# Fruit Juice Carotenol Fatty Acid Esters and Carotenoids As Identified by Matrix-Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry†

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Gradient and isocratic HPLC separated as many as 38 carotenoid components in extracts from fruit juices. Several oxygenated carotenoids (xanthophylls), hydrocarbon carotenoids, and carotenol mono- and bis-fatty acid esters were identified. Structural elucidation was based on UV/vis spectroscopy, matrix-assisted laser desorption ionization (MALDI) post-source-decay (PSD) mass spectrometry, and comparison with synthetic reference compounds. Straight-chain fatty acid esters (caprate, laurate, myristate, palmitoleate, palmitate, oleate) of β-cryptoxanthin were identified as major carotenoid constituents in tangerine juice concentrate. Fatty acid components were identified by MALDI PSD fragment ion analysis. The xanthophylls violaxanthin, luteoxanthin, neoxanthin, taraxanthin, antheraxanthin, mutatoxanthin, β-cryptoxanthin-5,6:5′,6′-diepoxide, lutein, zeaxanthin, α-cryptoxanthin, and β-cryptoxanthin were present in the extracts of saponified tangerine concentrate. The chromatographic profiles of orange juice concentrate showed marked differences from those of tangerine concentrate. In the saponified extract, the oxygenated carotenoid zeaxanthin predominated over β-cryptoxanthin.

**Keywords:** *Carotenoids; carotenol esters; carotenoid analysis; mass spectrometry; matrix-assisted laser desorption ionization*

## INTRODUCTION

Here we report on the use of a novel hybrid analytical protocol (Kaufmann et al., 1996) to determine the content and heterogeneity of carotenoids and their fatty acid esters extracted from natural sources such as tangerine and orange juice concentrates. This protocol involves comparison of HPLC retention times and UV/vis absorption spectra with reference compounds and analysis of isolated carotenoids by means of matrix-assisted laser desorption ionization (MALDI) post-source-decay (PSD) mass spectrometry.

Carotenoid uptake and distribution in human tissues has attracted considerable attention in recent years (Krinsky, 1993; Gerster, 1993). Carotenoids are among the most abundant micronutrients in fruits and vegetables. We recently investigated the uptake of β-cryptoxanthin into human chylomicrons after ingestion of tangerine juice concentrate, which is rich in β-cryptoxanthin esters (Wingerath et al., 1995). β-Cryptoxanthin is an important precursor of vitamin A (Zechmeister, 1962) and exceeds the amounts of α-carotene present in human serum and tissues (Stahl et al., 1992). Nutritional sources of β-cryptoxanthin are fruits such as mango, peach, papaya, orange, and tangerine (Mangels et al., 1993). Cryptoxanthin and other hydroxylated carotenoids in these fruits mainly occur as fatty acid esters. Leading carotenoid esters in tangerines and oranges were identified as β-cryptoxanthin laurate, myristate, and palmitate by Philip et al. (Philip, 1973; Philip et al., 1988, 1989).

Conventional analytical protocols for the identification of carotenoids and their fatty acid esters in biological samples rely on separation by reversed-phase HPLC and comparison of UV/vis absorption spectra with reference material. However, factors such as cis−trans isomerization, coelution, and the relative similarity of many absorbance spectra do not constitute unequivocal characterization (Schmitz et al., 1992; van Breemen et al., 1993). Characterization can be improved by using mass spectrometry, which provides molecular weight and characteristic fragment ions for unambiguous structural elucidation [Caccamese and Garozzo, 1990; Lusby et al., 1992; van Breemen et al., 1993, 1995; for review see Taylor et al. (1990); Enzell and Back, 1995].

With the introduction of new desorption techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), high molecular mass organic compounds have become accessible to mass spectrometry. MALDI (Karas and Hillenkamp, 1988) especially is an extremely sensitive (femtomole level) tool for mass analysis of peptides and proteins and has rapidly extended its applicability to other groups of "difficult compounds", such as oligosaccharides, oligonucleotides, and phospholipids (Kaufmann, 1995). Post-source decay (PSD) is a recent extension of the MALDI time-of-flight mass spectrometry and provides for true structural information on such molecules (Spengler et al., 1991; Spengler and Kaufmann, 1992; Kaufmann et al., 1994). This technique allows one to select a precursor ion of interest and to record fragment ions formed by unimolecular decay processes that occur during the passage of metastable precursor ions along the first field-free drift path (Kaufmann et al., 1994).

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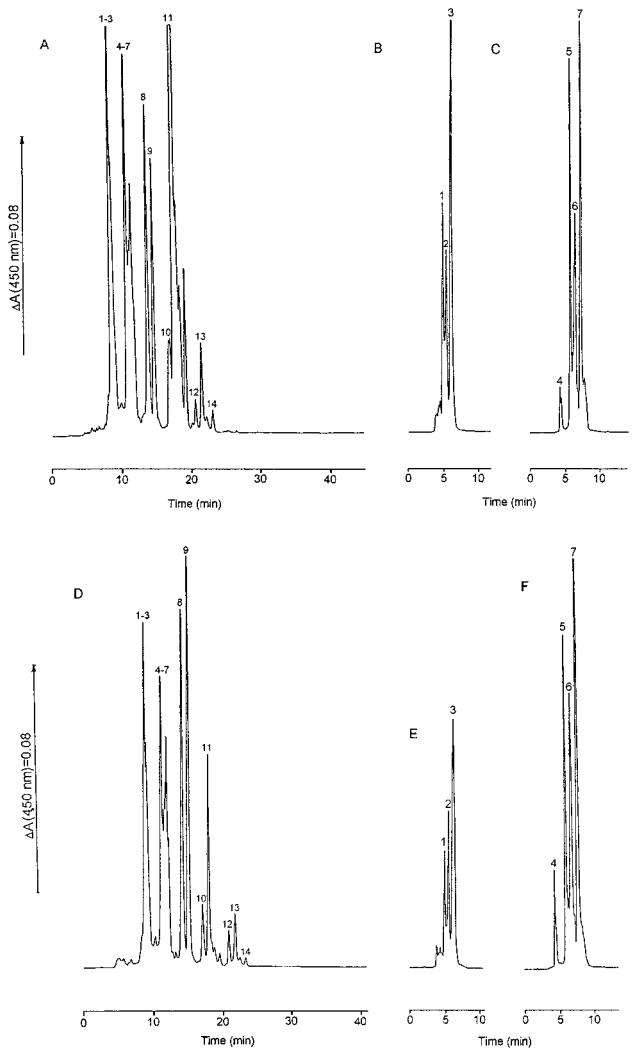


**Figure 1.** HPLC profiles of tangerine and orange concentrate extracts after saponification: (A) tangerine concentrate extract after methanolic potassium hydroxide saponification (eluent A); (B) extract A, peaks 1−3 (eluent B); (C) extract A, peaks 4−7 (eluent B); (D) orange concentrate extract after methanolic potassium hydroxide saponification (eluent A); (E) extract D, peaks 1−3 (eluent B); (F) extract D, peaks 4−7 (eluent B). For peak identification see Tables 1 and 2.

## MATERIALS AND METHODS

**Apparatus.** High-performance liquid chromatography was carried out with a Merck/Hitachi Model 655 A-12 ternary solvent delivery system equipped with a Merck/Hitachi Model L-4200 UV/vis detector and a Merck/Hitachi L-5000 LC controller (Merck, Darmstadt, Germany). For spectrophotometric peak identification, we used a Model 168 diode array detector (Beckman, Munich, Germany). The absorption spectra of carotenoids were recorded between 230 and 600 nm at the rate of 12 spectra/min. Mass spectra were obtained by positive matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).

As described in detail by Kaufmann et al. (1996), two instruments built in-house were employed. TOF-MS1 is a so-called linear machine that was utilized for obtaining survey mass spectra on the various HPLC fractions. TOF-MS2 is equipped with a reflectron, which not only gives higher precision and accuracy of molecular masses but, at the same time, provides for the option of structural elucidation by means of so-called post-source-decay (PSD) fragment ion mass analysis. Both instruments were equipped with a pulsed nitrogen laser ($\lambda = 337$ nm) for desorption. Kinetic energy of the ions was 16 kV; the initial acceleration field in the split acceleration stage is accurate to 4 kV cm$^{-1}$.

Samples for MALDI analyses were prepared by redissolving the HPLC fractions in about 20 $\mu$L of acetone. To this solution was added 5−10 $\mu$L of acetone saturated with 2,5-dihydroxybenzoic acid (DHB). A 5−10 $\mu$L aliquot of this mixture was pipetted onto the surface of a slightly preheated sample holder, where the solvent evaporated within a few seconds.

**Reagents and Materials.** Tangerine (*Citrus reticulata*, from Pakistan) and orange concentrates (*Citrus sinensis*, from Brazil) were kindly provided by Dr. H. Gründing, Krings Fruchtsaft AG (Mönchengladbach, Germany). The reference samples of *all-trans-β*-cryptoxanthin, lutein, zeaxanthin, α-carotene, and 13-*cis-β*-carotene were a gift from Dr. J. Bausch Hoffmann-La Roche (Basel, Switzerland). *all-trans-β*-Carotene was purchased from Fluka (Basel, Switzerland). *β*-Apo-8′-carotenol (internal standard) was prepared from *β*-apo-8′-carotenal (Fluka) upon reduction with lithium aluminum hydride (Merck, Darmstadt, Germany). All solvents used were HPLC-grade and obtained from Merck.

Straight, long-chain fatty acid esters were prepared by partial synthesis from the parent carotenoids and the appropriate fatty acid chlorides (Merck) according to published methods (Khachik and Beecher, 1988a). Epoxidized carotenoids (free or esterified) were prepared by epoxidation of the corresponding carotenoid fatty acid esters or free carotenoids

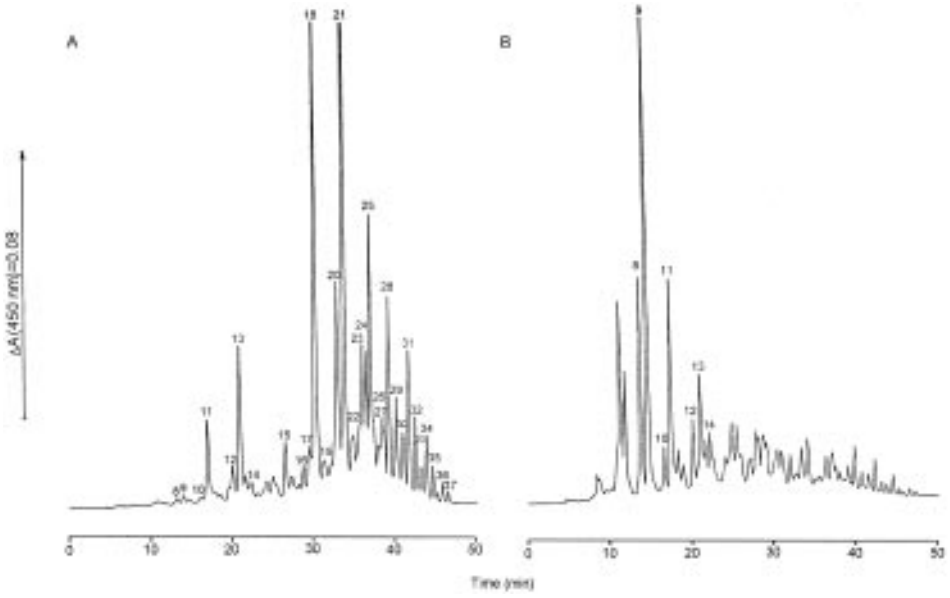


**Figure 2.** HPLC profiles of unsaponified tangerine and orange concentrate extracts: (A) tangerine concentrate extract (eluent A); (B) orange concentrate extract (eluent A). For peak identification see Tables 1 and 2.

**Table 1. Peak Identification of the Various Xanthophylls and Their Epoxides in Saponified Tangerine Juice Concentrate Extract Separated by HPLC (Eluents A and B)**

| peak | component | $\lambda_{max}$ (nm) | $\lambda_{max}$ after addition of traces of methanolic HCl (nm) | UV/vis absorption comparison with synthetic sample $r > 0.990$[b] | MALDI $M^{\bullet+}$ ($m/z$) | PSD analysis |
|---|---|---|---|---|---|---|
| 1 | violaxanthin | 414/439/468 | 380/400/425 | 0.998 | 600 | +[c] |
| 2 | luteoxanthin | 399/420/447 | 380/400/425 | −[a] | 600 | + |
| 3 | neoxanthin | 411/434/463 | 397/421/448 | − | 600 | + |
| 4 | taraxanthin | 421/445/472 | 399/421/447 | 0.998 | 584 | −[a] |
| 5 | antheraxanthin | 421/445/470 | 404/427/452 | 0.995 | 584 | + |
| 6 | mutatoxanthin | 404/427/452 | 404/427/452 | 0.998 | 584 | + |
| 7 | β-cryptoxanthin5,6:5′6′-diepoxide | 415/442/465 | 404/426/452 | − | 584 | − |
| 8 | lutein | 422/445/474 | − | 0.996 | 568 | + |
| 9 | zeaxanthin | 426/451/478 | − | 0.997 | 568 | + |
| 10 | α-cryptoxanthin | 421/446/475 | − | − | − | + |
| 11 | β-cryptoxanthin | 429/451/478 | − | 0.996 | 552 | + |

[a] Not measured. [b] $r$, Correlation coefficient comparing the UV/vis spectra of reference compounds with those of fruit juice carotenoids. Spectra were obtained by diode array spectroscopy. Calculation of $r$ was achieved with "gold software" (Beckman, Munich). [c] +, Structural elucidation based on PSD analysis.

with 4-chloroperbenzoic acid (MCPBA) (Merck). Upon the addition of traces of methanolic hydrochloric acid these compounds were converted to the corresponding 5,8-epoxides.

**Extraction.** Tangerine concentrate (0.5 g) was diluted in 100 mL of water (10% sodium chloride) and extracted five times with 50 mL of diethyl ether. The combined organic layers were washed several times with 50 mL of water and dried over sodium sulfate. The extract was filtered and the solvent was removed on a rotary evaporator at 28 °C. The residue was dissolved in 5 mL of dichloromethane/*n*-hexane (1/1) and stored at −20 °C until analysis. For HPLC analysis an appropriate volume was evaporated under a stream of nitrogen and redissolved in HPLC solvent A. For each analysis 50 μL was injected. All steps were performed under diminished light.

**Saponification.** An ethereal solution of tangerine concentrate extract was treated with satured methanolic potassium hydroxide for 30 min under nitrogen in the dark at room temperature. The solution was diluted with diethyl ether and washed several times with water (10% sodium chloride). The organic layer was dried over sodium sulfate and evaporated to dryness, and the residue was dissolved in the appropriate solvent for chromatographic analysis.

**Chromatographic Procedures.** The analytical separations were carried out under two sets of HPLC conditions employing eluents A and B; for preparative separations eluent C was employed. The chromatographic separation by eluent A was carried out as described by Khachik et al. (1989) with slight modifications.

*Eluent A.* A mixture of methanol/acetonitrile/dichloromethane/*n*-hexane (10/85/2.5/2.5) was applied isocratically for 5 min; from 5 to 40 min a linear gradient was applied to methanol/acetonitrile/dichloromethane/*n*-hexane (10/45/22.5/22.5). The final eluent was held for another 10 min. The flow rate was 0.7 mL/min.

*Eluent B.* This eluent consisted of a mixture of methanol/acetonitrile/2-propanol (54/44/2) and was used for the separation of the epoxidized carotenoids on the C-18 reversed-phase column. The flow rate was 1 mL/min.

*Eluent C.* Carotenol fatty acid esters prepared by partial synthesis were purified by preparative HPLC employing methanol/acetonitrile/dichloromethane/*n*-hexane (20/40/20/20) at a flow rate of 4 mL/min. Carotenoids were detected at 450 nm.

*Columns.* Analytical separations were performed on a 5 μm Suplex pKb 100 column (250 mm length × 4.6 mm i.d.) (Supelco, Bellefonte, PA) with a 20 mm guard column and on a 5 μm C-18 reversed-phase column (Lichrospher, 250 mm × 4.6 mm) (Merck) with a 20 mm guard column. The preparative separations were carried out on a 7 μm C-18 reversed-phase column (Lichrospher) (Merck).

**Partial Synthesis of Carotenol Fatty Acid Esters and Their Corresponding Epoxidation Products.** Synthesis of carotenol mono- and bis-fatty acid esters of β-cryptoxanthin, lutein, and zeaxanthin followed the procedure described by Khachik et al. (1988a,b) with slight modifications. Therefore, only the preparation of some model zeaxanthin fatty acid esters is described here.

A solution of zeaxanthin (10 μmol) in 100 μL of dichloromethane (dried over 4Å molecular sieves) was added to a

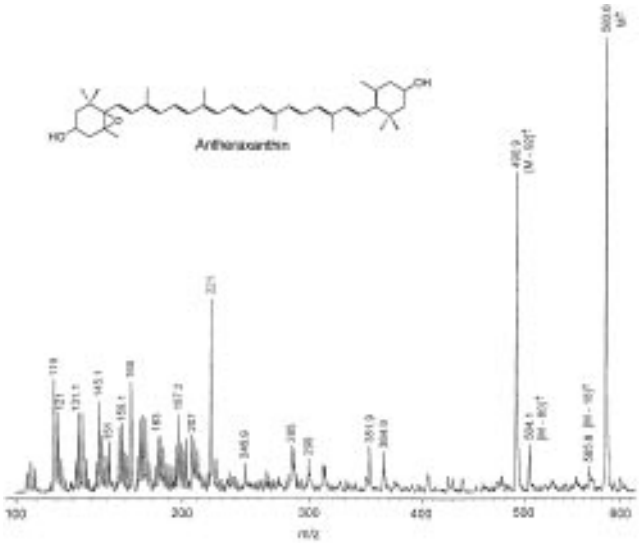


**Figure 3.** MALDI post-source-decay (PSD) fragment ion mass analysis of HPLC peak 5. Peak 5 of the HPLC trace shown in Figure 1C was identified as *all-trans*-antheraxanthin; see text.

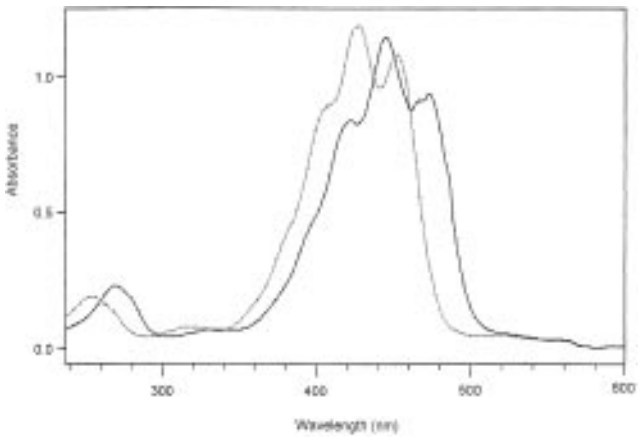


**Figure 4.** UV/vis absorption spectra of HPLC peak 5 before and after treatment with catalytic amounts of acid. Peak 5 was identified as *all-trans*-antheraxanthin [(—) $\lambda_{max}$ 421/445/470 nm] and the acid-catalyzed rearrangement product as *all-trans*-mutatoxanthin [(···) $\lambda_{max}$ 404/427/452 nm].

**Table 2. Peak Identification of the Various Carotenoids of Tangerine Juice Extract Separated by HPLC (Eluent A)**

| chemical class | peak | component | $\lambda_{max}$ (nm) | UV/vis absorption comparison with synthetic sample $r > 0.990$[c] | MALDI $M^{\bullet+}$ ($m/z$)[a] | PSD analysis |
|---|---|---|---|---|---|---|
| xanthophylls | 8 | *all-trans*-lutein | 445 | 0.995 | 568 | +[d] |
| | 9 | *all-trans*-zeaxanthin | 451 | 0.997 | 568 | + |
| | 10 | *all-trans*-α-cryptoxanthin | 446 | –[b] | – | + |
| | 11 | *all-trans*-β-cryptoxanthin | 451 | 0.996 | 552 | + |
| hydrocarbon | 12 | *all-trans*-α-carotene | 442 | 0.993 | 536 | + |
| carotenoids | 13 | *all-trans*-β-carotene | 450 | 0.995 | 536 | + |
| | 14 | 13-*cis*-β-carotene | 445 | – | 536 | – |
| carotenol | 15 | β-cryptoxanthin caprate | 451 | 0.999 | 706 | + |
| mono-fatty acid esters | 16 | lutein monomyristate | 444 | 0.999 | 778 | – |
| | 17 | α-cryptoxanthin laurate | 445 | – | 734 | – |
| | 18 | β-cryptoxanthin laurate | 449 | 0.999 | 734 | + |
| | 19 | zeaxanthin monopalmitate | 451 | 0.999 | 804 | + |
| | 20 | β-cryptoxanthin palmitoleate | 452 | – | 788 | + |
| | 21 | β-cryptoxanthin myristate | 449 | 0.999 | 762 | + |
| | 23 | β-cryptoxanthin oleate | 452 | 0.999 | 816 | + |
| | 25 | β-cryptoxanthin palmitate | 450 | 0.999 | 790 | + |
| carotenol | 22 | zeaxanthin dicaprate | 449 | – | 876 | – |
| bis-fatty acid esters | 24 | mutatoxanthin laurate myristate | 427 | – | 977 | – |
| | 26 | mutatoxanthin laurate oleate | 426 | – | 1031 | – |
| | 27 | lutein caprate laurate | 445 | – | 905 | – |
| | 28 | mutatoxanthin dimyristate | 429 | – | 1005 | – |
| | | antheraxantin dimyristate | 445 | – | 1005 | – |
| | 29 | zeaxanthin laurate myristate | 450 | 0.996 | 961 | + |
| | 30 | antheraxanthin myristate oleate | 446 | – | 1059 | + |
| | 31 | antheraxanthin myristate palmitate | 446 | 0.999 | 1033 | + |
| | 32 | zeaxanthin dimyristate | 450 | 0.999 | 989 | – |
| | 33 | taraxanthin palmitate stearate | 445 | – | 1089 | – |
| | 34 | antheraxanthin dipalmitate | 445 | 0.993 | 1061 | + |
| | 35 | zeaxanthin myristate palmitate | 452 | 0.992 | – | – |
| | 36 | lutein dipalmitate | 448 | 0.994 | – | – |
| | 37 | zeaxanthin dipalmitate | 451 | 0.998 | – | – |

[a] Mass assignments represent average masses of the componds rounded to the next integer. [b] Not measured. [c] $r$, Correlation coefficient (see Table 1). [d] +, Structural elucidation based on PSD analysis.

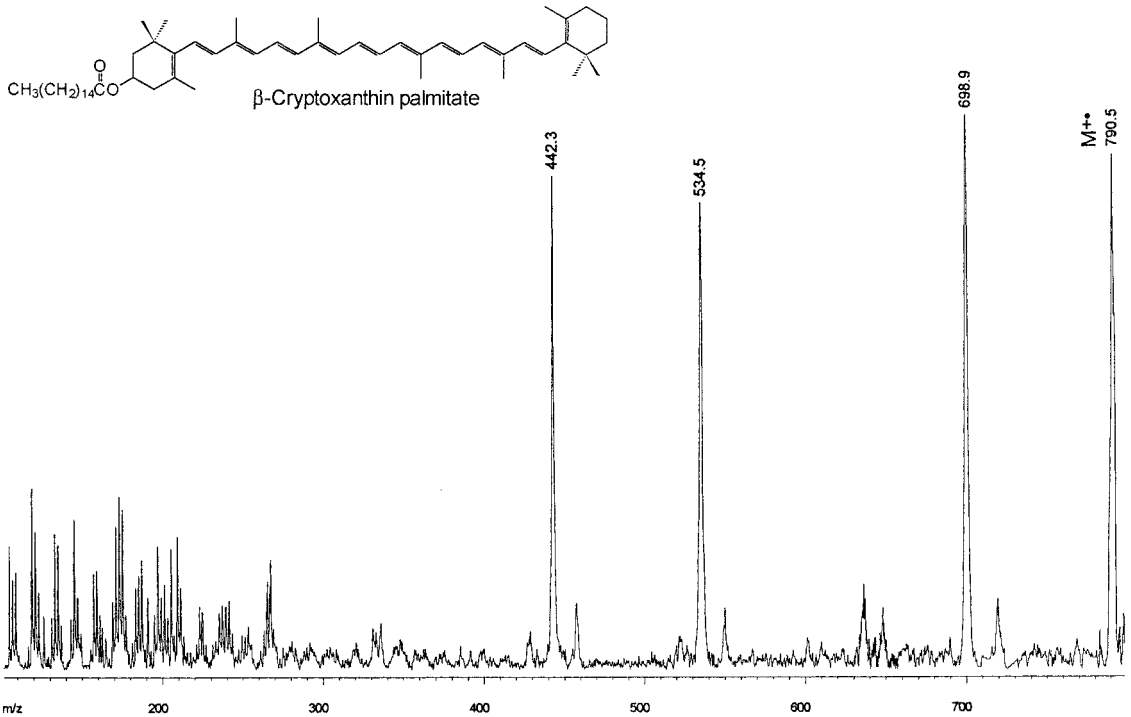


**Figure 5.** MALDI PSD fragment ion mass analysis of HPLC fraction peak 25. The MALDI PSD mass spectrum of the precursor analyte in HPLC peak 25 shows the parent ion at $m/z$ 790. The analyte was confirmed to be β-cryptoxanthin palmitate.

mixture of myristoyl chloride (200 μmol), palmitoyl chloride (200 μmol) (1/1), and triethylamine (300 μmol) in 400 μL of dichloromethane. The mixture was stirred under an atmosphere of nitrogen at 32−36 °C for 55 min. The progress of the reaction was controlled by C-18 reversed-phase thin-layer chromatography (aluminum sheets, silica gel 60 $F_{254}$ precoated, Merck), employing cyclohexane/ether (4/1) as the mobile phase. After completion of the reaction, the product was diluted with ether and washed with water (10% sodium hydrogen carbonate) several times. The organic layer was dried over sodium sulfate, and the solvent was evaporated. Six reaction products (three minor and three major) were shown to be present by preparative HPLC, employing eluent C, and collected by hand for further identification. They were identified from HPLC retention times, UV/vis absorption, and mass spectra as (1) unreacted zeaxanthin, (2) zeaxanthin monomyristate, (3)

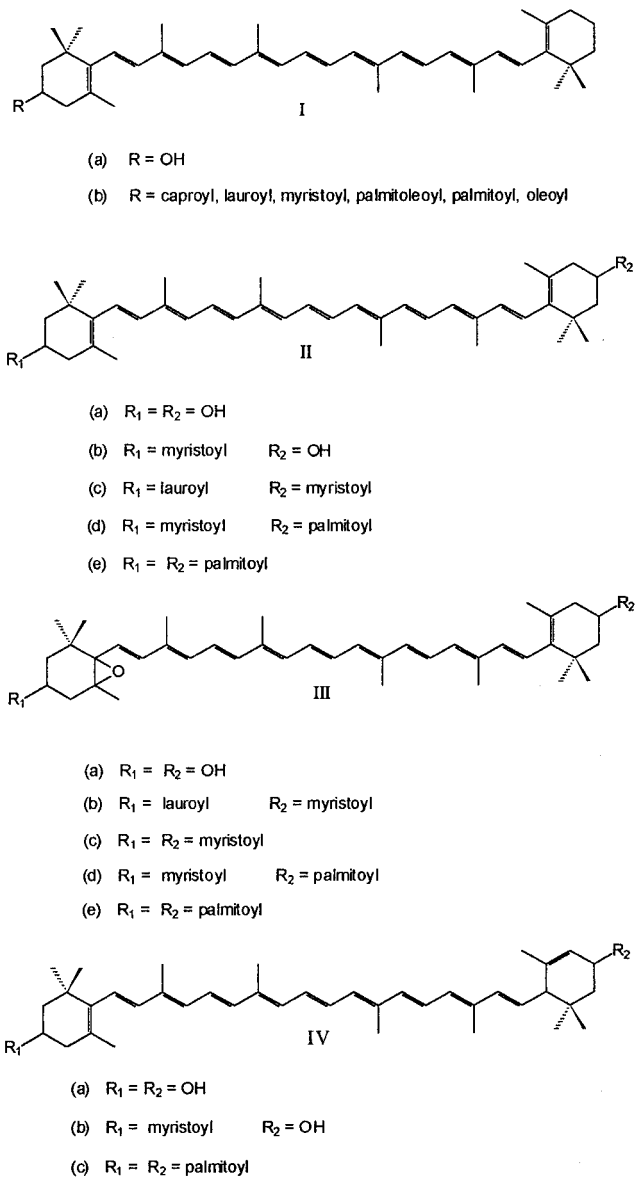


**Figure 6.** Chemical structures of some of selected carotenoids and carotenol fatty acid esters in tangerine concentrate: (Ia) $\beta$-cryptoxanthin; (Ib) $\beta$-cryptoxanthin fatty acid esters; (IIa) zeaxanthin; (IIb) zeaxanthin mono-fatty acid ester; (IIc,d) mixed zeaxanthin bis-fatty acid esters; (IIe) zeaxanthin bis-fatty acid ester; (IIIa) antheraxanthin; (IIIb,d) mixed antheraxanthin bis-fatty acid esters; (IIIc,e) antheraxantin bis-fatty acid esters; (IVa) lutein; (IVb) lutein mono-fatty acid ester; (IVc) lutein bis-fatty acid ester. Only the *all*-trans isomers are shown.

zeaxanthin monopalmitate, (4) zeaxanthin dimyristate, (5) zeaxanthin myristate palmitate, and (6) zeaxanthin dipalmitate.

A solution of the zeaxanthin myristate palmitate ester (0.06 $\mu$mol) in 400 $\mu$L of dichloromethane was allowed to react with 4-chloroperbenzoic acid (0.12 $\mu$mol) at room temperature for 50 min. The product was worked up as mentioned earlier and was shown by HPLC to consist of three components identified from UV/vis absorption spectra as (1) violaxanthin myristate palmitate, (2) antheraxanthin myristate palmitate, and (3) unreacted zeaxanthin myristate palmitate.

**Quantification of the Carotenoids and Related Fatty Acid Esters.** The concentrations of carotenoids and related fatty acid esters were calculated from calibration curves (slope of the regression line) generated from the ratios of the peak height of the carotenoids and fatty acid esters to the peak height of the internal standard ($\beta$-apo-8′-carotenol) in calibration samples and expressed as nanomoles/gram tangerine or orange concentrate.

## RESULTS AND DISCUSSION

The HPLC chromatograms of carotenoids after saponification are presented for extracts of tangerine (Figure 1A−C) and orange concentrates (Figure 1D−F). The concentration of $\beta$-cryptoxanthin was found to be much higher in the tangerine than in the orange, in which the leading carotenoid after saponification was zeaxanthin. Peak assignments based on MALDI and UV/vis spectroscopy of the various xanthophylls and their epoxides in saponified tangerine concentrate extracts (as given in Table 1) are basically in agreement with published data (Gross et al., 1972; Lin and Cheng, 1995).

The HPLC chromatograms of carotenoids in unsaponified extracts of tangerine and orange concentrates are shown in Figure 2A,B. The chromatographic profiles show marked differences in the intensity ratios of carotenoids and carotenol fatty acid esters. While free carotenoids dominate in the orange, carotenol fatty acid esters are predominant in tangerine (Figure 2A), eluting after $\beta$-carotene between 25 and 48 min. $\beta$-Cryptoxanthin was found to be esterified with capric, lauric, myristic, palmitoleic, palmitic, and oleic acids. Myristic acid was by far the most abundant esterified fatty acid in tangerine. $\beta$-Cryptoxanthin esters were high in tangerines compared to oranges [see also Philip et al. (1989)]. Tables 1 and 2 list all major constituents (carotenoids and related carotenol fatty acid esters) separated by HPLC from tangerine concentrate extract in the order of elution (Suplex pKb 100) during a 48 min run.

The classes of chemical compounds identified in tangerine juice concentrate are (a) xanthophylls (peaks 1−11), (b) hydrocarbon carotenoids (peaks 12−14), (c) carotenol mono-fatty acid esters (peaks 15−21, 23, 25), and (d) bis-fatty acid esters (peaks 22, 24, 26−37). The tentative identification of these compounds was initially based on the comparison of HPLC retention times and UV/vis absorption spectra with those of authentic samples. Subsequently, in most of the samples structural elucidation was performed by MALDI and MALDI PSD mass spectrometry. In some cases the structure was further verified by means of a rearrangement reaction catalyzed by small amounts of HCl (see Figure 4).

**Xanthophylls.** Saponification of carotenol fatty acid esters regenerates the parent hydroxycarotenoids. The major xanthophylls found in the saponified tangerine extract are listed in order of chromatographic elution in Table 1.

In the PSD spectra of these xanthophylls, we found characteristic fragments such as $[M - 18]^{\bullet+}$, indicating the presence of a hydroxyl group, and $[M - 92]^{\bullet+}$ (M − toluene) and $[M - 106]^{\bullet+}$ (M − xylene) as products of typical chain eliminations. This is consistent with observations in FAB-MS (van Breemen et al., 1995). The epoxyxanthophylls were identified from the fragmentation pattern of their PSD spectra, which showed characteristic fragments at $[M - 80]^{\bullet+}$ ($[M - C_6H_8]^{\bullet+}$), as well as a fragment at 221 ($[204 + \text{hydroxyl group}]^{\bullet+}$) (Vetter et al., 1971; Enzell et al., 1995; Kaufmann et al., 1996). Figure 3 shows the MALDI PSD mass spectrum of antheraxanthin. The position of the epoxide structures could not be identified unequivocally since the 5,6-epoxides and their rearrangement products (5,8-epoxides) showed the same fragmentation pattern (Vetter et al., 1971; Johannes et al., 1974). Coupling of the mass spectral data with the information obtained

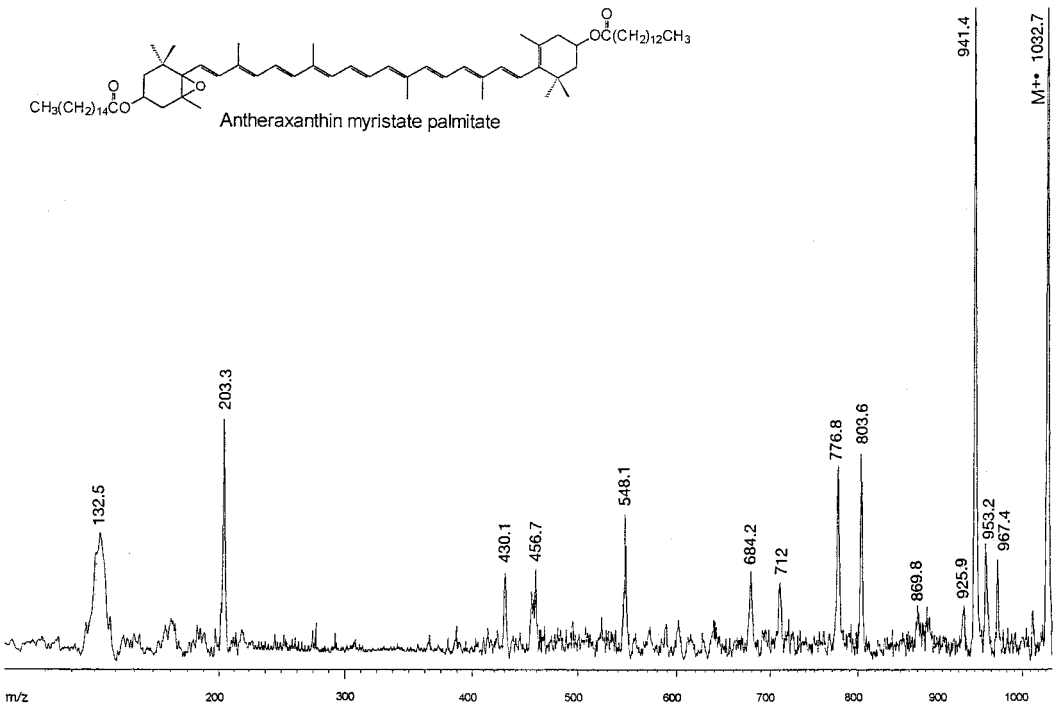


**Figure 7.** MALDI PSD fragment ion mass analysis of HPLC fraction peak 31. The MALDI PSD mass spectrum of the precursor analyte in HPLC peak 31 shows the precusor ion at $m/z$ 1033. The analyte was unequivocally assigned to be antheraxanthin myristate palmitate.

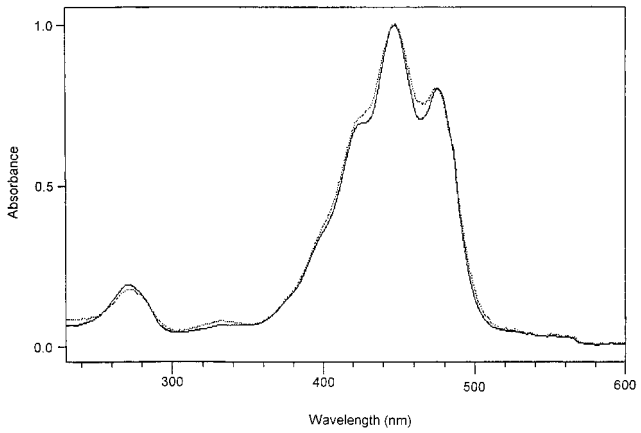


**Figure 8.** Comparison of the UV/vis absorption spectra of HPLC peak 31 with synthetic *all-trans*-antheraxanthin myristate palmitate. Antheraxanthin myristate palmitate from peak 31 (Table 2; Figure 7) [(···) $\lambda_{max}$ = 447 nm] is compared with synthetic *all-trans*-antheraxanthin myristate palmitate [(—) $\lambda_{max}$ = 447 nm]; monitored in the HPLC system (eluent A).

**Table 3. Quantitative Distribution of Some Xanthophylls, Hydrocarbon Carotenoids, and Mono- and Bis-Fatty Acid Esters in Tangerine Juice Concentrate before and after Saponification (Level of Carotenoids in Orange Concentrate Given in Parentheses)**

| compound | unsaponified concentrate (nmol/g) | ratio T/O[b] | saponified concentrate (nmol/g) | ratio |
|---|---|---|---|---|
| $\beta$-cryptoxanthin | 10 (3.6) | 2.8 | 156 (5.6) | 27.9 |
| lutein | 0.3 (2.7) | 0.1 | 26 (5.7) | 4.6 |
| zeaxanthin | 0.6 (6.8) | 0.1 | 22 (10) | 2.2 |
| α-carotene | 2.5 (0.9) | 2.8 | 2 (0.7) | 2.9 |
| $\beta$-carotene | 14 (2.3) | 6.1 | 14 (1.8) | 7.8 |
| $\beta$-cryptoxanthin caprate | 6 | | nd[a] | |
| $\beta$-cryptoxanthin laurate | 50 | | nd | |
| $\beta$-cryptoxanthin myristate | 56 | | nd | |
| $\beta$-cryptoxanthin oleate | 10 | | nd | |
| $\beta$-cryptoxanthin palmitate | 21 | | nd | |
| zeaxanthin dimyristate | 36 | | nd | |
| zeaxanthin myristate palmitate | 1.3 | | nd | |
| zeaxanthin dipalmitate | 0.3 | | nd | |
| lutein dipalmitate | 0.3 | | nd | |

[a] nd, not detectable (detection limit < 0.1 nmol/g concentrate). [b] T/O, Ratio of level in tangerine concentrate/orange concentrate.

by UV/vis absorption provided information on the nature of the end group and the chromophore involved. Table 1 shows the UV/vis absorption maxima of 11 xanthophylls present in tangerine or orange concentrate. Upon the addition of methanolic hydrogen chloride, isomerization of the 5,6-epoxides into 5,8-epoxides occurs rapidly. This well-known epoxide−furanoxide rearrangement (Karrer and Jucker, 1946) resulted in a 20 nm (one 5,6-epoxide group) or a 40 nm (two 5,6-epoxide groups) hypsochromic shift in the absorption maximum of the 5,6-epoxide carotenoid.

Figure 4 shows the UV/vis absorption spectra of peak 5 [$\lambda_{max}$ = 421/445/470 nm in HPLC solvent A] and its hydrogen chloride-catalyzed rearrangement product [$\lambda_{max}$ = 404/427/452 nm in HPLC solvent A]. By coupling these data with the information provided by mass spectrometry, peak 5 was identified as native *all-trans*-antheraxanthin.

**Hydrocarbon Carotenoids.** α-Carotene, *all-trans*-$\beta$-carotene, and its 13-cis isomer were shown to be the major hydrocarbon carotenoids in tangerine concentrate (see Table 2). Identification was based on UV/vis absorption and comparison of HPLC retention times of unknowns with those of reference compounds. Fragment ions observed in the MALDI PSD spectras are identical to those reported in the literature (van Breemen et al., 1995; Kaufmann et al., 1996).

**Carotenol Mono-Fatty Acid Esters.** The fatty acid esters of $\beta$-cryptoxanthin with caprate, laurate, myristate, palmitoleate, palmitate, and oleate, as well as α-cryptoxanthin laurate, were identified in tangerine concentrate extract. The structural identification of the mono-

fatty acid esters by means of MALDI TOF (PSD) mass spectrometry turned out to be unambiguous due to the following signature found in the PSD spectra: (1) cleavage of the fatty acid to form $[M - \text{fatty acid}]^{\bullet+}$; (2) loss of either $[M - 92]^{\bullet+}$ or $[M - 80]^{\bullet+}$ (diepoxides) or both (monoepoxides) from both the precursor fragment ion and the precursor minus fatty acid fragment ion; and (3) presence of a prominent fragment at $m/z$ 203 ($[m/z\ 221 - 18]^{\bullet+}$), indicative of the epoxidized species. The structure was confirmed by UV/vis absorption and comparison of these spectra with the obtained absorption spectra of synthetic carotenol fatty acid esters (see Table 2). PSD mass spectrometric analysis of peak 25 isolated by HPLC yielded a leading precursor ion signal at $m/z$ 790, corresponding to the quasi molecule of $\beta$-cryptoxanthin palmitate (see Figures 2A and 5). Characteristic fragment ions were observed at 698 $[M - \text{toluene}]^{\bullet+}$, 534 $[M - \text{palmitic acid}]^{\bullet+}$, and 442 $[M - \text{palmitic acid} - \text{toluene}]^{\bullet+}$, thus confirming the structure of $\beta$-cryptoxanthin palmitate.

The chemical structures of some of the predominant carotenoids and related carotenol fatty acid esters in tangerine concentrate are shown in Figure 6.

**Carotenol Bis-Fatty Acid Esters.** Structural elucidation of the carotenol bis-fatty acid esters followed basically the same protocol, combining mass spectrometry with a comparative UV/vis absorption spectroscopy and relating the absorption spectra of unknowns to those of reference compounds. As an example, we present here the data obtained in the case of peak 31 of the HPLC run in tangerine concentrate extract (see Figure 2A, Table 2).

Although this HPLC fraction turned out to be far from homogeneous, a leading precursor ion signal at $m/z$ 1033 was selected for further PSD fragment ion analysis (see Figure 7). Product ions formed by the elimination of $[M - 80]^{\bullet+}$ at $m/z$ 953 and $[M - 92]^{\bullet+}$ at $m/z$ 941 indicated that the xanthophyll present must be a monoepoxide. This notion is further supported by the observation of a prominent fragment ion at 203 ($[221 - 18]^{\bullet+}$) (see also preceding paragraphs). Product ions at 776 ($[M - 256]^{\bullet+}$) and 804 ($[M - 228]^{\bullet+}$), together with their even more abundant elimination of a toluene molecule to produce the ions 684 ($[M - 256 - 92]^{\bullet+}$) and 712 ($[M - 228 - 92]^{\bullet+}$) identify the fatty acids as palmitic and myristic acids, respectively. This is in line with the finding of a fragment ion at 548 ($[M - 256 - 228]^{\bullet+}$), which was obviously formed by the cleavage of both fatty acids. With this fragment apparently representing the xanthophyll moiety (minus two $H_2O$), one can easily deduce that this xanthophyll molecule—if present as a monoepoxide—can be only antheraxanthin or mutatoxanthin.

This ambiguity was clarified by finding $\lambda_{max}$ at 446 nm in the absorption spectra (see Figure 8). Thus, our hybrid structural elucidation protocol finally resulted in an unambiguous assignment, identifying the main constituent of the HPLC peak 31 as antheraxanthin myristate palmitate (in the case of a mutatoxanthin as a base residue, $\lambda_{max}$ would have been around 427 nm). Finally, an epoxide−furanoxide rearrangement reaction was carried out by adding methanolic HCl, which resulted in the expected −20 nm shift of $\lambda_{max}$ due to a transformation of antheraxanthin into mutatoxanthin.

Although many carotenoid fragment ions are identified in this study for the first time, some of the fragment ions by MALDI PSD MS are identical to those reported (van Breemen et al., 1995; Johannes et al., 1974). The absolute configurations of naturally occurring carotenol mono- and bis-fatty acid esters in tangerine concentrate shown here are not known.

**Quantitative Distribution of Some Carotenoids and Carotenol Fatty Acid Esters in Tangerine and Orange Juice Concentrates.** The quantitative distributions of some carotenoids and related carotenol fatty acid esters in tangerine concentrate before and after saponification are presented in Table 3 (concentration of carotenoids in orange concentrate extracts is given in parentheses). The carotenoid levels in tangerine concentrate are significantly higher than those in orange concentrate, although both concentrates had about the same dry weight (≈65%). $\beta$-Apo-8′-carotenol was used as internal standard for the quantitative determination of some carotenoids and carotenol fatty acid esters. Under the HPLC conditions (eluent A) employed, $\beta$-apo-8′-carotenol elutes between $\beta$-cryptoxanthin 5,6:5′,6′-epoxide and lutein. The absorption maximum ($\lambda_{max}$ = 403/426/451 nm) in HPLC solvent A is reasonably close to those of carotenoids and carotenol fatty acid esters. Under saponification and the HPLC conditions employed, the recovery of the internal standard was more than 96% as determined by the peak height of $\beta$-apo-8′-carotenal before and after saponification. Recovery of $\beta$-cryptoxanthin was >90% following the saponification of known amounts of synthetic $\beta$-cryptoxanthin esters.

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