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HPLC analysis of geometrical isomers of lutein epoxide isolated from dandelion (*Taraxacum officinale* F. Weber ex Wiggers)

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Abstract

Lutein epoxide has been isolated from petals of dandelion (*Taraxacum officinale* F. Weber ex Wiggers) by thin-layer chromatography (TLC) on silica to be used for the accurate identification of this carotenoid in other sources. The extract was analyzed by high-performance liquid chromatography (HPLC) using a C_{30} column, as a result of which six geometrical isomers were separated. The identification of these isomers was performed on the basis of their UV/vis spectroscopic features in the mobile phase. In quantitative terms, it was observed that all-*E*-lutein epoxide was the major carotenoid and that there were also high amounts of the (9*Z*)- and (9′*Z*)-isomers, although the latter may be an artifact.

Keywords: *Taraxacum officinale* F. Weber ex Wiggers; Asteraceae; Dandelion; Carotenoids; Geometrical isomers; Lutein epoxide; Taraxanthin

1. Introduction

Carotenoid pigments occur in plants, animals, algae, fungi and bacteria (Rodriguez-Amaya, 1997; Britton et al., 2004). Chemically they are basically C40 tetraterpenoids with a long chromophore of conjugated double bonds, which is responsible for their red to yellow coloration (Weedon and Moss, 1995; Britton, 1995a). Aside from being responsible for the colour of a wide variety of structures, interest is being focused on food carotenoids due to their likely health benefits (Bramley, 2000; Fraser and Bramley, 2004; Meléndez-Martínez et al., 2004), so they are being extensively studied currently (Mercadante et al., 1999a,b; Fraser et al., 2001; Burns et al., 2003; Meléndez-Martínez et al., 2005b).

To date more than 700 carotenoids have been reported (Britton et al., 2004). From an analytical point of view, and due to the growing interest in these compounds, it is important to reduce the tentativeness with which they have been identified in many foodstuffs. Therefore, isolation of standards from natural sources must be encouraged for accurate identifications. In this sense, green leaves are used for the isolation of neoxanthin, violaxanthin and lutein, palm oil for the isolation of α-carotene and β-carotene or red peppers for the isolation of capsanthin, capsorubin and other carotenoids (Mínguez-Mosquera and Hornero-Ménde, 1993; Britton, 1995b; Olatunde Farombi and Britton, 1999). Many flowers are also good sources of carotenoids. Thus, eschscholtzxanthin can be isolated from Californiam poppy (*Eschscholtzia californica* Cham.), neoxanthin from laburnum (*Laburnum* sp.), lutein from marigold (*Tagetes erecta* L.), etc (Leigh Hadden et al., 1999; Britton et al., 2004). The petals of dandelion (*Taraxacum officinale* F. Weber ex Wiggers) contain high amounts of

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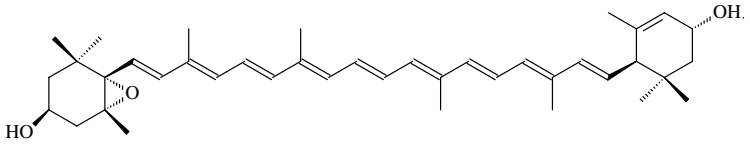
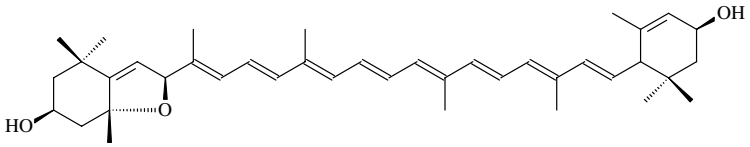
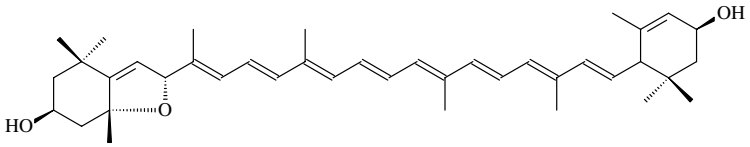


lutein epoxide ((*3S,5R,6S,3'R,6'R*)-5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3'-diol)



chrysanthemaxanthin ((3*S,5R,8S,3'R,6'R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3'-diol)



flavoxanthin ((3*S,5R,8R,3'R,6'R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3'-diol)

Fig. 1. Chemical structures of lutein epoxide, chrysanthemaxanthin and flavoxanthin.

lutein epoxide ((*3S,5R,6S,3′R*,6′*R*)-5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3′-diol) (Britton et al., 2004) (Fig. 1), also known as taraxanthin, whose occurrence has been reported, for instance, in flowers and leaves of many plants, and in foods such as tangerines, olive fruits, peas, pumpkins, etc (Pfander, 1987; Britton, 1991; Wingerath et al., 1996; Gandul-Rojas et al., 1999; Rodriguez-Amaya, 2001; Edelenbos et al., 2001; Humphries and Khachik, 2003). One of the aims of this study was to describe a simple and rapid procedure for obtaining lutein epoxide from petals of dandelion, which can be used along with others described before (Toth and Szabolcs, 1981; Toth et al., 1988) for the screening of this carotenoid in other sources. Furthermore, this work is also aimed at providing information concerning the geometrical isomers of the pigment that occur in that material by means of HPLC with a C_{30} stationary phase, which allows their rapid separation.

2. Results and discussion

The band corresponding to lutein epoxide isolated from petals of dandelion was separated as a yellow band on the TLC silica gel plates. After a few minutes of exposition to air the band turned greenish as a result of the acidity of the stationary phase, which was checked on TLC silica gel aluminium sheets. This fact confirmed the presence of a 5,6-epoxy group in the molecule (Eugster, 1995). As for the co-chromatography with the mixture lutein + lutein epoxide on TLC silica gel aluminium sheets, it was seen that the location of the lower band of the mixture, the one corresponding to lutein epoxide, matched with that of the extract obtained from petals of dandelion.

The UV/vis spectra in ethanol and acetone of the extract isolated from petals of dandelion showed three well-defined peaks with absorption maxima at 416, 438 and 466 nm and 416, 440 and 468 nm respectively, which were coherent with those reported in the literature (Britton, 1995a; Rodriguez-Amaya, 2001).

After adding four drops of ethanolic HCl (0.1 M) in the cuvette used to record the spectra, a hypsochromic shift of around 18 nm was observed (Fig. 2), which confirmed the presence of one 5,6-epoxy group in the molecule (Eugster, 1995).

The mass spectrum showed a strong molecular ion of 584 mass units (m.u.), consistent with the formula $C_{40}H_{56}O_3$. The fragments at m/z 566 $[M-18]^+$ and 548 $[M-18-18]^+$ indicated losses of two molecules of water, due to the presence of two hydroxy groups in the molecule. The fragments at m/z 504 $[M-80]^+$ and 486 $[M-80-18]^+$ are typical of epoxycarotenoids, whereas those at m/z 352, 221 and 181 indicated that the epoxy group was in a ring with a 3-hydroxy group (Enzell and Back, 1995).

The molecular weight of the carotenoid isolated (584 Da) determined from the electron-impact mass spectrum (EI-MS) confirmed the preliminary identification of lutein epoxide made on the basis of its chromatographic behaviour on silica plates, visible absorption spectra and the result of the text for detecting 5,6-epoxy groups by chemical derivatization. Thus, the minimum criteria for identification of carotenoids (co-chromatography with standard, UV/vis spectrum and mass spectrum) (Liaaen-Jensen, 1995) were met.

A C_{30} column, a type of stationary phase specifically developed for the separation of geometrical isomers of carotenoids (Sander et al., 1994, 2000), was used for the

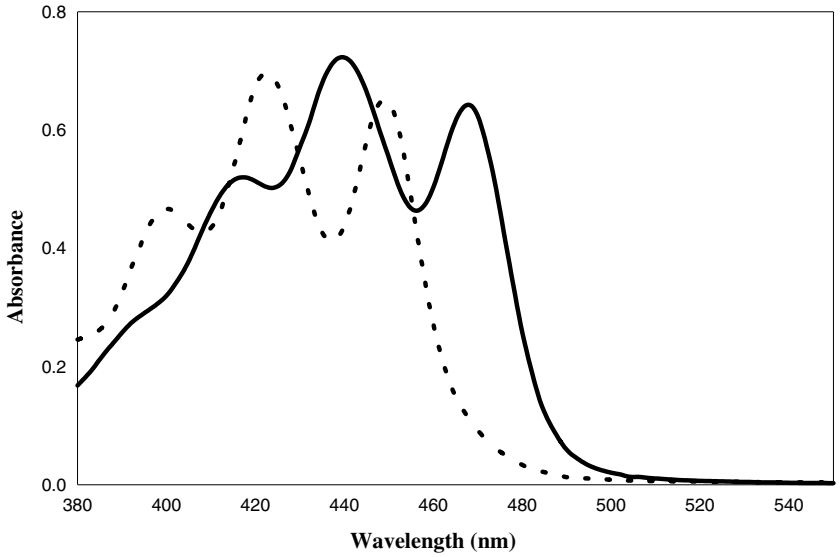


Fig. 2. Spectra of the extract of lutein epoxide from petals of dandelion before (—) and after (---) the treatment with ethanolic HCl (0.1 M).

HPLC analysis of the extract, since it is being increasingly used to identify different isomers of carotenoids in foods (Lessin et al., 1997; Godoy and Rodriguez-Amaya, 1998; Pott et al., 2003) and other materials (Emenhiser et al., 1996; Dachtler et al., 1998). The chromatogram revealed the presence of six geometrical isomers of lutein epoxide (Fig. 3), whose chromatographic and spectral features in the mobile phase are summarized in Table 1.

An aliquot of the extract of lutein epoxide was subsequently treated with ethanolic HCl (0.1 M) and analyzed by HPLC. As a result of the acidic treatment, the isomers of lutein epoxide disappeared, whereas several isomers of its 5,8-epoxyderivatives, the stereoisomers chrysanthemaxanthin ((3*S*,5*R*8*S*,3′*R*,6′*R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3′-diol) and flavoxanthin ((3*S*,5*R*,-8*R*,3′*R*,6′*R*)-5,8-epoxy-5,8-dihydro-β,ε-carotene-3,3′-diol) (Britton et al., 2004) were formed.

To identify the geometrical isomers of lutein epoxide tentatively, their spectral properties in the mobile phase were taken into account. On one hand, the hypsochromic shift of the absorption maxima of the *Z* isomers of carotenoids relative to those of the *all-E* isomer, around 2-6 nm in the case of mono-*Z* isomers and 10-12 nm in the case of di-*Z* isomers, were considered. On the other hand, the intensities of the "*cis* peaks" in the spectra, which appear in the ultraviolet region, were also taken into consideration. In this sense, it is well-known that the intensity of the *cis* peak is greater as the *cis* double bond is nearer the centre of the molecule (Zechmeister, 1962; Rodriguez-Amaya, 2001). For that purpose, the ratio of the absorbance of the *cis* peak to the absorbance of the second absorption band in the visible region, known as *Q* ratio or D_B/D_{II} (Deli et al., 1988; Saleh and Tan, 1991; Phillip et al., 1999; Breitenbach et al., 2001), was calculated.

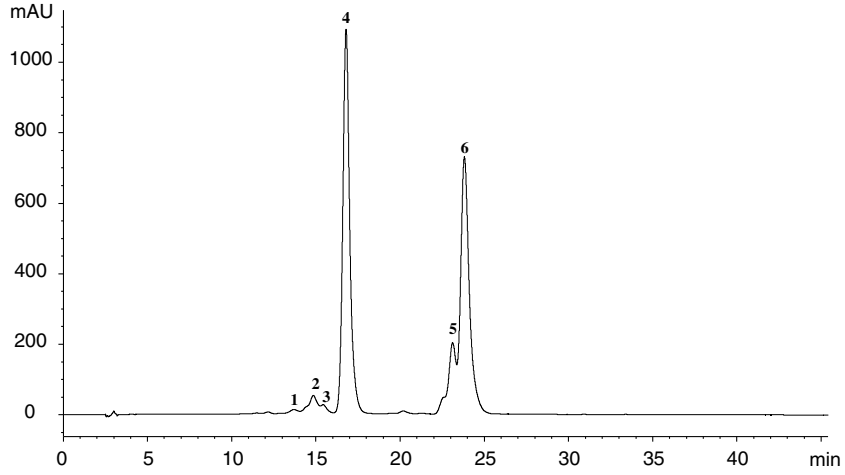


Fig. 3. Chromatogram at 430 nm of the extract of lutein epoxide isolated from petals of dandelion (peak identification in Table 1).

Table 1
Chromatographic and spectral features of the different geometrical isomers of lutein epoxide separated in the HPLC system

Peak	r_t (min)[a]	Isomer identification	Absorption maxima (nm)				D_B/D_{II}
1	13.69	Di-*Z*-lutein epoxide	326	410	430	458	0.337
2	14.85	Di-*Z*-lutein epoxide	326	408	428	456	0.290
3	15.44	(13*Z*)-Lutein epoxide, (13′*Z*)-lutein epoxide or mixture of both	328	410	432	460	0.464
4	16.79	All-*E*-Lutein epoxide		416	438	468	
5	23.12	(9′*Z*)-Lutein epoxide	326	414	436	462	0.082
6	23.82	(9*Z*)-Lutein epoxide	326	412	434	462	0.077

[a] r_t: retention time.

Peak 4 was identified as all-*E* lutein epoxide, on the basis of the absence of *cis* peak in its UV/vis spectra and the location of its absorption maxima (Table 1) in comparison to those of the remaining isomers. To confirm the identification of this peak, an aliquot of all-*E*-lutein standard was treated with 3-chloroperoxybenzoic acid (Barua, 2001; Barua and Olson, 2001) and analyzed by HPLC (Fig. 4), and a mixture of diastereoisomers of lutein epoxide (peaks 3 and 4) was obtained (Britton et al., 2004). As it can be observed in Table 2, the chromatographic and spectral features of the peak 4, matched with those of the peak identified as all-*E*-lutein epoxide in the extract from petals of dandelion. Peaks 1 and 2 (Fig. 4, Table 2) may be trihydroxy-derivatives formed as a consequence of the acidic treatment (Deli and Molnar, 2002), while peak 6 was a rest of the lutein standard.

Peak 3 was identified as (13*Z*)- or (13′*Z*)-lutein epoxide, or a mixture of both, on the basis of the intensity of its *cis* peak ($D_B/D_{II} = 0.464$), the hypsochromic shift of its absorption maxima (6 nm with respect to the all-*E* isomer), and the findings of other authors (Toth and Szabolcs, 1981; Toth et al., 1988). Peaks 1 and 2 showed almost identical absorption maxima and *cis* peaks of lower intensity ($D_B/D_{II} = 0.337$ and 0.290, respectively) than that corresponding to peak 3. This fact, along with the hypsochromic shifts of their absorption maxima relative to the all-*E* isomer (8 and 10 nm, respectively) may indicate that they corresponded to di-*Z* isomers, which have been reported in other sources (Kishimoto et al., 2004). As for peaks 5 and 6, they both showed smooth *cis* peaks ($D_B/D_{II} = 0.082$ and 0.077, respectively) and virtually identical absorption maxima (326, 414, 436 and 462; 326, 412, 434 and 462 nm), being identified as the peripheral (9*Z*)- or (9′*Z*)-lutein epoxide isomers.

In quantitative terms, it was clearly observed that the major isomer was all-*E*-lutein epoxide (peak 4), although high amounts of the isomers identified as (9*Z*)- or (9′*Z*)-lutein epoxide were also found (Fig. 3). According to the findings of Toth and Szabolcs (1981), who did not find (9′*Z*)-lutein epoxide in the non-photosynthetic tissues of several higher plants, (9′*Z*)-lutein epoxide may be an artifact formed during the analysis.

Table 2
Chromatographic and spectral features of the compounds obtained by treating lutein with 3-chloroperoxybenzoic acid

Peak	r_t (min)[a]	Carotenoid	Absorption maxima (nm)		
1	12.99	Trihydroxy-derivative	416	438	468
2	13.92	Trihydroxy-derivative	416	438	468
3	16.04	Lutein epoxide diastereoisomer	416	438	468
4	16.73	Lutein epoxide diastereoisomer	416	438	468
5	20.06	Unidentified	422	444	470
6	24.08	Rest of lutein standard	422	444	472

[a] r_t: retention time.

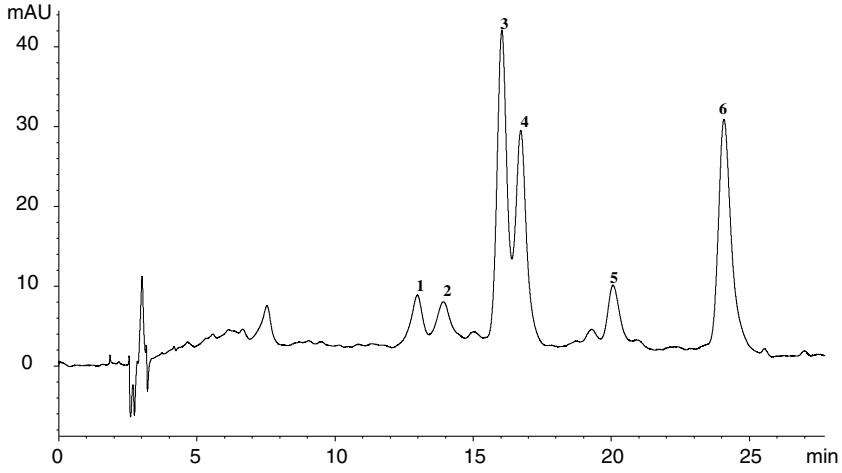


Fig. 4. Chromatogram at 430 nm of an extract of lutein standard treated with 3-chloroperoxybenzoic acid (peak identification in Table 2).

3. Experimental

3.1. Samples

The flowers of dandelion (*Taraxacum officinale* F. Weber ex Wiggers) were collected at lawn fields located in the same area of Liverpool (United Kingdom), during the summer of 2004. The samples were kept in a freezer at −18 °C until analysis. Before extraction, the petals were carefully separated and the remaining material (sepals, flower stalks, etc.) was discarded.

3.2. Pigment extraction and saponification

The petals of dandelion (10 g approximately) were cut up and subsequently transferred to a round flask containing 250 ml of acetone and 250 ml of a methanolic solution of KOH (10% w/v) under a nitrogen atmosphere. The methanolic solution was used not only for the hydrolysis of the esterified carotenoids, but also to contribute to the breakage of the petals to enhance the extraction of pigments. After 12 hours, the pigments were transferred to diethyl ether in a separatory funnel and the extract was washed four times with distilled water to remove any trace of alkali. Finally, the ethereal extract was taken to dryness in a rotary evaporator at a temperature below 35 °C.

3.3. Thin-layer chromatography (TLC)

A primary evaluation of the carotenoid profile was carried out by using silica gel TLC aluminium sheets (Merck, Darmstadt, Germany) and a mixture of diethyl ether and petroleum ether (40–60 °C) (3:1) as mobile phase. An aliquot of the extract was dissolved in diethyl ether and co-chromatographed with lutein standard, obtained from spinach leaves according to standard procedures (Britton, 1995b), to determine the location of the band corresponding to dihydroxycarotenoids. After half an hour of elution the TLC aluminium sheet was left to dry exposed to air. A few minutes later it was clearly seen that the band below the one corresponding to dihydroxycarotenoids turn greenish, which revealed the presence of monoepoxycarotenoids, whereas the band below the latter turn bluish, which revealed the presence of diepoxycarotenoids (Eugster, 1995).

The rest of the extract was chromatographed on laboratory-made $60GF_{254}$ (Merck, Darmstadt, Germany) silica gel plates (20 × 20 cm, 1 mm thickness) using the same mobile phase. The inherent acidity of silica was reduced to some extent by adding a pellet of NaOH and by moving the plate over a flask containing NH_3. To determine the location of the dihydroxycarotenoid fraction, a little amount of lutein standard was co-chromatographed on every plate. After one hour, the band located between the one corresponding to dihydroxycarotenoids and the bluish one was scraped from the plates. To remove rests from other bands, the band of interest was re-chromatographed on laboratory-made aluminium G type E (Merck, Darmstadt, Germany) plates (20 × 20 cm, 0.5 mm thickness) using a mixture acetone: petroleum ether (40–60 °C) (3:7). After one hour, the main band was scraped from the plate. The extract was kept dry at −18 °C and under a nitrogen atmosphere until analysis.

3.4. Identification

Identification of lutein epoxide was carried out by routine procedures. For that purpose the chromatographic behaviour and the colour on silica gel plates were taken into account, as well as the test for detecting 5,6-epoxy groups by chemical derivatization (Eugster, 1995) and its UV/vis and mass spectra. The extract was also co-chromatographed with a mixture of lutein and lutein epoxide, obtained by treating lutein with 3-chloroperoxybenzoic acid (Barua, 2001; Barua and Olson, 2001). The co-chromatography was carried out on silica gel TLC aluminium sheets (Merck, Darmstadt, Germany) using a mixture diethyl ether:petroleum ether (40–60 °C) as solvent system. The UV/vis spectra ($\Delta\lambda = 2$ nm) were recorded on a HP 8452 diode array spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). The electron impact mass spectrum (EI-MS) was obtained with a Micromass AutoSpec instrument (Micromass, Manchester, UK) with a direct insertion probe system at 70 eV. The temperature of the ion source chamber was 230–240 °C. Immediately before mass spectrometry, the extract was purified through an alumina minicolumn (Brockmann activity grade III) as recommended (Britton and Young, 1993; Mercadante et al., 1998), and concentrated to dryness.

3.5. High-performance liquid chromatography

HPLC analyses were carried out by means of an Agilent 1100 system, equipped with a quaternary pump, a photodiode array detector and a column temperature control module (Agilent, Palo Alto, CA, USA). A 20 μl loop and a C_{30} column (5 μm, 250 × 4.6 mm) (YMC, Wilmington, NC, USA) 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 (Mouly et al., 1999; Meléndez-Martínez et al., 2005a,b): 0 min: 90% MeOH + 5% TBME + 5% water; 12 min: 95% MeOH + 5% TBME; 25 min: 89% MeOH + 11% TBME; 40 min: 75% MeOH + 25% TBME; 60 min: 50% MeOH + 50% TBME; 62 min: 90% MeOH + 5% TBME + 5% water. MeOH and TBME contained a small proportion of butylated hydroxytoluene (BHT) (0.1%) and triethylamine (0.05%) in order to protect the carotenoids during the chromatographic analysis (Hart and Scott, 1995).

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