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ORIGINAL PAPER

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Determination of free and bound carotenoids in paprika (*Capsicum annuum* L.) by LC/MS

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Abstract A protocol for extraction and chromatographic separation with a C30-reversed-phase column for analysis of non–saponified lipid extracts of paprika fruits by LC/MS has been developed. Using this procedure it was possible to identify the main mono- and diesterified derivatives of capsanthin, capsorubin, β-cryptoxanthin, and zeaxanthin naturally occuring in red peppers. LC/MS analyses proved that xanthophylls of red peppers were exclusively acylated with saturated C12, C14, and C16 fatty acids whereas unsaturated as well as C18 fatty acids were generally absent. However, saponification experiments on paprika lipid extracts showed that approximately 75% of the total fatty acids of red peppers are C18 fatty acids. These findings can only be explained by selective esterification of xanthophylls during maturation of red peppers. In contrast, direct extracts of green peppers comprised only free carotenoids, while the fatty acid distributions of green and red peppers did not differ significantly.

Key words *Capsicum annuum* · Red peppers · Carotenoid · Carotenoid esters · Liquid chromatography/mass spectrometry

Introduction

Red peppers (*Capsicum annuum* L.), which belong to the oldest and most important natural colorants of foods, provide a wide range of various carotenes and hydroxylated carotenoids (xanthophylls). During maturation, the free (non–esterified) carotenoids become more and more acylated by fatty acids, rendering them more liposoluble. This process leads to partially as well as completely esterified carotenoids [1]. Classical methods of carotenoid analysis involve extraction of the lipids, chemical saponification of the extract and chromatographic separation of the free carotenoids [2, 3]. By these procedures the characteristics of the naturally binding forms are lost. Since in literature the identification of acylation products has not been proven unequivocally [4–6], little is known about naturally occuring esterified carotenoids e.g. in paprika. For this reason, we have established a method for extraction of the plant tissue as well as for chromatographic separation and characterization of the major carotenoid esters by LC/MS analysis without saponification.

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Materials and methods

Materials

Red and green peppers (cultivars were not known) were obtained from local retail shops. The preparation was the same as that for consumption. To obtain homogenous samples, 80–100 g (fresh weight) of each fruit were minced by an Ultra Turrax.

Chemicals

Lutein, zeaxanthin, β-cryptoxanthin and capsanthin were generously provided by Hoffmann-La Roche (Basel, Switzerland). Trivial names of carotenoids are used instead of the complex IUPAC nomenclature [7] throughout this text. Light petroleum (40–60 °C), methanol, ethyl acetate, n–hexane, and butylated hydroxytoluene (BHT) were purchased from Merck (Darmstadt, Germany), *tert*-butyl methyl ether, ethanol, sodium sulfate (anhydrous), and β-carotene from Fluka (Neu–Ulm, Germany), boron trifluoride–methanol reagent (15%) and fatty acid methyl esters (FAMEs) (saturated C10:0-C22:0 and C16:1, C18:1, C18:2, C18:3) from Sigma (Steinheim, Germany). The solvents used were of analytical grade and were distilled before use. For LC/MS analysis, ultrapure water from a Milli-Q 185 plus apparatus (Millipore, Eschborn, Germany) was employed.

Equipment

Liquid chromatography/mass spectrometry. LC/MS was run on an HP1100 HPLC system which comprised an HP1100 autosampler, HP1100 gradient pump, HP1100 column thermoregulator and HP1100 diode array detector (DAD) module (Hewlett Packard; Waldbronn, Germany), coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer. MS parameters: atmospheric pressure chemical ionisation (APcI),+; source temperature, 150 °C; APcI probe temperature, 400 °C; corona, 3.7 kV; high voltage lens, 0.5 kV; cone voltage, 30 V. The MS system was operated in full scan mode (*m/z* 200–1200). UV absorbance of the carotenoids was maintained at 450 nm by using a DAD. For data acquisition and processing, MassLynx 3.2 software was used. A YMC analytical column (YMC, Schermbeck, Germany) with 5 µm C30-reversed phase material (250 × 4.6 mm) including a precolumn (Nucleosil 5 µm C_{18}, 10 × 4.6 mm, Bischoff, Leonberg, Germany) was used and kept at 35 °C. The mobile phase consisted of mixtures of methanol, *tert* butyl methyl ether, and water at 81 : 15 : 4, v/v/v (A) and 6 : 90 : 4, v/v/v (B), starting with 10 min isocratic at 100% A, followed by a gradient to obtain 50% B at 40 min, 100% B at 50 min, 100% A at 55 min and isocratic 100% A from 55 min to 60 min at a flow rate of 1 ml/min. The injection volume was 20 µl.

Gas liquid chromatography. GLC was performed using a Carlo Erba (Hofheim, Germany) Vega Series 2 gas chromatograph GC 6000 equipped with a flame ionization detector (FID) and a J&W (Folsom, Calif., USA) fused-silica capillary column (30 m × 0.53 mm) wall-coated with DB-23 (0.5 µm film thickness). The oven temperature was programmed to hold at 80 °C, for 2 min, followed by an increase at 5 °C/min to 220 °C, held for 8 min. Injector and detector (FID) temperatures were set at 230 °C; injection (1 µl) was in the split mode (1 : 10). Helium was used as the carrier gas with a column head pressure of 20 kPa and a linear velocity of 40 cm/s (determined by the injection of methane, oven temperature 80 °C). Chromatograms were recorded with a Carlo Erba (Hofheim, Germany) Mega Series integrator.

Methods

Preparation of samples. This was carried out in two stages: extraction, and transesterification. Carotenoids and carotenoid esters were extracted by shaking 2 g of homogenized samples with 25 ml methanol/ethyl acetate/light petroleum (1 : 1 : 1, v/v/v) and filtered through a glass–wool plug. This procedure was repeated twice. The combined filtrates were dried with 10 g of anhydrous sodium sulfate, filtered, and evaporated to dryness in vacuo at 30 °C. The residue was dissolved in 2 ml methanol/*tert*-butyl methyl ether (1 : 1, v/v) which contained 1% BHT. After membrane filtration (0.45 µm) an aliquot of the solution was subjected to the LC/MS system. The whole procedure was performed in dim light and repeated thrice.

Transesterification was carried out by taking the residue resulting from the extraction procedure and suspending it in 1 ml of 0.5 M methanolic potassium hydroxide solution, transferring it to a Pyrex tube, sealing tightly, and keeping it at 80 °C for 5 min. Boron trifluoride-methanol reagent (1 ml) was added and the mixture kept at 80 °C for 5 min. After cooling to room temperature, 2 ml hexane and 2 ml saturated sodium chloride solution were added. After phase separation, 1 µl of the super-

Table 1 Fatty acid composition, LC/MS data ($[M+H]^+$ and main fragment ions) of carotenoids and carotenoid esters in direct extracts of red peppers. *RRT* Relative retention time, calculated in relation to β-carotene

Peak no. in Fig. 1	RRT	Carotenoid	Fatty acid	*m/z* $[M+H]^+$ (Main fragment ions)[a]
1	0.27	Capsanthin	–	585
2	0.33	Zeaxanthin	–	567
3	0.59	Capsorubin	C12 : 0	783 (583)
4	0.61	β-Cryptoxanthin	–	553
5	0.68	Capsorubin	C14 : 0	811 (583)
6	0.69	Capsanthin	C12 : 0	767 (567)
7	0.73	Capsanthin	C12 : 0	767 (567)
8	0.75	Capsorubin	C16 : 0	839 (583)
9	0.78	Capsanthin	C14 : 0	795 (567)
10	0.83	Capsanthin	C14 : 0	795 (567)
11	0.87	Capsanthin	C16 : 0	823 (567)
12	1.00	β-Carotin	–	537
13	1.04	Capsorubin	C12 : 0, C12 : 0	965 (765, 565)
14	1.11	Capsorubin	C12 : 0, C14 : 0	993 (793, 765, 565)
15	1.16	Capsorubin	C14 : 0, C14 : 0	1021 (793, 565)
16	1.19	Capsanthin	C12 : 0, C12 : 0	949 (749, 549)
17	1.24	Capsorubin	C14 : 0, C16 : 0	1049 (821, 793, 565)
18	1.26	Ca psanthin	C12 : 0, C14 : 0	977 (777, 749, 549)
19	1.31	Capsorubin	C16 : 0, C16 : 0	1077 (821, 565)
20	1.33	Capsanthin	C14 : 0, C14 : 0	1005 (777, 549)
21	1.39	Capsanthin	C14 : 0, C16 : 0	1033 (805, 777, 549)
22	1.41	Zeaxanthin	C12 : 0, C14 : 0	961 (761, 733, 533)
23	1.45	Capsanthin	C16 : 0, C16 : 0	1061 (805, 549)
24	1.48	Zeaxanthin	C14 : 0, C14 : 0 [b]	989 (761, 533)
25	1.54	Zeaxanthin	C14 : 0, C16 : 0	1017 (789, 761, 533)
26	1.59	Zeaxanthin	C16 : 0, C16 : 0	1045 (789, 533)

[a] Values in parentheses represent fragment ions, indicating the loss of any fatty acid(s) as neutral molecules from the parent ion $[M+H]^+$ (loss of C12 : 0 = Δ*m/z* 200; C14 : 0 = Δ *m/z* 228; C16 : 0 = Δ *m/z* 256)

[b] Since the presence of lutein can be definitely excluded from hydrolysis experiments (not detailed in the experimental section), the analytes were identified as zeaxanthin esters and not as isomeric lutein esters

natant organic phase was used for analysis of the FAMEs by GLC.

Identification of carotenoids by LC/MS. Carotenoids were identified by their quasimolecular ions $[M+H]^+$ (Table 1) and, if authentic reference material was available, by their UV spectra and specific retention times. Peaks were assigned to carotenoid esters if daughter ions with *m/z* $[M+H\text{-}CH_3(CH_2)_nCOOH]^+$ in the case of monoesterified carotenoids and *m/z* $[M+H\text{-}CH_3(CH_2)_nCOOH\text{–}CH_3(CH_2)_mCOOH]^+$ in the case of diesterified carotenoids were present (*n,m* indicating the number of methylene groups of the respective fatty acids, Table 1). For preparation of reference solutions, 2 mg lutein, zeaxanthin, and β-cryptoxanthin were dissolved in 100 ml methanol each; capsanthin and β-carotene were dissolved in ethanol and light petroleum/ethyl acetate (1 : 1, v/v), respectively. The retention times were as follows: capsanthin, 7.3 min; lutein, 8.2 min; zeaxanthin, 9.1 min; β-cryptoxanthin, 16.7 min; β-carotene, 27.4 min. Since esterification does not influence the absorption spectra of xanthophylls, the absorption pattern of capsanthin (maximum at 474–478 nm) was used for identification of capsanthin esters as well.

Identification of FAMEs by GLC. The FAMEs were identified by comparison of their retention times with a standard mixture in hexane (1 mg/ml each). The retention times were as follows: C10 : 0, 9.4 min; C12 : 0, 13.6 min; C14 : 0, 17.6 min; C16 : 0, 21.2 min; C16 : 1, 21.5 min; C18 : 0, 24.5 min; C18 : 1, 24.8 min; C18 : 2, 25.5 min; C18 : 3, 26.4 min; C20 : 0, 27.5 min; C22 : 0, 30.3 min. This mixture was also used for the calculation of response factors relative to methyl palmitate. Since absolute quantitative data were not necessary, the fatty acid distribution was calculated after area correction as area% FAME in relation to the total area amount of all FAMEs.

Results and discussion

Due to its capability of separating a broad range of carotenoids a recently commercially available C30-reversed-phase column was used for LC/MS analysis. Figure 1 shows a typical chromatogram of a direct extract of red pepper.

The unequivocal identification of each compound is problematic because only a few carotenoids are available as reference standards. Since all compounds are highly sensitive towards temperature and light, isolation of authentic reference material by preparative HPLC can not easily be achieved in many cases. Therefore, LC/MS is the method of choice to determine the degree of esterification of paprika carotenoids. As most compounds bear hydroxyl or ester groups, an APcI interface can be employed for ionization. Operating the MS system in the positive ion mode, the molecular ion signal of xanthophylls and carotenoid esters thus appears as quasimolecular ion $[M+H]^+$. The fragmentation of carotenoid esters is dominated by the loss of fatty acids from $[M+H]^+$. That way, the nature of acylation can be determined. Compared to β-carotene, the diesterified derivatives of capsanthin, capsorubin, and zeaxanthin were detected at higher retention times, and the free and monoesterified compounds at lower retention times. An example of a mass spectrum obtained from

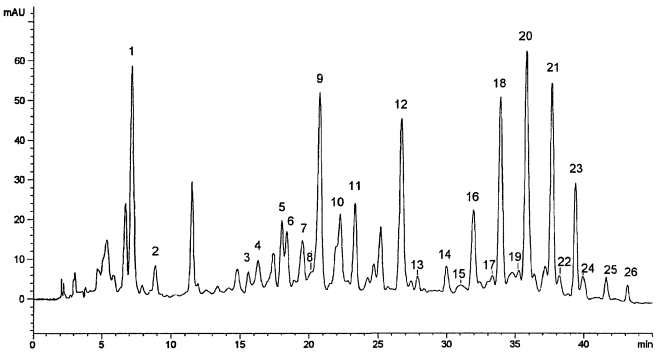

Fig. 1 Typical HPLC chromatogram (DAD, 450 nm) of a direct carotenoid extract of red peppers (for conditions, see Materials and methods, for assignment of the peaks see Table 1)

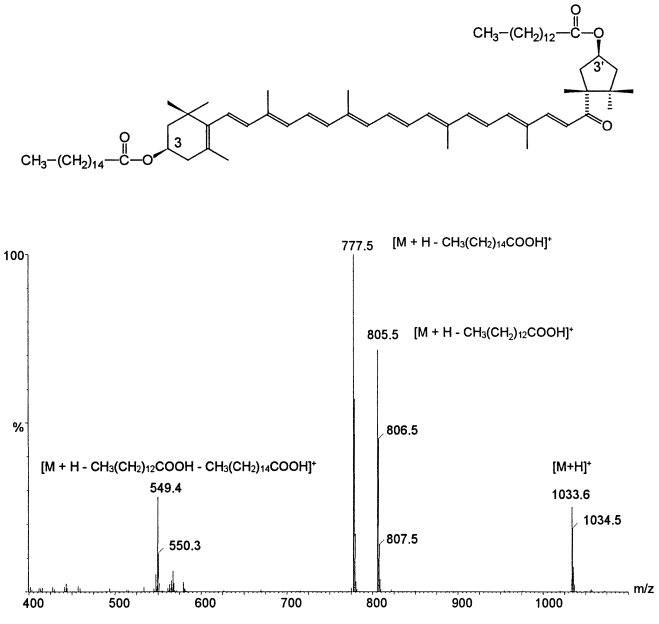

Fig. 2 Structure and MS spectrum of C14 : 0,C16 : 0-capsanthin (see Fig. 1, peak no. 21; *m/z* 1033 $[M+H]^+$) as example for a diesterified capsanthin. The positions of the fatty acids cannot be established by the MS data and may have to be reversed

C14 : 0,C16 : 0–capsanthin (Fig. 1, peak 21) is presented in Fig. 2.

Capsanthin appeared in the direct extracts of red peppers as free xanthophyll, but mainly as mono- and diesters, comprising only lauric acid (C12 : 0), myristic acid (C14 : 0), and palmitic acid (C16 : 0) (Fig. 1, Table 1). Generally two peaks could be assigned each to monoesterified C12 : 0 and C14 : 0 capsanthin. These double peaks presumably represent the 3′-O and 3-O isomers, which cannot be differentiated by means of MS. This assumption is in contrast to the results of Goda et al. [6], who analyzed the monoester fraction directly by ^{1}H-NMR and found that monoesterification occurs at the 3′-hydroxyl group of the capsanthin cyclopentane ring, exclusively.

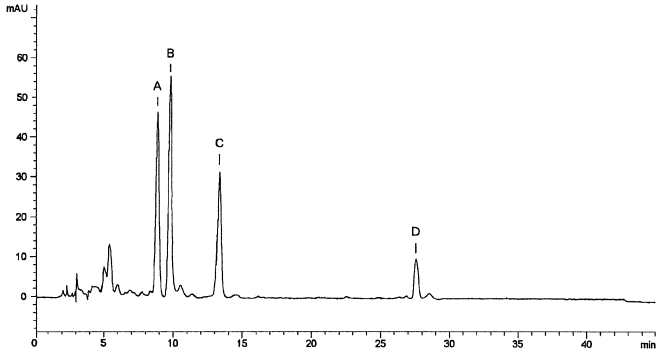

Fig. 3 HPLC chromatogram (DAD, 450 nm) of a direct carotenoid extract of green peppers (for conditions, see Materials and Methods). Peaks: **A**, lutein (8.2 min), *m/z* 569 $[M+H]^+$, UV (nm): 422/446/474; **B**, unknown (9.9 min), *m/z* 585 $[M+H]^+$, UV [nm]: 311/344/468; **C**, unknown (13.5 min), *m/z* 571 $[M+H]^+$, UV (nm): 432; **D**, β-carotene (27.4 min), *m/z* 537 $[M+H]^+$; UV (nm): 424/452/480

Capsorubin was detected as mono- and diesters, comprising the same fatty acids as the corresponding capsanthin esters; free capsorubin (*m/z*, 601 $[M+H]^+$) was not detected by LC/MS. Zeaxanthin was present only as free and diesterified carotenoid. Violaxanthin and neoxanthin (peaks with retention times from 5 min to 6 min, Figs. 1 and 3, respectively), were tentatively identified on the basis of their molecular mass and absorption spectra described in the literature [8]. Since both xanthophylls were not available as reference compounds, the data could not be confirmed by authentic material.

Chromatograms of direct unsaponified extracts of green peppers, however, showed no peaks with higher retention times than β-carotene, indicating the absence of xanthophyll esters at the green stage of ripening (Fig. 3). Instead of non–esterified zeaxanthin, free lutein, which is typical for green peppers, was present. The xanthophylls capsanthin, capsorubin, and β-cryptoxanthin, peculiar to the red fruits, were not detected by LC/MS in extracts of green peppers.

Unexpectedly, the esterified xanthophylls of red peppers only contain saturated C12, C14, and C16 fatty acids. Both unsaturated fatty acids and C18–fatty acids were generally absent. These results, which are in contrast to several reports [9, 10], are amazing since oleic, linoleic, and linolenic acids represent the main fatty acids of paprika. Our own experiments based on saponification of paprika lipids combined with GLC anaylses of the FAMEs showed that approximately 75% of the total fatty acids of red peppers are C18 fatty acids (Table 2). These findings also apply to green peppers (78% C18 fatty acids) which shows that the fatty acid composition does not change significantly during maturation of green fruits. The exclusive occurrence of saturated fatty acids with chain lengths below C18 in the carotenoid fraction of red peppers can therefore only be explained by the selectivity of the biochemical esterification reactions.

Table 2 Fatty acid distribution of red and green peppers after saponification calculated as area% FAME/total FAMEs. Values are arithmetic means ± standard deviations of three independent determinations

Fatty acid methyl ester	Red pepper	Green pepper
Lauric (C12:0)	0.6 ± 0.1	– [a]
Myristic (C14:0)	2.3 ± 0.4	0.8 ± 0.2
Palmitic (C16:0)	17.4 ± 1.0	16.7 ± 0.3
Palmitoleic (C16:1)	1.5 ± 0.2	1.5 ± 0.2
Stearic (C18:0)	4.3 ± 0.5	8.4 ± 0.6
Oleic (C18:1)	7.6 ± 1.5	0.8 ± 0.1
Linoleic (C18:2)	46.4 ± 2.5	47.3 ± 2.5
Linolenic (C18:3)	16.5 ± 0.1	21.4 ± 1.6
Arachidic (C20:0)	0.8 ± 0.2	1.8 ± 0.2
Behenic (C22:0)	0.6 ± 0.1	1.7 ± 0.6

[a] <0.5 area% of total FAMEs

In this study a suitable extraction method for carotenoids as well as carotenoid esters is presented. Optimum chromatographic performance for these compounds in direct extracts of red and green peppers was achieved with a C30–reversed–phase column. While xanthophylls in green peppers appear only in free form, these compounds are mainly esterified in red peppers. This raises the question, if and to what extent xanthophyll esters are bioavailable for the human digestive system. With regard to the health effects assigned to several free carotenoids in recent years, it is important to know whether acylated carotenoids have comparable properties.

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