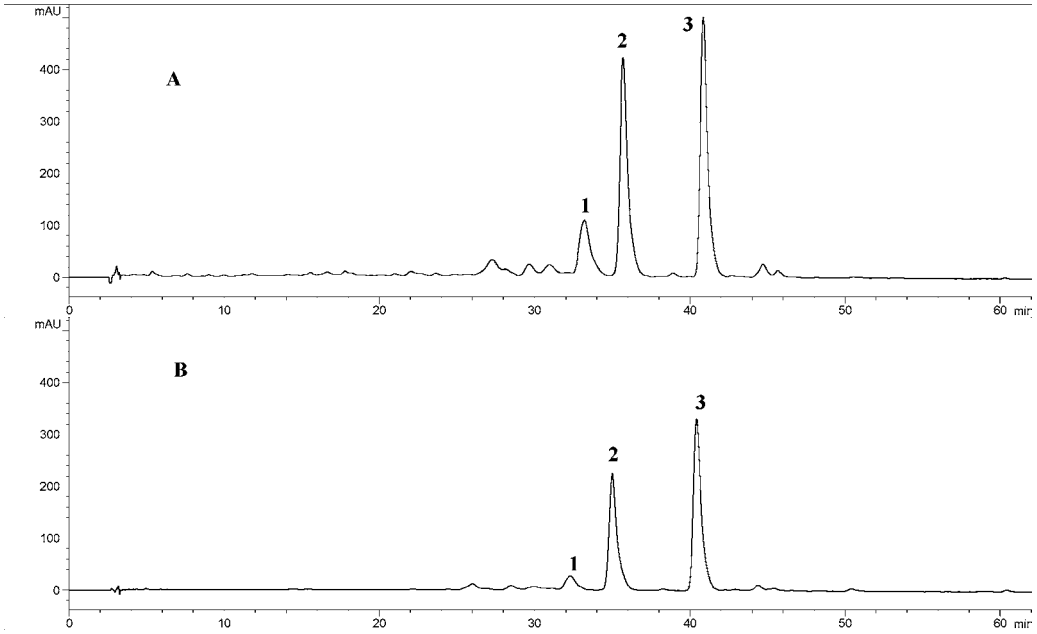

Figure 3. Chromatograms at 430 nm of the monohydroxycarotenoid fraction of orange juice before (**A**) and after treatment with methanolic HCl (0.2 M) for 3 h (**B**). Peak identification is given in **Table 2**.

smooth cis-peaks and identical absorption maxima, being tentatively identified as 9- or 9-*cis*-β-cryptoxanthin isomers. The characteristics of peak 3 matched completely with the ones of the β-cryptoxanthin standard prior to isomerization.

Identification of the Monohydroxycarotenoid Accompanying β-Cryptoxanthin in Orange Juices as Xeinoxanthin. The chromatogram of the monohydroxycarotenoid fraction of orange juice is shown in **Figure 3A**.

The spectrum in the mobile phase of peak 1 showed a marked cis-peak at 338 nm (**Table 2**), being tentatively identified as 13- or 13′-*cis*-β-cryptoxanthin as a result of the comparison of its spectral and chromatographic properties with those corresponding to the different geometrical isomers obtained from the β-cryptoxanthin standard.

The spectral and chromatographic features of the peaks identified as β-cryptoxanthin in the monohydroxycarotenoid fraction of orange juice and in the samples matched completely with those of the all-trans-standard analyzed under the same HPLC conditions.

Table 2. Chromatographic and Spectral Data of the Monohydroxycarotenoids of Orange Juice in the HPLC System

peak	carotenoid	retention time (min)	spectral maxima (nm)
1	13- or 13′-*cis*-β-cryptoxanthin	32.18	338, 444, 466
2	zeinoxanthin	34.68	424, 444, 472
3	β-cryptoxanthin	39.90	452, 476

The electron impact mass spectrum of the whole fraction (**Figure 4**) was similar to that of the β-cryptoxanthin standard, which confirmed the presence of monohydroxycarotenoids in it. Thus, a strong molecular ion at 552 mass units was observed, consistent with the formula $C_{40}H_{56}O$. The fragment at *m*/*z* 534

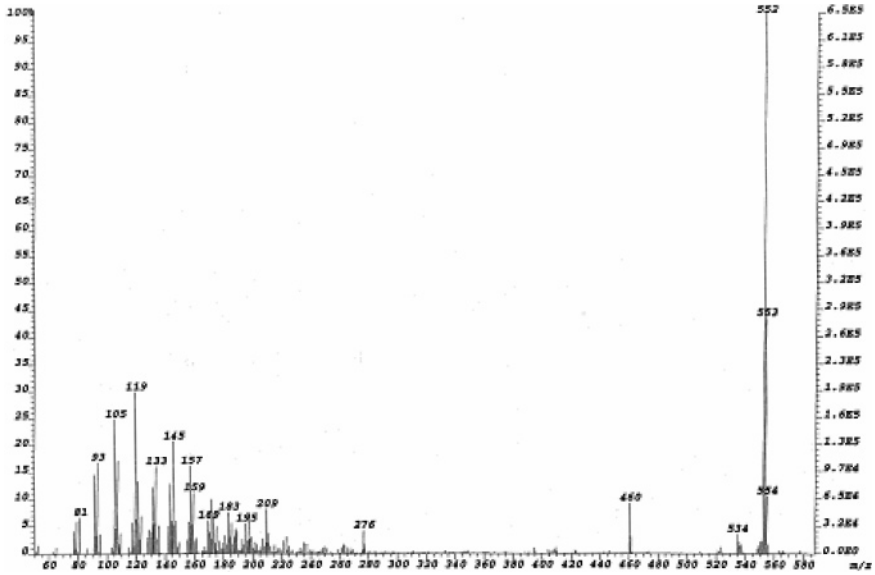

Figure 4. Electron impact mass spectra of the monohydroxycarotenoid fraction of orange juice.

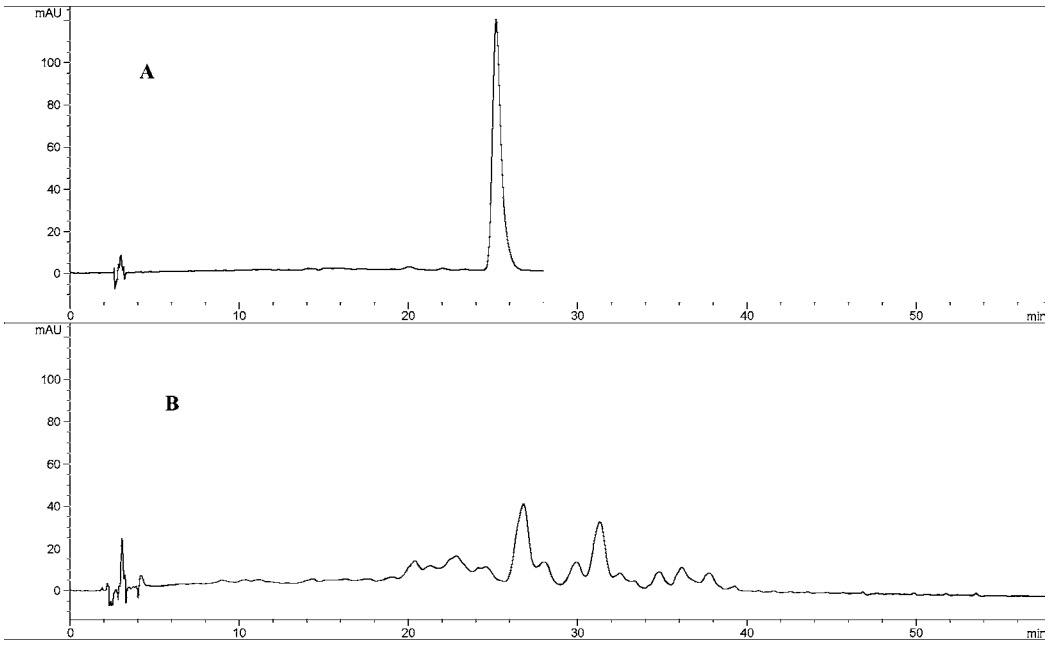

Figure 5. Chromatograms at 430 nm of the lutein standard before (**A**) and after treatment with methanolic HCl (0.2 M) for 3 h (**B**).

$[M - 18]^+$ indicated the loss of water due to the presence of one hydroxyl group in the molecule.

To find out if the monohydroxycarotenoid accompanying β-cryptoxanthin isomers (peak 2, **Figure 3A**) was α-cryptoxanthin (β,ϵ-caroten-3′-ol) or zeinoxanthin (β,ϵ-caroten-3-ol), the methylation test with acidified methanol was carried out. Traditionally, the monohydroxycarotenoid α-cryptoxanthin, which has been tentatively identified in orange juices in many studies (*12*, *20*, *22*, *31*), has not been considered as a provitamin A carotenoid due to the fact that it was assigned the chemical structure of zeinoxanthin (**Figure 1**), an isomer of the former one that is becoming increasingly reported (*4*, *32*).

Differentiation between α-cryptoxanthin and zeinoxanthin is difficult because they both have the same spectra and chromatographic behavior (*4*). Nevertheless, a correct identification is of great importance due to the fact that α-cryptoxanthin has provitamin A activity, whereas zeinoxanthin lacks that property. The only structural difference between them is the position of the hydroxyl group, which is located in the ϵ ring, that is, in allylic position, in the case of α-cryptoxanthin (**Figure 1**). As a result of this, α-cryptoxanthin reacts positively to the methylation test with acidified methanol, and a methylated more apolar derivative is formed.

Because isolation of pure α-cryptoxanthin or zeinoxanthin is difficult in materials containing also β-cryptoxanthin, the test can be carried out by using the monohydroxycarotenoid fraction of orange juice isolated either by TLC or by column chromatography, because the hydroxyl group of β-cryptoxanthin is not in allylic position and, therefore, does not react with acidified methanol to give a methylated derivative. Thus, the extract was concentrated to dryness, redissolved in methanol, and treated with five drops of methanolic hydrochloric acid (0.2 M) under nitrogen for 3 h.

The HPLC analysis of the treated extract (**Figure 3B**) revealed that no more apolar derivatives were formed, which indicated that zeinoxanthin, and not α-cryptoxanthin, was present in the extract, because methylation did not take place.

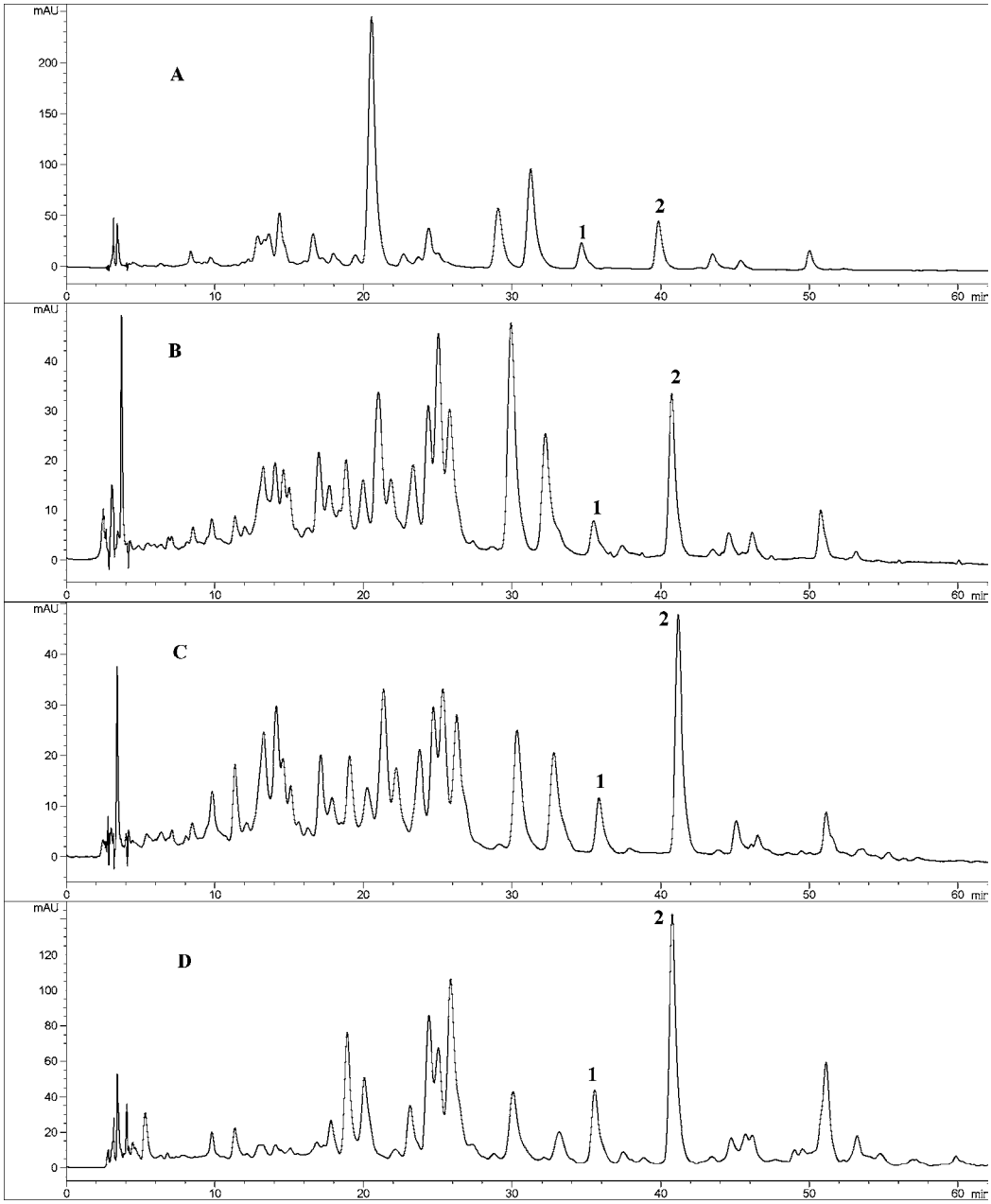

Figure 6. Chromatograms at 430 nm of UFOJ (**A**), OJFC (**B**), OJFSO (**C**), and OJFEA (**D**).

To confirm the identification, the extract was treated for a further 3 h, after which time no more apolar compounds were observed.

To carry out this test it is important to bear in mind that if too much diluted acid is added, both β-cryptoxanthin and zeinoxanthin disappear, whereas new peaks corresponding to breakdown products are formed.

To test the validity of the methylation test, this was carried out at the same time with the lutein standard, because this pigment does have one hydroxyl group in allylic position (**Figure 1**). After 3 h of treatment, the expected results were seen; that is, there were substantial changes in the chromatogram as a result of the formation of methylated derivatives (**Figure 5A**,**B**).

The occurrence of the peak identified as zeinoxanthin was observed in the different types of orange juices analyzed, as is shown in **Figure 6**.

As for how much total vitamin A activity is lost as a result of the identification of α-cryptoxanthin as zeinoxanthin, it was observed that, in ultrafrozen orange juices, the levels of the compound now identified as zeinoxanthin were clearly higher than those of the provitamin A carotenoids α-carotene and β-carotene. The β-cryptoxanthin (the main provitamin A carotenoid in orange juices) to zeinoxanthin ratio in these juices was ~3 (*12*).

In conclusion, evidence has been given concerning the occurrence of the non-provitamin A carotenoid zeinoxanthin in orange juice. Because isolation of α-cryptoxanthin and zeinoxanthin is difficult, a novel approach was used. Thus, the whole monohydroxycarotenoid fraction of orange juice was used to carry out the methylation test after checking that the other carotenoid occurring in the fraction was β-cryptoxanthin, which would not interfere with the results.

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