HPLC and Mass Spectrometric Analysis of Carotenoids from Mango

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β-Carotene (*all-trans*), β-cryptoxanthin (*all-trans* and *cis*), zeaxanthin (*all-trans*), luteoxanthin isomers, violaxanthin (*all-trans* and *cis*), and neoxanthin (*all-trans* and *cis*) were identified in mango cultivar Keitt by means of HPLC data (retention time, co-injection, and chemical reactions). Mass spectrometry (molecular ion, fragmentation pattern, m/z relative intensity) confirmed the identity of these carotenoids (not the geometric form); additionally, lutein and mutatochrome were detected when a large amount of sample was utilized. The quantitative composition was determined by HPLC, Sudan I being used as internal standard. *all-trans*-Violaxanthin (21.1 ± 2.9 μg/g), *all-trans*-β-carotene (15.1 ± 1.5 μg/g), and a *cis*-violaxanthin (10.1 ± 0.2 μg/g), tentatively identified as 9-*cis*, were the principal carotenoids.

Keywords: *Mango carotenoids; HPLC; MS*

INTRODUCTION

The mango (*Mangifera indica* L.) is native to South and Southeast Asia. Among the tropical and subtropical fruits, it is one of the most important in terms of production, acreage, and popularity. The principal producing country is India, but Brazil is among the other major producing countries (Wu *et al.*, 1993).

The yellow-orange characteristic color of mango is due to the presence of carotenoids. Jungalwala and Cama (1963) found 16 carotenoids in the mango cultivar Alphonso from India. John *et al.* (1970) followed the carotenoid distribution during ripening of mango cv. Badami (also from India), detecting 15, 14, and 17 carotenoids at mature-green, partially ripe, and fully ripe stages, respectively. Godoy and Rodriguez-Amaya (1989) quantified eight carotenoids in five mango cultivars grown in Brazil. In the first two studies, 1 kg samples were analyzed, saponification was performed under rather drastic conditions, repetitive column chromatography was used to separate the carotenoids, and the relative percentages were presented. In the third work, much smaller samples were used, saponification conditions were milder, only one column chromatography step was employed, and the concentrations in micrograms per gram were reported. However, the epoxy carotenoids had to be separated by thin-layer chromatography (TLC) on silica. Recently, Cano and Ancos (1994) reported the separation of a series of esters and *cis*−*trans* isomeric carotenoids in mango cv. Alphonso by high-performance liquid chromatography (HPLC). The major carotenoids were reported as antheraxanthin and β-carotene. Surprisingly, on saponification, antheraxanthin disappeared completely and luteoxanthin, which was in minute amount in the original extract, became the principal carotenoid. No quantitative data were presented. Although natural variation among mango samples is expected because of cultivar differences, climatic effects, stage of maturity at harvest, and time after harvest, some of the discrepancies in reported results are apparently due to the analytical procedures.

Conclusive identification of the carotenoids and demonstration that they occur naturally and are not artifacts are needed, especially for the epoxycarotenoids, as well as determination of the quantitative composition. HPLC and mass spectrometry (MS) were used for this purpose in the present paper.

MATERIALS AND METHODS

Materials. Three different sample lots of mango, cultivar Keitt, produced in Bahia, Brazil, were bought at the fully ripe stage in Campinas, São Paulo, Brazil. For each sample, peel and seed of three fruits were removed, the pulp was homogenized, and 100 g was taken for identification and 15 g for quantification.

Identification of the Carotenoids. Initial provisional identification of the carotenoids was carried out by HPLC through the combined use of the retention time, visible absorption spectrum obtained with a photodiode array detector, co-injection with carotenoid standards, and response to chemical tests, as monitored by HPLC. Iodine-catalyzed isomerization, methylation, and acid-catalyzed epoxide−furanoxide rearrangement (Davies, 1976; Eugster, 1995) were undertaken on the whole extract and also on fractions separated by open column chromatography, according to the method of Rodriguez-Amaya *et al.* (1988).

A Varian liquid chromatograph equipped with a Waters photodiode array detector Model 994 was used. The separation was carried out with a 150 × 4.6 i.d. mm Spherisorb nitrile column (5 μm particle size), protected by a 30 × 4.6 mm Varian guard column (10 μm particle size). A multilinear gradient with increasing concentration of acetone in *n*-hexane from 0 to 15% in 10 min, to 20% in 20 min, to 30% in 10 min, and to 40% in 2 min was used with a flow rate of 1 mL/min. The re-equilibration time to the initial conditions was 8 min.

Identification was confirmed by mass spectrometry. The carotenoids were isolated either by accumulation of the carotenoids separated by HPLC through several runs or by a procedure involving several chromatography steps. In this, separation on a neutral alumina open column (activity grade III) gave three broad fractions, each of which was separated by TLC on silica, developed with petroleum ether, petroleum ether/diethyl ether (3:2), and ether according to increasing polarity of the fractions. Each carotenoid isolated by silica TLC was further purified by TLC on MgO/kieselguhr (1:1) with combinations of acetone and petroleum ether as mobile phase.

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Immediately before mass spectrometry, all carotenoids were purified through a neutral alumina minicolumn.

The electron impact mass spectra were taken with a VG Quattro instrument. The carotenoids were introduced directly with an insertion probe. The ionizing voltage was 70 eV, and the temperature of the ion source chamber was 240 °C. The mass spectra obtained were compared with those presented in the literature (Enzell *et al.*, 1969; Vetter *et al.*, 1971; Enzell and Back, 1995).

Quantitative Analysis. The carotenoids were exhaustively extracted with cold acetone in a blender, transferred to diethyl ether/petroleum ether, saponified overnight at room temperature with 10% KOH in methanol under a N_2 atmosphere, washed free of alkali, concentrated in a rotary evaporator (T < 35 °C), and adjusted to a known volume (50 or 100 mL). An appropriate aliquot (3−9 mL) of this extract was taken and, after addition of Sudan I, evaporated under N_2 and dissolved in 1 mL of hexane.

The carotenoids were analyzed quantitatively by use of internal calibration with Sudan I as internal standard. The curves were constructed with a minimum of four concentration levels, each in triplicate, the concentrations bracketing those of the mango samples. With the aid of the software Energraphic, the curves were traced and the straight line equations and their coefficients of correlation (ranging from 0.9762 to 1.0000) were obtained.

Precision of the HPLC method was verified for the principal carotenoids, β-carotene and violaxanthin, in triplicate analyses. Coefficients of variation of 3 and 10%, respectively, were obtained, which are comparable to those reported in the literature for HPLC analysis of carotenoids (Stewart, 1977; Bushway and Wilson, 1982; Hsieh and Karel, 1983).

β-Carotene, β-cryptoxanthin, and zeaxanthin standards were kindly provided by Hoffmann-La Roche (Basel, Switzerland). Since the standards were not used immediately after the vials were opened, purification on MgO/HyfloSupercel (1:1) or alumina columns was carried out immediately before the construction of the standard curves. *all-trans*-Violaxanthin, 9-*cis*-violaxanthin, and luteoxanthin were accumulated through several HPLC runs. 9′-*cis*-Neoxanthin was extracted from kale and isolated by MgO/HyfloSupercel (1:2) and alumina open column chromatography prior to use. Because standards were not available, and the compounds were present at very low concentration in the sample, the calibration curves of the corresponding *all-trans* carotenoids were used for the *cis* isomer of β-cryptoxanthin and 13-*cis*-violaxanthin. *cis*-Neoxanthin was used for *all-trans*-neoxanthin.

RESULTS AND DISCUSSION

The following carotenoids were identified on the basis of HPLC data in mango cv. Keitt: *all-trans*-β-carotene, *cis*- and *all-trans*-β-cryptoxanthin, *all-trans*-zeaxanthin, luteoxanthin isomers, *cis*- (tentatively 9-*cis* and 13-*cis*) and *all-trans*-violaxanthin, *cis*- and *all-trans*-neoxanthin. The identity of these carotenoids was confirmed by MS. Additionally, lutein and mutatochrome were also detected when large amounts of sample were utilized and when isolation was done by open column and TLC. The HPLC chromatogram is presented in Figure 1, and the peak identification and characteristics are in Table 1. The mass spectrometric data, i.e. molecular ion and characteristic mass fragment, are shown in Table 2.

A nitrile column was employed because it allowed better separation of the xanthophylls (mango presented only one carotene) and Sudan I could be used as internal standard. Sudan I would coelute with polyhydroxycarotenoids, such as neoxanthin, in the commonly used reversed-phase chromatography on C_{18} columns (Quackenbush and Smallidge, 1986).

β-Carotene (β,β-Carotene). β-Carotene (peak 1) was identified by comparing the spectrum (λ_{max} and fine structure) with those given in the literature (Vetter *et al.*, 1971; Davies, 1976; Britton, 1995), by coelution with β-carotene standard (Hoffmann-La Roche), and by a positive isomerization reaction for the *all-trans* isomer (hypsochromic shift). Its rapid elution on the nitrile column indicated the absence of polar substituents in the structure. The mass spectrum showed the characteristic molecular ion ($C_{40}H_{56}$) of 536 mass units (m.u.), and distinct terminal group fragmentations were absent since the loss of the β-ring results in a very low intensity peak. The mass spectrum showed the same characteristic peaks as those given in the literature (Vetter *et al.*, 1971). The ratio $[M - 92]^{\bullet+}/[M - 106]^{\bullet+}$ was 13.0, similar to the 12.9 ratio obtained by Schwieter *et al.* (1965). This value indicates a dicyclic carotenoid (Britton and Young, 1993).

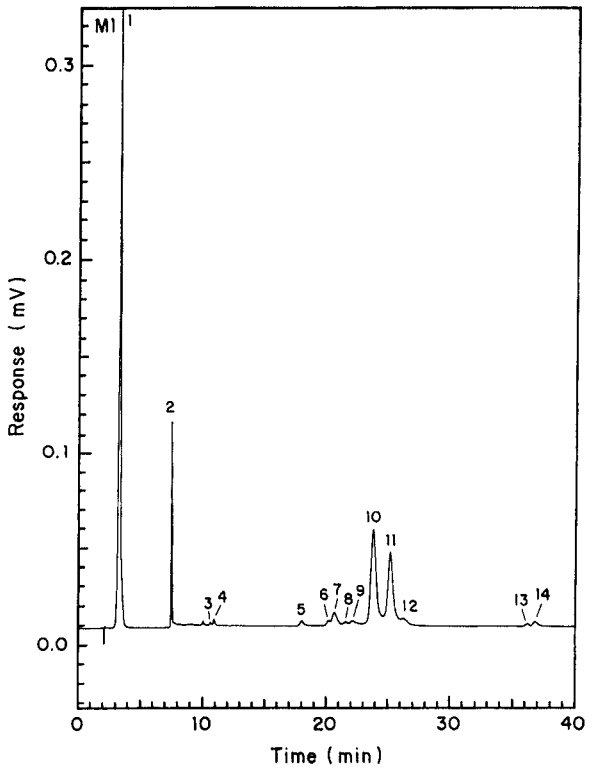

Figure 1. Chromatogram of the carotenoids of fully ripe mango cv. Keitt. Carotenoid identification: **1**, *all-trans*-β-carotene; **2**, Sudan (internal standard); **3**, *cis*-β-cryptoxanthin; **4**, *all-trans*-β-cryptoxanthin; **5**, *all-trans*-β-zeaxanthin; **6**−**9**, luteoxanthin isomers; **10**, *all-trans*-violaxanthin; **11**, 9-*cis*-violaxanthin; **12**, 13-*cis*-violaxanthin; **13**, *cis*-neoxanthin; **14**, *all-trans*-neoxanthin.

Mutatochrome (5,8-Epoxy-5,8-dihydro-β,β-carotene). This carotenoid was not detected by HPLC. Its mass spectrum showed the molecular ion of 552 m.u. ($C_{40}H_{56}O$). The loss of 80 m.u. to give a peak at m/z 456, which was more intense than the molecular ion, and the peaks at m/z 336, 205, and 165 indicated the presence of an epoxide group located on an unsubstituted β-ring. The visible spectrum supported the identification as the 5,8-epoxide, not the 5,6-epoxide, which would have given an identical MS. Since the purification was done on silica TL, where rearrangement of 5,6- to 5,8-epoxide-β-carotene could occur, it could not be ascertained which type of epoxide group was present in the natural compound.

β-Cryptoxanthin (*cis* and *all-trans*) (β,β-Caroten-3-ol). Peak 3 was tentatively identified as *cis*-β-cryptoxanthin by the visible absorption spectrum which was similar to that of *trans*-β-cryptoxanthin, but with the maxima at lower wavelengths (difference of 6 nm), a positive isomerization reaction for *cis* isomer (batho-

Table 1. Principal Properties of Mango Carotenoids Obtained by HPLC

peak no.[a]	carotenoid	λ_{max}[b] (nm)	reaction to chemical tests
1	*all-trans*-β-carotene	(422), 450, 477	trans +
2	Sudan I		
3	*cis*-β-cryptoxanthin	(420), 445, 473	cis +, epoxide −
4	*all-trans*-β-cryptoxanthin	(423), 450, 478	trans +
5	*all-trans*-zeaxanthin	(423), 450, 478	trans +
6−9	luteoxanthin isomers	400, 423, 450	epoxide +
		398, 421, 449	
		408, 432, 462	
10	violaxanthin	416, 440, 469	trans +, epoxide +
11	9-*cis*-violaxanthin[c]	412, 437, 465	cis +, epoxide +
12	13-*cis*-violaxanthin[c]	410, 434, 461	cis +, epoxide +
13	*cis*-neoxanthin	412, 436, 465	cis +, epoxide +
14	*all-trans*-neoxanthin	416, 438, 468	trans +, epoxide +

[a] Numbered according to the chromatogram shown in Figure 1. [b] Obtained with diode array detector in a gradient of acetone in hexane. Value in parentheses indicates shoulder instead of peak. [c] Tentative identification.

Table 2. Molecular Ion and Characteristic Mass Fragments of Mango Carotenoids

carotenoid	*m/z* (% relative abundance)	
	molecular ion	fragments
β-carotene	536 (7)	105 (100), 399 (0.8), 430 (0.3), 444 (3.9)
monoepoxy-β-carotene	552 (18)	69 (100), 105 (94), 165 (43), 205 (70), 336 (28), 472 (21)
β-cryptoxanthin	552 (0.6)	69 (99), 442 (0.4), 460 (0.4), 534 (0.4)
zeaxanthin	568 (0.3)	55 (100), 476 (0.5), 532 (0.2), 550 (0.2)
lutein	568 (0.3)	105 (100), 458 (0.2), 476 (0.1), 532 (0.3), 550 (1.4)
luteoxanthin	600 (6)	105 (97), 181 (90), 221 (98), 352 (27), 440 (1.4), 520 (7)
violaxanthin	600 (0.6)	181 (14), 221 (23), 352 (2.6), 440 (1.4), 520 (0.8)
neoxanthin	600 (0.9)	105 (95), 221 (37), 352 (2.7), 502 (3.1), 582 (1.2)

chromic shift), and a negative epoxide test, both reactions monitored by HPLC diode array detector.

all-trans-β-Cryptoxanthin (peak 4) was identified by its visible spectrum (similar to that of *trans*-β-carotene), a positive *trans* isomer reaction, and coelution with the β-cryptoxanthin standard. The mass spectrum showed the molecular ion ($C_{40}H_{56}O$) of 552 m.u. and fragments at *m/z* 534 $[M - H_2O]^{+}$, corresponding to the loss of a hydroxyl group, and at *m/z* 460 $[M - 92]^{\bullet+}$, due to toluene elimination from the isoprenic chain. The ratio $[M]^{\bullet+}/[M - 18]^{\bullet+}$ obtained was higher than 1.0; that is, the intensity of the molecular ion peak exceeded that of H_2O elimination, indicating that the hydroxyl group was not allylic.

Zeaxanthin (β,β-Carotene-3,3-diol) and Lutein (β,ϵ-Carotene-3,3′-diol). The visible spectrum, which was similar to that of β-carotene, coelution with the zeaxanthin standard, and a positive *trans* isomer reaction identified peak 5 as *trans*-zeaxanthin. Lutein, not shown in the chromatogram, was also found when a large amount of sample was utilized.

Zeaxanthin and lutein have the same chemical formula, $C_{40}H_{56}O_2$, and therefore the same molecular ion (*m/z* 568). Both presented typical mass fragments at *m/z* 550 $[M - 18]^{\bullet+}$ and 532 $[M - 18 - 18]^{\bullet+}$, corresponding to loss of one and two water molecules, respectively. The ratio $[M]^{\bullet+}/[M - 18]^{\bullet+}$ found for zeaxanthin (1.25) was 6 times higher than that of lutein (0.21), confirming the location of a hydroxy group in an allylic position in lutein. The ratios reported by Vetter *et al.* (1971) were 8.3 and 0.74 and by Britton and Young (1993) 2.5 and 0.60 for zeaxanthin and lutein, respectively.

Luteoxanthin (5,6:5′,8′-Diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol). The luteoxanthin isomers (peaks 6−9), violaxanthin, and neoxanthin all showed molecular ions of 600 m.u. ($C_{40}H_{56}O_4$). The mass spectrum of luteoxanthin was virtually the same as that of violaxanthin, presenting characteristic mass fragment ions at *m/z* 440 $[M - 80 - 80]^{\bullet+}$, due to the presence of two epoxy groups, and at *m/z* 221, showing that these were located in rings with hydroxy groups. The mass spectra cannot differentiate between the 5,6- and 5,8-epoxy groups because the high ionization temperature by itself promotes this rearrangement. The 5,8-furanoid structure was verified by the absorption spectrum and the 5,6-epoxide by the hypsochromic shift of 20 nm caused by addition of HCl.

Violaxanthin (*all-trans* and *cis*) (5,6:5′,6′-Diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol). *all-trans*-Violaxanthin (peak 10) was identified by its visible spectrum, positive *trans* isomer reaction, and epoxide−furanoxide rearrangement (hypsochromic shift of 40 nm and shifting of the retention time from 24 to 18 min due to the epoxide−furanoxide rearrangement), both reactions being monitored by HPLC and spectrophotometry. The mass spectrum showed fragments at *m/z* 564 $[M - 18 - 18]^{\bullet+}$ due the elimination of two hydroxyls, at 520 $[M - 80]^{\bullet+}$ and 440 $[M - 80 - 80]^{\bullet+}$, representing losses of one and two epoxide groups, respectively. The peaks at *m/z* 352, 221, and 181 indicated that the epoxy substituents were in rings with a hydroxy group.

The *cis* isomers of violaxanthin (peaks 11 and 12) showed positive *cis* isomerization reaction and furanoid rearrangement of the epoxy groups. These *cis* forms were tentatively identified as 9-*cis*-violaxanthin (peak 11) and 13-*cis*-violaxanthin (peak 12) according to the characteristics presented by Mólnar and Szabolcs (1980), who carried out a detailed NMR study of the violaxanthin isomers of *Viola tricolor*. The proposed 9-*cis* isomer showed a 3 nm difference in the λ_{max} compared to the *trans* isomer, and the proposed 13-*cis* isomer had a λ_{max} difference of 6 nm and a *cis* peak at 365 nm.

Neoxanthin (*all-trans* and *cis*) (5′,6′-Epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol). *all-trans*-Neoxanthin (peak 14) and *cis*-neoxanthin (peak 13) showed the characteristic visible spectra. The *cis* and *trans* configurations were verified by the isomerization reaction, and the presence of one 5,6-epoxide was proved by a hypsochromic shift of 20 nm after HCl addition. The identification was supported by co-injection with neoxanthin isolated from kale.

Table 3. Carotenoid Composition of Mango Cultivar Keitt from Bahia, Brazil

carotenoid	concentration (μg/g) range	mean[a]
all-trans-β-carotene	13.4−16.2	15.1 ± 1.5
unidentified compound		0.2 ± 0.0
cis-β-cryptoxanthin	tr−0.1	0.1 ± 0.1
all-trans-β-cryptoxanthin	0.3−0.3	0.3 ± 0.0
all-trans-zeaxanthin	0.6−0.9	0.8 ± 0.2
luteoxanthin isomers	3.1−4.1	3.8 ± 0.6
all-trans-violaxanthin	18.2−23.9	21.1 ± 2.9
9-*cis*-violaxanthin[b]	9.9−10.3	10.1 ± 0.2
13-*cis*-violaxanthin[b]	1.3−1.5	1.4 ± 0.1
cis-neoxanthin	tr−0.2	0.1 ± 0.1
all-trans-neoxanthin	1.0−3.6	2.1 ± 1.3
total	49.9−59.8	55.0 ± 5.0
vitamin A value	222−270	251 ± 26

[a] Mean and standard deviation of three sample lots. [b] Tentative identification.

Neoxanthin was distinguished from violaxanthin by its characteristic mass fragments at *m/z* 582 $[M - 18]^{\bullet+}$, 564 $[M - 18 - 18]^{\bullet+}$, 520 $[M - 80]^{\bullet+}$, and 502 $[M - 18 - 80]^{\bullet+}$, the latter being more intense than the molecular ion, and by the lack of any fragment ion at *m/z* 420 due to the loss of two epoxide groups.

Mass spectrometry confirmed the initial identification of the mango carotenoids made on the basis of the combined use of retention time/cochromatography, visible absorption spectra obtained by diode array detector, and some specific chemical reactions. Thus, the correct use of these other parameters (other than MS) can provide strong evidence for the identification of some common carotenoids, but it is now considered that the following minimum criteria for identification should be fulfilled: cochromatography with an authentic sample and UV−visible spectrum and mass spectrum that allow identification of the molecular ion (Liaaen-Jensen, 1995). These criteria have been met in the present work.

Quantitative Composition. As shown in Table 3, *all-trans*-violaxanthin, *all-trans*-β-carotene, and 9-*cis*-violaxanthin are the principal carotenoids of mango cv. Keitt accounting for 38, 27, and 18% of the total carotenoid content, respectively. In the three previous papers (Jungalwala and Cama, 1963; John *et al.*, 1970; Godoy and Rodriguez-Amaya, 1989), β-carotene was found to be the major carotenoid, with violaxanthin a minor component. The cultivars analyzed were different, but it is reasonable to suspect that violaxanthin was present but underwent transformation and degradation during the repetitive open column and thin-layer chromatography utilized previously. In fact, auroxanthin, the product of epoxide−furanoxide rearrangement of violaxanthin, was detected in all of these studies.

Phytoene, phytofluene, ζ-carotene, γ-carotene, and other 5,8-epoxy carotenoids that have been reported previously as mango carotenoids were not encountered in the present study.

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