

# Characterization of carotenoids and carotenoid esters in red pepper pods (*Capsicum annuum* L.) by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry

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Carotenoids and carotenoid esters were extracted from red pepper pods (*Capsicum annuum* L.) without saponification. Among the 42 compounds detected, 4 non-esterified, 11 mono- and 17 diesters were characterized based on their retention times, UV/Vis spectra and their fragmentation patterns in collision-induced dissociation experiments in atmospheric pressure chemical ionization mass spectrometry (APCI-MS). Positive and negative ion mode measurements were used for the characterization of major and minor carotenoids and their esters. Capsanthin esterified with lauric, palmitic and myristic acids represented the predominant compounds in the red pepper extracts. Additionally, three  $\beta$ -cryptoxanthin and one zeaxanthin monoester were tentatively identified in red pepper pods for the first time. Furthermore, the specific fragmentation patterns of capsanthin-laurate-myristate and capsanthin-myristate-palmitate were used for the distinction of both regioisomers. The results obtained from LC-DAD-APCI-MS<sup>n</sup> experiments demonstrated that the carotenoid profile of red pepper pods is considerably more complex than considered hitherto. Copyright © 2005 John Wiley & Sons, Ltd.

Red pepper pods (*Capsicum annuum* L.) are commonly used as vegetables and for the production of natural food colorants. The red color is due to the presence of carotenoids. Their composition in *Capsicum* species has frequently been studied.<sup>1–3</sup> Capsanthin and capsorubin are characteristic of the genus *Capsicum*,<sup>4</sup> but other carotenoids such as  $\beta$ -cryptoxanthin, zeaxanthin and  $\beta$ -carotene may also contribute to the red color. During pepper fruit ripening, selective xanthophyll esterification with fatty acids increases with a gradual decrease of free pigments and is directly linked to the transformation of chloroplasts into chromoplasts.<sup>5</sup> In the fully ripe stage, a balance between free, partially and totally esterified fractions is reached, which seems to be largely independent of variety and could be used as indices of the physiological maturity of the fruit.<sup>6,7</sup>

Usually, a saponification step is used after the extraction to facilitate carotenoid isolation. It is an effective means of removing unwanted lipids and degrading chlorophylls, which may interfere with the chromatographic separation.

Therefore, separation, identification, and quantification are simplified, since free carotenoids are analyzed instead of their esters, which are difficult to separate and usually occur as a complex mixture of compounds esterified with a variety of fatty acids.<sup>8</sup> However, since the saponification of carotenoid extracts results both in the degradation and structural transformation of some carotenoids,<sup>9</sup> high-performance liquid chromatography (HPLC) methods for the separation of the various classes of carotenoids without saponification are highly desirable, thus providing valuable information on the genuine profile and the amounts of these compounds in plant foods.<sup>10</sup>

Most methods for the separation and identification of alkenes, such as distillation, gas chromatography and infrared spectroscopy, have proven inadequate for the analysis of carotenoids in biological tissues because of their structural similarity, their thermolability and the presence of interfering compounds. In contrast, HPLC diode-array detection (DAD) analysis of carotenoids allows a continuous collection of spectrophotometric data. However, the complexity of the mixtures and the structural similarity compromise unequivocal identification of individual compounds solely based on chromatographic behavior and UV/Vis spectrometric data. For this reason, much effort has been made to develop LC/MS interfaces that add the specificity of mass spectrometric detection to the HPLC determination

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of carotenoids.<sup>11</sup> High-resolution and accurate mass measurements provide not only molecular masses, but also unequivocal molecular formulae for the carotenoids and for important fragment ions. Structures of many new carotenoids and carotenoid conjugates have been established with the aid of mass spectrometry. Several LC/MS methods have been reported for carotenoid analysis, including particle beam, continuous-flow fast atom bombardment, electrospray and atmospheric pressure chemical ionization (APCI) techniques.<sup>11–14</sup> The latter provides high sensitivity and a superior linearity of detector response, suggesting that this LC/MS technique should become the standard method for carotenoid analysis.<sup>14</sup> While LC/APCI-MS analysis of free carotenoids has comprehensively been reported,<sup>11,14–18</sup> the characterization of carotenoid esters in plant tissues has only emerged in the last few years. Other authors established a method for the extraction and characterization of the major carotenoid esters in red pepper by tandem mass spectrometry.<sup>16</sup> In continuation of our studies on the carotenoid contents of red pepper and chilli powder and on the processing and storage stability of free and esterified compounds, the carotenoids of red pepper pods were investigated by LC/APCI-MS. Major and minor carotenoids and carotenoid esters were characterized in positive and negative ion mode by their specific fragmentation patterns, revealing a more complex profile than described so far. Additionally, selective collision-induced dissociation (CID) experiments were used to differentiate between regioisomeric compounds.

## EXPERIMENTAL

### Materials

All reagents and solvents used were purchased from VWR (Darmstadt, Germany) and were of analytical or HPLC grade. Zeaxanthin and all-*trans*- $\beta$ -carotin were from Sigma (St. Louis, MI, USA); capsanthin and capsorubin were obtained from Extrasynthèse (Lyon, France);  $\beta$ -cryptoxanthin was purchased from CaroteNature (Lipsingen, Switzerland); lutein was kindly provided by Hoffmann-La Roche (Basel, Switzerland).

Cellulolytic and pectinolytic enzyme preparations (Cellubrix<sup>®</sup> L and Ultrazym<sup>®</sup> AFP-L) were kindly provided by Novozymes Switzerland (Dittingen, Switzerland). Density measurements of enzyme preparations were performed using a DMA 48 density meter (Paar Physica, Graz, Austria). Red pepper pods (*Capsicum annuum* L.) of unknown cultivar were from organic production in Israel. The whole fruits were lyophilized and kept at  $-20^{\circ}\text{C}$  until analysis.

### Sample preparation

Sample preparation was carried out according to a modified procedure described previously.<sup>19</sup> Lyophilized pepper pods were ground using a Retsch ZM 1 Ultra Centrifugal mill (Haan, Germany) with a 1 mm screen insert. To avoid degradation and isomerization, amber glassware was used and processing was performed under dim light conditions. Before extraction, aliquots of 500 mg of powdered red pepper were digested enzymatically by adding both 1000 ppm Cellubrix L and Ultrazym AFP-L in a graduated flask, which was made up to 50 mL with deionized water. After flushing with

nitrogen, enzymatic hydrolysis was performed at ambient temperature for 1 h under continuous stirring.

Free carotenoids and carotenoid esters were extracted from the suspension in a glass separatory funnel with a mixture of 50 mL of acetone and hexane (1:1, v/v) containing butylated hydroxytoluene (62.5 mg/100 mL) and butylated hydroxyanisole (62.5 mg/100 mL) as antioxidants. After shaking the solution was washed with 50 mL of sodium chloride solution (10 g/100 mL) and twice with 50 mL of water to remove acetone. The aqueous phase was re-extracted with ethyl acetate until it was colorless. The combined organic phases were dried with sodium sulfate (2 g) and evaporated in vacuo ( $T < 30^{\circ}\text{C}$ ). The residue was dissolved in 2-propanol and made up to 10 mL, membrane-filtered (0.2  $\mu\text{m}$ ), and used for LC/MS analyses.

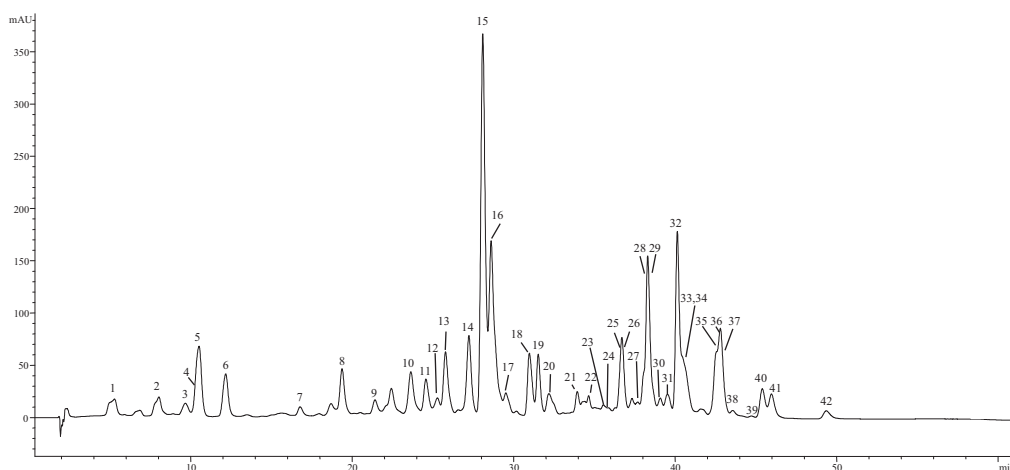
### HPLC system

Carotenoid analyses were performed using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1322A degasser, a model G1312A binary pump, a model G1313A autosampler, a model G1316A column oven, and a model G1315A DAD system. The column used was a  $150 \times 3.0$  mm i.d., 3  $\mu\text{m}$  particle size, analytical scale YMC C30 reversed-phase column (Wilmington, MA, USA), with a YMC C30 guard column ( $10 \times 3.0$  mm, i.d.), operated at  $25^{\circ}\text{C}$ . The mobile phase consisted of methanol/methyl *tert*-butyl ether (MTBE)/water (81:15:4, v/v/v; eluent A) and methanol/MTBE/water (4:92:4, v/v/v; eluent B) using a gradient program as follows: 0% B to 30% B (22 min), 30% B to 51.3% B (10 min), 51.3% B to 62.7% B (23 min), 62.7% B to 100% B (5 min), 100% B isocratic (5 min), 100% B to 0% B (5 min). Total run time was 80 min. The injection volume was 10  $\mu\text{L}$ . All carotenoids and carotenoid esters were monitored at 450 nm, at a flow rate of 0.42 mL/min. Additionally, UV/Vis spectra were recorded in the range of 200–600 nm at a spectral acquisition rate of 1.25 scans/s (peak width 0.2 min).

### Mass spectrometry

LC/MS analyses were performed with the HPLC system described above coupled on-line to a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an APCI source. Data acquisition and processing were performed using Esquire Control software. Positive and negative ion mass spectra of the column eluate were recorded in the range of  $m/z$  100–1100 at a scan speed of 13 000 Th/s (peak width 0.6 Th, FWHM). Nitrogen was used both as the drying gas at a flow rate of 3.5 L/min and as the nebulizing gas at a pressure of 50 psi. The nebulizer temperature was set at  $350^{\circ}\text{C}$  and a potential of  $+2779/-2779$  kV was used on the capillary. Corona was set at 2000 nA both in positive and negative ion mode, and the vaporizer temperature was set at  $400^{\circ}\text{C}$ .

Helium was used as the collision gas for CID at a pressure of  $4.9 \times 10^{-6}$  mbar. CID spectra were obtained with an isolation width of 2.0 Th for precursor ions and a fragmentation amplitude of 1.0 V. Capsanthin and all-*trans*- $\beta$ -carotin were used for the optimization of ionization and fragmentation parameters.



**Figure 1.** Separation of non-esterified carotenoids and carotenoid esters from red pepper pods by HPLC (450 nm). For peak assignment, see Tables 1, 2 and 3.

## RESULTS AND DISCUSSION

One of the major objectives of the present study was to characterize the genuine profile of free and esterified carotenoids in red pepper fruits. Therefore, sample preparation was performed by simple acetone/hexane extraction of red pepper powder after enzymatic digestion without saponification before HPLC analysis. The chromatogram of a non-saponified red pepper extract is presented in Fig. 1.

APCI-MS analyses of carotenoids showed unexpected ion heterogeneity. Molecular ions  $[M]^+$  and protonated species  $[M+H]^+$  for both xanthophylls and even hydrocarbon carotenes were observed in the positive ion mode, whereas mainly molecular ions  $[M]^-$  were detected in the negative ion mode, which is in accordance with literature reports.<sup>15,20</sup> Studies using deuteriochloroform as solvent had shown that the mobile phase is the source of hydrogen for protonation.<sup>15</sup> The relative abundance of molecular ions and protonated molecules varies with the mobile phase composition. For instance, polar solvents such as alcohols increase the abundance of protonated carotenoids (even protonated  $\beta$ -carotene), whereas less polar solvents such as MTBE facilitate the formation of molecular ions. Because both methanol and MTBE were used for the separation on a C30 column in the present study, a mixture of molecular ions and protonated molecules was observed in the mass spectra of the carotenoids. The  $[M]^+$  to  $[M+H]^+$  ratios ranged from 0–0.09 for non-esterified carotenoids to 0.34–0.55 for carotenoid diesters which were eluted under different conditions as concerns eluent composition, thus confirming the assumption of another study.<sup>14</sup>

The results presented in the following were obtained by positive ionization experiments, whereas negative ion measurements were only used for confirmation.

### Non-esterified carotenoids

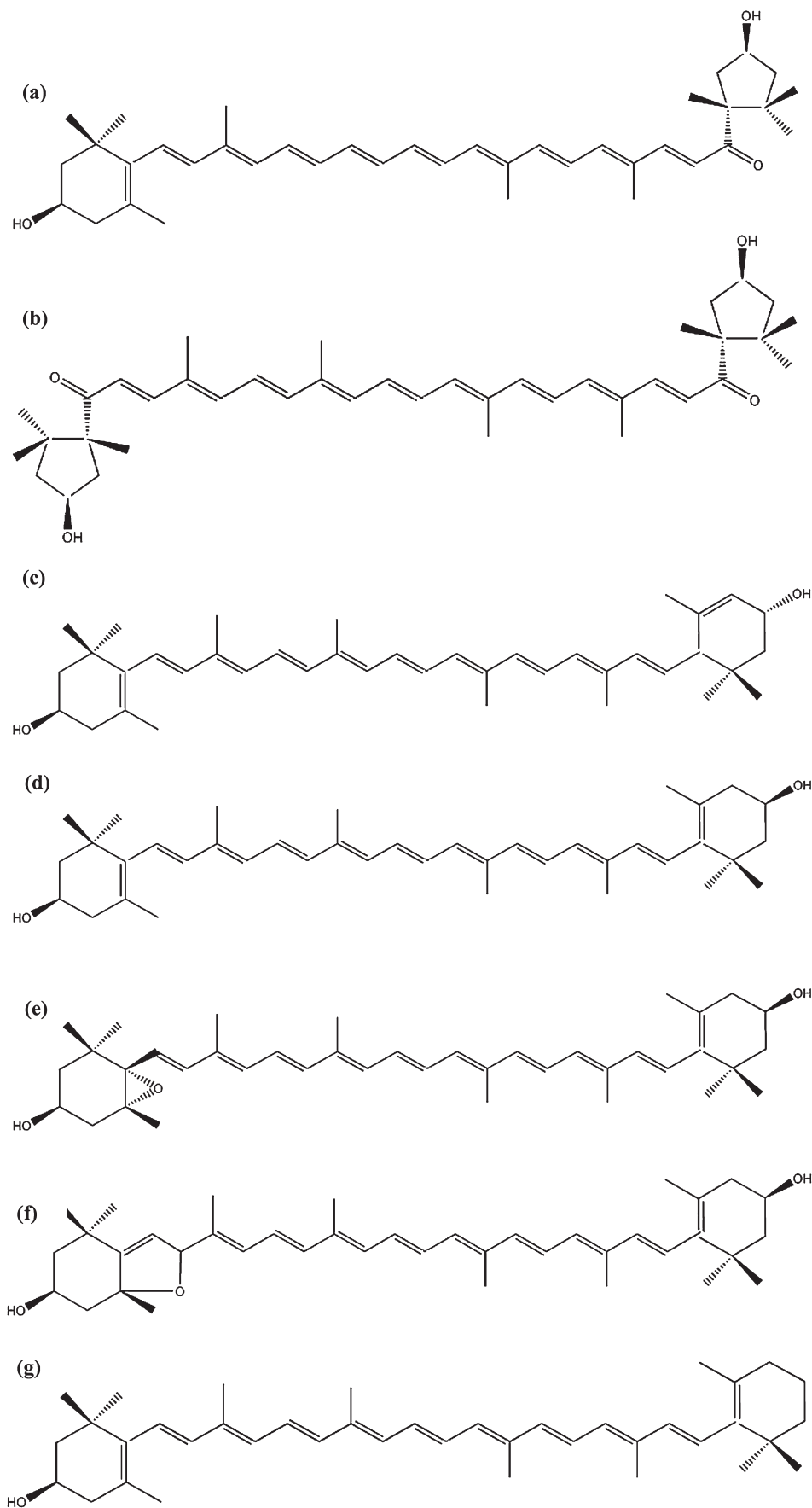
The nature and biosynthesis of the carotenoids of differently colored *Capsicum* varieties and their changes during ripening have already been investigated.<sup>3,4</sup> The major qualitative difference between green and mature red pods is the presence of

mainly lutein and violaxanthin in unripe pepper fruits. In contrast, the characteristic 'paprika ketones' capsanthin and capsorubin are the major carotenoids in the red fruits which are completely devoid of lutein.<sup>4</sup>

Expectedly, since the red pepper pods used in this study were brightly red-colored, lutein was detected neither in its free nor in esterified form. Free capsorubin was also absent in the crude extracts, whereas some mono- and diacylated derivatives of this xanthophyll were detected (see below).

Figure 2 shows the structures of the free xanthophylls characterized in this study. The identification of capsanthin, zeaxanthin,  $\beta$ -cryptoxanthin and  $\beta$ -carotene (5, 6, 8 and 15) was based on the comparison of retention times and UV/Vis spectra with those of reference substances. These assignments were corroborated by their mass spectrometric behavior. Fragmentation of  $m/z$  585 for capsanthin (5),  $m/z$  569 for zeaxanthin (6) and  $m/z$  553 for  $\beta$ -cryptoxanthin (8) yielded dehydrated product ions at  $m/z$  567, 551 and 535, respectively (Table 1). In the  $MS^3$  experiment the loss of a second  $H_2O$  molecule was observed for capsanthin and zeaxanthin. Dehydrated fragment ions from protonated molecules  $[M+H-nH_2O]^+$  have been described for all hydroxylated carotenoids.<sup>21,22</sup> Relative intensities of the dehydrated fragment ions may differ and thus reflect the structural characteristics of the hydroxylated end groups of isomeric carotenoids. This different mass spectrometric behavior was used for the distinction of lutein and zeaxanthin.<sup>22</sup> Consequently, compound 6 was identified as zeaxanthin, since it showed a base peak at  $m/z$  569 in the  $MS^1$  experiment with a dehydrated product ion at  $m/z$  551 of low abundance, which is in accordance with the behavior of the zeaxanthin reference compound. In contrast, the lutein reference compound, possessing a 3-hydroxy- $\epsilon$  end group, showed an  $[M+H-H_2O]^+$  ion at  $m/z$  551 as the most abundant signal and another product ion at  $m/z$  533 ( $[M+H-2H_2O]^+$ ) in the  $MS^1$  experiment, which was hardly observed for zeaxanthin (data not shown), thus confirming the data of another study.<sup>23</sup>

The UV/Vis spectrum of  $\beta$ -cryptoxanthin exhibited additional absorption maxima at 331, 348 and 367 nm, as reported for phytofluene in hexane and petroleum ether, respec-



**Figure 2.** Structures of capsanthin (a), capsorubin (b), lutein (c), zeaxanthin (d), antheraxanthin (e), mutatoxanthin (f) and  $\beta$ -cryptoxanthin (g).

**Table 1.** UV/Vis spectra and characteristic ions of non-esterified carotenoids from red pepper pods (*Capsicum annuum* L.)

Compound	Retention time (min)	Identity	HPLC-DAD		[M+H] <sup>+</sup> <i>m/z</i>	HPLC/APCI(+)-MS <sup>n</sup> experiment <i>m/z</i> (% base peak)
			UV/Vis spectrum $\lambda_{\max}$ (nm)			
1	5.0	n.i.	325, 420sh, 441, 469		585	MS <sup>2</sup> [585]: 567 (100), 566 (62), 525 (23), 491 (12) MS <sup>3</sup> [585 → 567]: 549 (100), 444 (78), 566 (56), 548 (24)
2	8.0	n.i.	420sh, 445, 472		585	MS <sup>2</sup> [585]: 567 (100), 549 (12), 577 (12), 431 (10), 565 (10), 511 (9), 527 (9), 432 (9) MS <sup>3</sup> [585 → 567]: 549 (100), 403 (23), 567 (20), 411 (19), 427 (17), 509 (17), 493 (16)
3	9.7	n.i.	406, 427, 453		585	MS <sup>2</sup> [585]: 567 (100), 549 (14), 565 (10), 379 (7), 511 (6) MS <sup>3</sup> [585 → 567]: 549 (100), 567 (46), 511 (28), 236 (10)
4	10.7	n.i.	—		601	MS <sup>2</sup> [601]: 527 (100), 545 (82), 528 (70), 546 (50), 319 (44), 509 (43), 583 (32), 565 (4) MS <sup>3</sup> [601 → 583]: 565 (100), 491 (79), 485 (58), 397 (35), 439 (28), 527 (21)
5	10.8	Capsanthin	474		585	MS <sup>2</sup> [585]: 567 (100), 568 (27), 529 (12), 415 (11), 511 (9), 549 (7), 473 (5), 492 (4) MS <sup>3</sup> [585 → 567]: 567 (100), 568 (50), 511 (41), 549 (39), 493 (19), 397 (16), 483 (16), 429 (12), 550 (12)
6	12.2	Zeaxanthin	420sh, 450, 476		569	MS <sup>2</sup> [569]: 551 (100), 569 (13), 476 (12), 429 (10), 415 (8), 533 (7) MS <sup>3</sup> [569 → 551]: 533 (100), 551 (75), 495 (35), 411 (22), 481 (16), 536 (16)
7	16.8	n.i.	450sh, 472		569	MS <sup>2</sup> [569]: 551 (100), 399 (73), 569 (59), 513 (44), 445 (31), 443 (25), 413 (21) MS <sup>3</sup> [569 → 551]: 551 (100), 495 (76), 533 (31), 393 (28), 427 (27), 429 (22)
8	19.4	$\beta$ -Cryptoxanthin (Phytofluene)	425, 450, 477 (331, 348, 367)		553	MS <sup>2</sup> [553]: 535 (100), 553 (57), 460 (56), 497 (19), 399 (16), 429 (13), 415 (12) MS <sup>3</sup> [553 → 535]: 535 (100), 479 (80), 520 (63), 411 (31), 453 (27), 455 (24), 465 (23)
15	28.1	$\beta$ -Carotene	427sh, 452, 476		537	MS <sup>2</sup> [537]: 537 (100), 400 (44), 413 (43), 399 (41), 414 (34), 401 (31), 482 (30)

n.i. not identified.

tively.<sup>24</sup> This finding was corroborated by the MS<sup>1</sup> experiment, where another molecular ion at *m/z* 543 was detected. Therefore, the coeluting compound was tentatively identified as phytofluene.

CID of the [M+H]<sup>+</sup> ions of compounds 5, 7 and 8 resulted in product ions at *m/z* 529, 513 and 497, respectively, CID of the [M+H-H<sub>2</sub>O]<sup>+</sup> ion of compound 6 produced a fragment at *m/z* 495, each corresponding to a loss of 56 Da, which is usually attributed to retro-Diels-Alder fragmentation reactions in  $\epsilon$ -ionone rings releasing methyl propene units or to  $\beta$ -ionone rings containing a carbonyl function.<sup>15,25</sup> Since capsanthin and  $\beta$ -cryptoxanthin are composed of  $\beta$ -ionone rings without a carbonyl group, this fragmentation behavior was not expected. The loss of 56 Da from zeaxanthin was observed after the expulsion of water, producing a conjugated polyene system which enables this fragmentation pathway. Additionally, [M+H-74]<sup>+</sup> ions were found for compounds 2, 3, 4 and 5 in the MS<sup>2</sup> experiment caused by consecutive losses of water and the neutral molecule methyl propene with a molecular weight of 56 Da.

A loss of 92 Da was observed for compounds 2, 4 and 5 in the MS<sup>2</sup> and MS<sup>3</sup> experiments, respectively, which originates from in-chain fragmentation by the loss of toluene, a characteristic elimination of the polyene system<sup>26-28</sup> or from the sequential losses of 56 Da and two H<sub>2</sub>O molecules (5).

Furthermore, three compounds (1, 2 and 3) displayed [M+H]<sup>+</sup> ions at *m/z* 585. Their fragmentation in the MS<sup>2</sup> and MS<sup>3</sup> experiments yielded base peaks resulting from the dehydrated fragment ions at *m/z* 567 and 549, indicating the presence of two hydroxylated end groups. Compounds 2 and 3 exhibited the same absorption maxima as reported for antheraxanthin and mutatoxanthin in ethanol,<sup>24</sup> those of compound 3 differed only slightly. Since the spectra were recorded during the HPLC run using methanol, MTBE and water as the eluent, these differences may be attributed to solvent effects. An ultimate factor for the differentiation of the fine structure of the UV/Vis spectrum is the ratio between III and II, where the peak height of the most bathochromic absorption band is designated as III and that of the middle absorption band (usually  $\lambda_{\max}$ ) as II. The baseline or zero value is taken as the minimum between the two peaks. The spectral fine structure is then expressed as a percentage of the ratio of the peak heights III/II. Thus, antheraxanthin and mutatoxanthin give % III/II values in ethanol of 55 and 50, respectively.<sup>24</sup> In the present study identical ratios were also found for compounds 2 and 3. Furthermore, the [M+H]<sup>+</sup> ions corroborate our tentative assignments.

The absence of near-UV (about 330 nm) absorbance ('cis-peak') is characteristic of the all-*E* configuration of the double bonds. The absorption maxima of compound 1 showed a hypsochromic shift of 3 and 4 nm, respectively, which can be observed with the introduction of a *Z*-bond compared to the all-*E* configuration.<sup>29</sup> Additionally, a small absorption band at 325 nm was detected for compound 1, indicating a *Z*-isomer probably of antheraxanthin. The occurrence of antheraxanthin in plants containing zeaxanthin can be explained by the precursor function of zeaxanthin in the enzymatic pathway of the xanthophyll biosynthesis.<sup>30</sup>

The [M+H]<sup>+</sup> ion at *m/z* 601 (4) might be indicative of the presence of violaxanthin, exhibiting an additional epoxide

function. The fragmentation of compound 4 showed common losses of 74, 56, 92, 18, 36 Da and 18, 92, 56 Da in the MS<sup>2</sup> and MS<sup>3</sup> experiments, respectively. However, due to coelution with capsanthin and the quantitative predominance of the latter, the absorption maxima of compound 4 could not be determined. The presence of capsorubin ( $m/z$  601) could be excluded by comparison of the retention time with that of the authentic substance. Compound 7 exhibited an  $[M+H]^+$  ion at  $m/z$  569 as observed for zeaxanthin, characterized by the expulsion of two water moieties in the MS<sup>2</sup> and MS<sup>3</sup> experiments, respectively, indicating the presence of two hydroxyl groups. However, the structure of this compound could not be elucidated by its fragmentation pattern and UV/Vis spectrum.

### Carotenoid monoesters

Esterification mainly occurs at the beginning of pigment *de novo* synthesis during ripening, but it also involves pigments previously present in the green fruit. The mechanisms and biosynthetic pathways of xanthophyll esterification have scarcely been studied.<sup>6</sup> In the positive ion mode protonated molecules  $[M+H]^+$  and in the negative ion mode molecular ions  $[M]^-$  were detected for carotenoid monoesters. Fragmentation both in the positive and in the negative mode is dominated by the loss of the fatty acid moiety,<sup>16,20</sup> which was also observed in the present study. Although ions of monoacylated compounds as in-source fragments of carotenoid diesters were detected in high abundance, the signals of carotenoid monoesters were of very low abundance under these conditions. In the positive ion mode carotenoid monoesters produced protonated molecules  $[M+H]^+$  and fatty acids were released as neutral molecules, whereas, in the negative mode, the deacylated ions and the fatty acids were detected as  $[M-acyl]^-$  ions and deprotonated molecules  $[M-H]^-$ , respectively (Fig. 3). Negative ion mode experiments were performed to confirm the results obtained by the positive measurements. Since hydroxylation of the carotenoids and their esterification does not affect the chromophore and therefore have virtually no effect on the absorption spectrum,<sup>8</sup> the characteristic UV/Vis spectra of the non-esterified carotenoids were also used for the identification of their esters.

Compounds 9 and 11 (Table 2) were tentatively identified as capsorubin monoesters ( $m/z$  783 and 811), which both showed the expulsion of water and lauric and myristic acid moieties, respectively, in the MS<sup>2</sup> experiment (18, 200 and

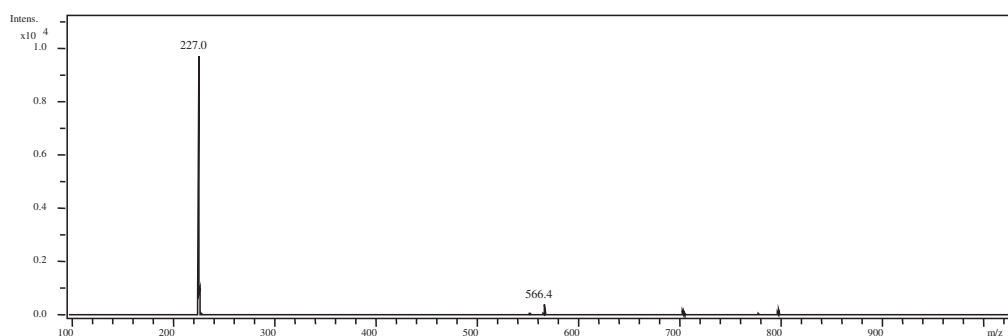
228 Da). Occasionally, these losses were also observed in the MS<sup>1</sup> experiment due to in-source fragmentation. In the MS<sup>3</sup> experiment (fragmentation of the dehydrated product ions) the release of the fatty acid moiety was observed. In all cases the formation of dehydrated product ions seemed to be favored, indicating the presence of a non-esterified hydroxyl function.

Compounds 13, 16, 19 and 20 were characterized as capsanthin monoesters, mainly based on a loss of 200, 228 and 256 Da and a signal at  $m/z$  567 in the MS<sup>2</sup> experiment. Dehydrated molecular ions were of lower signal abundances than observed for capsorubin monoesters. Additionally, CID yielded product ions at  $m/z$  511 and 493, which is in accordance with the fragmentation behavior of free capsanthin (Table 1). Compounds 19 and 20 could not unambiguously be differentiated based on their fragmentation patterns. Therefore, they probably represent the 3-*O* and 3-*O'* isomers. These results confirm the assumption of other authors<sup>16</sup> that monoesterification does not exclusively occur at the 3'-hydroxyl group of the cyclopentane ring.

In contrast to the above-mentioned study,<sup>16</sup> two additional groups of carotenoid esters were found in red pepper pods. Beside capsanthin, zeaxanthin occurred in the extracts of red peppers as free xanthophyll, but also as monoester of myristic and palmitic acid (18 and 21). The fragmentation patterns were similar to those of capsanthin monoesters. Other authors have found zeaxanthin-palmitate in orange and zeaxanthin-myristate in both orange and red pepper (*Capsicum annuum* L.).<sup>31</sup> They additionally identified zeaxanthin esterified with lauric acid in orange peppers, which could not be detected in this study.

Fragmentation of compounds 22, 26 and 31 obtained by CID of isolated pseudomolecular ions and partial in-source fragmentation showed patterns similar to capsorubin, capsanthin and zeaxanthin monoesters. Comparison of the UV/Vis spectra and the product ions at  $m/z$  535 and 479 with the free carotenoids (Table 1) allowed their identification as  $\beta$ -cryptoxanthin derivatives esterified with lauric (22), myristic (26) and palmitic acid (31), respectively. The occurrence of  $\beta$ -cryptoxanthin and zeaxanthin monoesters in *Capsicum* fruits has been reported previously,<sup>5,7,32</sup> but, to the best of our knowledge, the identification both of  $\beta$ -cryptoxanthin and zeaxanthin monoesters in red pepper pods by means of LC/MS has not yet been described.

Two further compounds (10 and 12) were detected exhibiting identical  $[M+H]^+$  ions ( $m/z$  767) and a similar



**Figure 3.** Fragmentation pattern of capsanthin-myristate at  $m/z$  794 in the negative ion mode (16).

**Table 2.** UV/Vis spectra and characteristic ions of carotenoid monoesters from red pepper pods (*Capsicum annuum* L.)

Compound	Retention time (min)	Identity	HPLC-DAD		[M+H] <sup>+</sup> m/z	HPLC/APCI(+)-MS <sup>n</sup> experiment m/z (% base peak)
			UV/Vis spectrum $\lambda_{\max}$ (nm)			
9	21.4	Capsorubin-laurate (C12:0)	479		783	MS <sup>2</sup> [783]: 765 (100), 583 (49), 565 (14), 527 (13), 575 (12) MS <sup>3</sup> [783 → 765]: 565 (100)
10	23.7	n.i.	—		767	MS <sup>2</sup> [767]: 567 (100), 749 (67), 566 (58), 509 (21), 549 (14) MS <sup>3</sup> [767 → 567]: 549 (100), 567 (49), 510 (38), 511 (35), 566 (27), 431 (23)
11	24.6	Capsorubin-myristate (C14:0)	479		811	MS <sup>2</sup> [811]: 581 (100), 793 (97), 583 (31), 582 (21), 565 (21), 431 (12), 471 (10), 799 (10) MS <sup>3</sup> [811 → 793]: 565 (100)
12	25.3	n.i.	425sh, 450, 475sh		767	MS <sup>2</sup> [767]: 567 (100), 749 (83), 565 (40), 566 (34), 549 (13), 559 (9), 485 (8), 673 (8) MS <sup>2</sup> [749]: 549 (100), 731 (29), 599 (16), 611 (16) MS <sup>3</sup> [767 → 567]: 549 (100), 567 (49), 510 (38), 511 (35), 431 (23)
13	25.8	Capsanthin-laurate (C12:0)	474		767	MS <sup>2</sup> [767]: 567 (100), 749 (45), 549 (16), 511 (14), 565 (11), 493 (5), 566 (5), 413 (3) MS <sup>2</sup> [749]: 549 (100), 550 (73), 493 (24), 551 (22), 694 (18), 494 (16), 693 (10) MS <sup>3</sup> [749 → 549]: 493 (100), 549 (60)
14	27.2	n.i.	420sh, 446, 472		795	MS <sup>2</sup> [795]: 567 (100), 777 (45), 565 (13), 566 (13), 549 (11), 511 (5) MS <sup>2</sup> [777]: 549 (100), 759 (25), 531 (10), 547 (9) MS <sup>3</sup> [795 → 777]: 549 (100)
16	28.6	Capsanthin-myristate (C14:0)	475		795	MS <sup>2</sup> [795]: 567 (100), 777 (30), 565 (17), 549 (13), 511 (10), 566 (9), 493 (7) MS <sup>2</sup> [777]: 549 (100), 550 (75), 551 (30), 493 (16), 397 (13) MS <sup>3</sup> [795 → 777]: 549 (100)
17	29.4	n.i.	420sh, 445, 474		795	MS <sup>3</sup> [795 → 567]: 511 (100), 549 (37), 567 (28), 195 (25) MS <sup>2</sup> [795]: 567 (100), 777 (30), 549 (20), 566 (12), 511 (9), 565 (8), 493 (6) MS <sup>2</sup> [777]: 549 (100), 550 (75), 551 (30), 493 (16), 397 (13)
18	30.7	Zeaxanthin-myristate (C14:0)	425sh, 450, 474		779	MS <sup>3</sup> [795 → 567]: 549 (100), 511 (82), 345 (67), 392 (53) MS <sup>2</sup> [779]: 551 (100), 761 (39), 550 (24), 686 (19), 458 (12), 429 (10), 533 (9) MS <sup>2</sup> [761]: 533 (100), 531 (64), 532 (63), 742 (50), 741 (43), 475 (10) MS <sup>2</sup> [551]: 533 (100), 531 (44), 495 (42), 493 (30), 551 (30)
19	31.5	Capsanthin-palmitate (C16:0)	472		823	MS <sup>2</sup> [823]: 567 (100), 805 (52), 549 (13), 511 (12), 415 (11), 565 (10), 493 (10) MS <sup>2</sup> [805]: 549 (100), 493 (41), 359 (21), 749 (20), 550 (11) MS <sup>3</sup> [823 → 567]: 511 (100), 567 (59), 549 (41), 429 (15), 455 (14)
20	32.2	Capsanthin-palmitate (C16:0)	—		823	MS <sup>2</sup> [823]: 567 (100), 805 (67), 565 (23), 566 (21), 549 (15), 509 (6) MS <sup>2</sup> [805]: 549 (100), 749 (23), 493 (19)
21	33.9	Zeaxanthin-palmitate (C16:0)	425sh, 450, 476		807	MS <sup>2</sup> [807]: 551 (100), 789 (55), 550 (18), 458 (15), 714 (13), 533 (12), 549 (12) MS <sup>3</sup> [807 → 789]: 533 (100), 238 (63), 733 (42), 293 (42), 533 (26) MS <sup>3</sup> [807 → 551]: 533 (100), 551 (77), 383 (65), 423 (59), 356 (58), 263 (42)
22	34.6	$\beta$ -Cryptoxanthin-laurate (C12:0)	425sh, 450, 478		735	MS <sup>2</sup> [735]: 535 (100), 642 (16), 534 (11), 442 (7), 533 (6), 321 (6) MS <sup>3</sup> [735 → 535]: 479 (100), 397 (90), 387 (48), 535 (45)
26	36.7	$\beta$ -Cryptoxanthin-myristate (C14:0)	(425sh), 451, 476		763	MS <sup>2</sup> [763]: 535 (100), 533 (15), 534 (13), 670 (9) MS <sup>3</sup> [763 → 535]: 479 (100)
31	39.5	$\beta$ -Cryptoxanthin-palmitate (C16:0)	428, 450, 478sh		791	MS <sup>2</sup> [791]: 535 (100), 549 (17), 442 (8), 699 (8) MS <sup>3</sup> [791 → 535]: 479 (100)

n.i. not identified.

fragmentation behavior as shown for capsanthin monoesters, but a different absorption spectrum. The UV/Vis spectrum of capsanthin showed a single band at 474 nm, whereas compound 12 exhibited the typical fine structure of most carotenoids. The two shoulders (425sh, 475sh) indicate the loss of this fine structure, resulting in a spectrum with less defined maxima, which is probably caused by changes in the ring systems, e.g. by an additional ring double bond or introduction of a carbonyl group in conjugation with the chromophore. Because of coeluting substances a UV/Vis spectrum of compound 10 could not be obtained.

The structures of two further compounds (14 and 17) with an  $[M+H]^+$  ion of  $m/z$  795 could only partially be characterized. They exhibited product ions at  $m/z$  567, 777 and 549, which is identical to the fragmentation pattern of capsanthin-laurate, showing the expulsion of myristic acid and a water moiety. Therefore, compounds 14 and 17 were assigned to monoesters. However, based on the UV/Vis spectra, the presence of two further capsanthin derivatives could be excluded. Further fragmentation of the dehydrated monoester ( $m/z$  777) of compound 14 led to the formation of a product ion at  $m/z$  759  $[\text{monoester}+H-2H_2O]^+$ . Therefore, the presence of at least three hydroxyl functions may be assumed.

Free xanthophylls are usually eluted prior to monoesters and diesters under reversed-phase conditions due to an increase in lipophilicity resulting from the acylation with fatty acids.  $\beta$ -Carotene has often been described being eluted between mono- and diesters.<sup>2,15,17,32-34</sup> However, in the present study, some zeaxanthin and  $\beta$ -cryptoxanthin monoesters (16-22, 27 and 33) showed retention times higher than  $\beta$ -carotene, confirming the results of another study.<sup>7</sup>

### Carotenoid diesters

During ripening of *Capsicum* fruits the contents of diesterified carotenoids increased until a balance between the three esterification fractions was reached.<sup>6</sup> Consequently, a large number of carotenoid diesters were found in the fully ripe red pepper pods investigated in this study (Table 3). In-source fragmentation, indicated by the preferred expulsion of one fatty acid moiety, complicated the identification of these compounds since the protonated diester molecules displayed signals of very low abundance in the positive ion mode. Additionally, intense background noise hampered the interpretation of the MS<sup>1</sup> experiments. These interferences may originate from residual triacylglycerides occurring in the red pepper extracts which may also form positive ions.<sup>18,20</sup> The experiments in the negative ion mode facilitated the characterization of carotenoid diesters, since molecular ions  $[M]^-$  of the diesters were detected in high abundance (Fig. 4).

The identification of the carotenoid diesters described below was based on their fragmentation behavior in the MS<sup>2</sup> experiments and on the comparison of their UV/Vis spectra with the non-esterified carotenoids, respectively. As mentioned previously, in-source fragmentation led to the formation of product ions revealing the loss of one of the fatty acid moieties. These ions were used for further fragmentation experiments.

Although no free capsorubin and only low amounts of capsorubin monoesters were detected, several capsorubin

diesters could tentatively be identified. Compounds 23 and 24 showed  $[M+H]^+$  ions at  $m/z$  993 and predominant fragments at  $m/z$  765 and 793, corresponding to the loss of lauric (200 Da) and myristic (228 Da) acids. Because of low absorbances, a UV/Vis spectrum of compound 24 could not be obtained. Compound 23 showed a hypsochromic shift of 5 nm and an additional absorption band at 377 nm. Therefore, the occurrence of a Z-isomer of the capsorubin diester could be excluded, since the 'cis-peak' appears at a characteristic wavelength in the UV/Vis region  $142 \pm 2$  nm below the longest wavelength absorption maximum in the spectrum of the all-*trans* compound.<sup>24</sup>

Compounds 28 and 30 exhibited  $[M+H]^+$  ions at  $m/z$  1021 and 1049. CID of these compounds produced fragments both at  $m/z$  793, corresponding to a loss of myristic acid (228 Da) and palmitic acid (256 Da), and at  $m/z$  565 indicating the capsorubin backbone. Additionally, fragmentation of  $m/z$  1049 led to a product ion at  $m/z$  821 resulting from the loss of myristic acid. These results and the UV/Vis spectra allowed the characterization as capsorubin esterified with two myristic acids and one myristic and palmitic acid moiety, respectively.

The occurrence of several capsanthin diesters in *Capsicum* species has previously been reported.<sup>2,5,7,16</sup> In accordance with these studies, compounds 25, 27, 29, 32, 33, 35, 36 and 40 were tentatively identified as capsanthin diesters of lauric, myristic and palmitic acids. The compounds were eluted from the C30 column with increasing chain length of the fatty acids. Compounds 25, 32 and 40 are homogeneous capsanthin diesters since they are characterized by the loss of two moieties of lauric (200 Da), myristic (228 Da) and palmitic (256 Da) acids and a signal at  $m/z$  549 in the MS<sup>2</sup> experiments, representing the dehydrated capsanthin backbone. In addition to previous findings,<sup>16</sup> another  $[M+H]^+$  ion at  $m/z$  1005 (33) was detected, the fragmentation of which produced ions at  $m/z$  805, 749 and 549, indicating the loss of a lauric and palmitic acid moiety. Therefore, compound 33 was tentatively identified as capsanthin-laurate-palmitate. In the MS<sup>1</sup> experiment of compound 33 (Table 4) the expulsion of lauric acid occurred due to in-source fragmentation, whereas the product ion at  $m/z$  749, indicating the loss of the palmitic acid moiety, could not be observed under these conditions. The elimination of the fatty acid linked to the 3-hydroxy group of the capsanthin  $\beta$ -cyclohexene ring ( $\beta$  end group) is energetically favored because of the elongation of the conjugated  $\pi$ -system. Therefore, it may be assumed that the 3-hydroxy group is esterified with lauric acid and palmitic acid is attached to the 3'-hydroxy group of the capsanthin cyclopentane ring ( $\kappa$  end group).

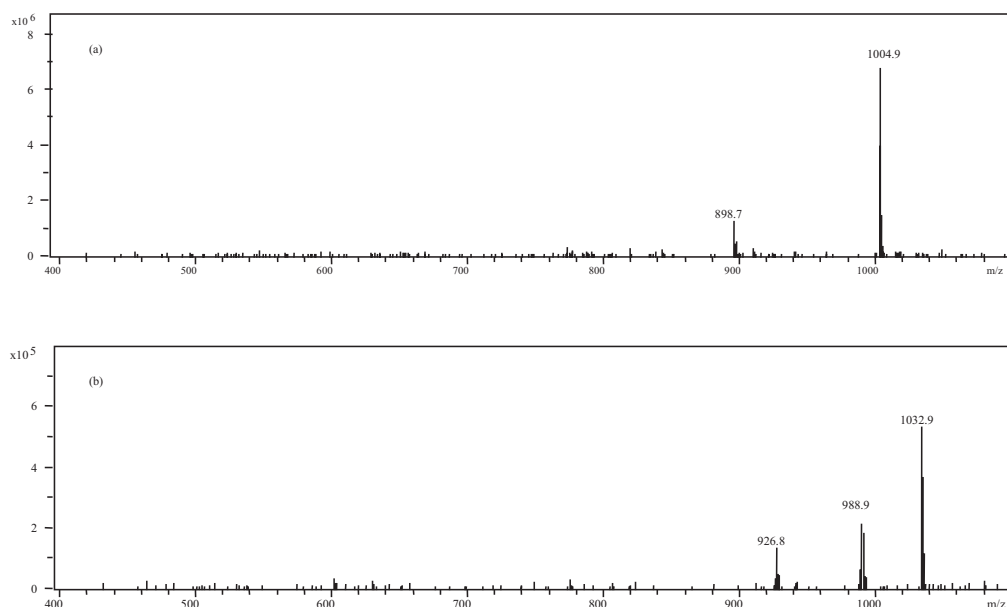
A similar behavior was observed for compounds 35 and 36. Both the MS<sup>1</sup> experiment (Table 4) and the total ion chromatograms of the prominent product ions at  $m/z$  777 and 805 in the MS<sup>2</sup> experiment (Figs. 5(a) and 5(b)) displayed the different in-source fragmentation behavior of compounds 35 and 36. It is reasonable to assume that compounds 35 and 36 represent the two regioisomers of capsanthin esterified with palmitic and myristic acid since the order of fatty acid elimination was different. The fragmentation behavior of the isolated intact diester ions at  $m/z$  1033 confirmed this assumption (Table 3).



**Table 3.** UV/Vis spectra and characteristic ions of carotenoid diesters from red pepper pods (*Capsicum annuum* L.)

Compound	Retention time (min)	Identity	HPLC-DAD		[M+H] <sup>+</sup> m/z	HPLC/APCI(+)-MS <sup>n</sup> experiment m/z (% base peak)
			UV/Vis spectrum $\lambda_{\max}$ (nm)			
23	35.5	n.i.	377, 474		993	MS <sup>2</sup> [993]: 765 (100), 793 (67), 974 (23), 902 (21), 764 (19) MS <sup>2</sup> [793]: 565 (100), 509 (26), 549 (20), 592 (20) MS <sup>2</sup> [765]: 565 (100)
24	35.6	Capsorubin-laurate-myristate (C12:0, C14:0)	—		993	MS <sup>2</sup> [993]: 793 (100), 765 (53), 565 (15) MS <sup>2</sup> [793]: 565 (100)
25	36.7	Capsanthin-di-laurate (C12:0, C12:0)	474		949	MS <sup>2</sup> [765]: 565 (100), 509 (15), 613 (9), 549 (7) MS <sup>2</sup> [949]: 749 (100), 549 (24), 748 (7), 721 (6), 397 (6) MS <sup>2</sup> [749]: 549 (100), 693 (18), 493 (17), 397 (5), 371 (3)
27	37.7	Capsanthin-laurate-myristate (C12:0, C14:0)	474		977	MS <sup>2</sup> [977]: 749 (100), 777 (19), 747 (16), 549 (12) MS <sup>2</sup> [749]: 549 (100), 693 (19), 493 (18), 397 (7), 427 (3)
28	38.3	Capsorubin-di-myristate (C14:0, C14:0)	480		1021	MS <sup>2</sup> [1021]: 565 (100), 793 (86), 791 (41), 821 (38) MS <sup>2</sup> [793]: 565 (100), 566 (95), 509 (61), 510 (52), 413 (32), 737 (21) MS <sup>2</sup> [977]: 749 (100), 777 (91), 748 (21), 549 (18), 977 (18)
29	38.3	Capsanthin-laurate-myristate (C12:0, C14:0)	474		977	MS <sup>2</sup> [777]: 549 (100), 547 (28), 721 (17), 548 (15), 493 (14) MS <sup>2</sup> [749]: 550 (100), 549 (76), 551 (29), 493 (22), 694 (22), 494 (21), 693 (15)
30	39.1	Capsorubin-myristate-palmitate (C14:0, C16:0)	479		1049	MS <sup>2</sup> [1049]: 793 (100), 821 (32), 565 (5) MS <sup>2</sup> [821]: 565 (100), 509 (31), 566 (27), 413 (20), 765 (6) MS <sup>2</sup> [793]: 565 (100)
32	40.1	Capsanthin-di-myristate (C14:0, C14:0)	474		1005	MS <sup>2</sup> [1005]: 777 (100), 776 (36), 775 (24), 549 (22) MS <sup>2</sup> [777]: 549 (100), 550 (97), 721 (37), 722 (34), 493 (30), 551 (26), 494 (21), 723 (12)
33	40.3	Capsanthin-laurate-palmitate (C12:0, C16:0)	—		1005	MS <sup>2</sup> [1005]: 805 (100), 749 (80), 549 (35) MS <sup>2</sup> [805]: 549 (100), 493 (17), 397 (11), 749 (4)
34	40.3	Zeaxanthin-laurate-myristate (C12:0, C14:0)	425, 450, 478		961	MS <sup>2</sup> [749]: 549 (100), 550 (98), 551 (36), 694 (28), 493 (27), 494 (27) MS <sup>2</sup> [961]: 761 (100), 733 (86), 533 (49), 760 (45), 668 (41) MS <sup>2</sup> [761]: 533 (100), 531 (37), 532 (22), 450 (10)
35	42.8	Capsanthin-palmitate-myristate (C16:0, C14:0)	474		1033	MS <sup>2</sup> [733]: 533 (100), 411 (5) MS <sup>2</sup> [1033]: 777 (100), 805 (74), 549 (16) MS <sup>2</sup> [777]: 549 (100), 550 (68), 493 (24), 551 (20), 721 (17), 494 (15), 722 (10)
36	42.8	Capsanthin-myristate-palmitate (C14:0, C16:0)	474		1033	MS <sup>2</sup> [805]: 549 (100), 493 (18), 749 (11), 411 (4) MS <sup>2</sup> [1033]: 805 (100), 777 (73), 549 (18), 1033 (6)
37	42.8	Zeaxanthin-di-myristate (C14:0, C14:0)	425, 450, 478		989	MS <sup>2</sup> [805]: 549 (100), 550 (97), 749 (27), 493 (27), 750 (25), 551 (23), 751 (12) MS <sup>2</sup> [777]: 549 (100), 493 (24), 721 (17), 550 (16), 397 (6), 551 (3), 494 (4), 722 (4)
38	43.6	Zeaxanthin-laurate-palmitate (C12:0, C16:0)	426sh, 450, 478		989	MS <sup>2</sup> [989]: 761 (100), 668 (31), 760 (19), 285 (16), 533 (14) MS <sup>2</sup> [761]: 533 (100), 411 (8)
39	44.7	n.i. (C16:0, C16:0)	404, 428, 454		1061	MS <sup>2</sup> [989]: 733 (100), 789 (45), 533 (35), 788 (32), 696 (16), MS <sup>2</sup> [789]: 533 (100), 411 (6) MS <sup>2</sup> [733]: 533 (100)
40	45.4	Capsanthin-di-palmitate (C16:0, C16:0)	474		1061	MS <sup>2</sup> [1061]: 805 (100), 615 (7), 549 (5) MS <sup>2</sup> [805]: 549 (100), 531 (4)
41	46.0	Zeaxanthin-myristate-palmitate (C14:0, C16:0)	426sh, 450, 478		1017	MS <sup>2</sup> [1061]: 805 (100), 549 (12), 804 (11), 1061 (5) MS <sup>2</sup> [805]: 549 (100), 550 (88), 551 (30), 493 (28), 749 (23), 750 (22)
42	49.4	Zeaxanthin-di-palmitate (C16:0, C16:0)	426sh, 450, 478		1045	MS <sup>2</sup> [1017]: 761 (100), 789 (65), 760 (34), 533 (32), 668 (30) MS <sup>2</sup> [789]: 533 (100), 411 (8), 397 (4), 531 (3), 477 (3) MS <sup>2</sup> [761]: 533 (100), 411 (7), 477 (4), 532 (3), 531 (3), 397 (2) MS <sup>2</sup> [1045]: 789 (100), 696 (30), 788 (25), 532 (8), 952 (7) MS <sup>2</sup> [789]: 533 (100), 411 (7), 477 (3), 397 (3), 532 (2)

n.i. not identified.



**Figure 4.** MS<sup>1</sup> experiment in the negative ion mode of capsanthin-di-myristate (a) (32) and capsanthin-myristate-palmitate (b) (36).

**Table 4.** Characteristic ions of carotenoid diesters from red pepper pods (*Capsicum annuum* L.) detected in the MS<sup>1</sup> experiment

Compound	Retention time (min)	HPLC/APCI(+)-MS <sup>1</sup> experiment <i>m/z</i> (% base peak)
33 and 34	40.3	805 (100), 806 (48), 733 (17), 734 (13), 533 (11), 761 (9), 1005 (7), 961 (7)
35	42.7	777 (100), 778 (49), 549 (20), 1033 (12), 686 (6)
36 and 37	42.7	805 (100), 777 (22), 761 (15), 778 (13), 549 (9), 533 (8), 989 (5)

In addition to capsanthin-di-palmitate, another  $[M+H]^+$  ion at *m/z* 1061 (39) was detected, showing a similar fragmentation behavior as the homogeneous capsanthin ester. CID of this compound led to the loss of two palmitic acid moieties and the UV/Vis spectra showed the characteristic fine structure of most carotenoids. Therefore, this compound represents a xanthophyll with two hydroxyl groups esterified with two palmitic acid moieties.

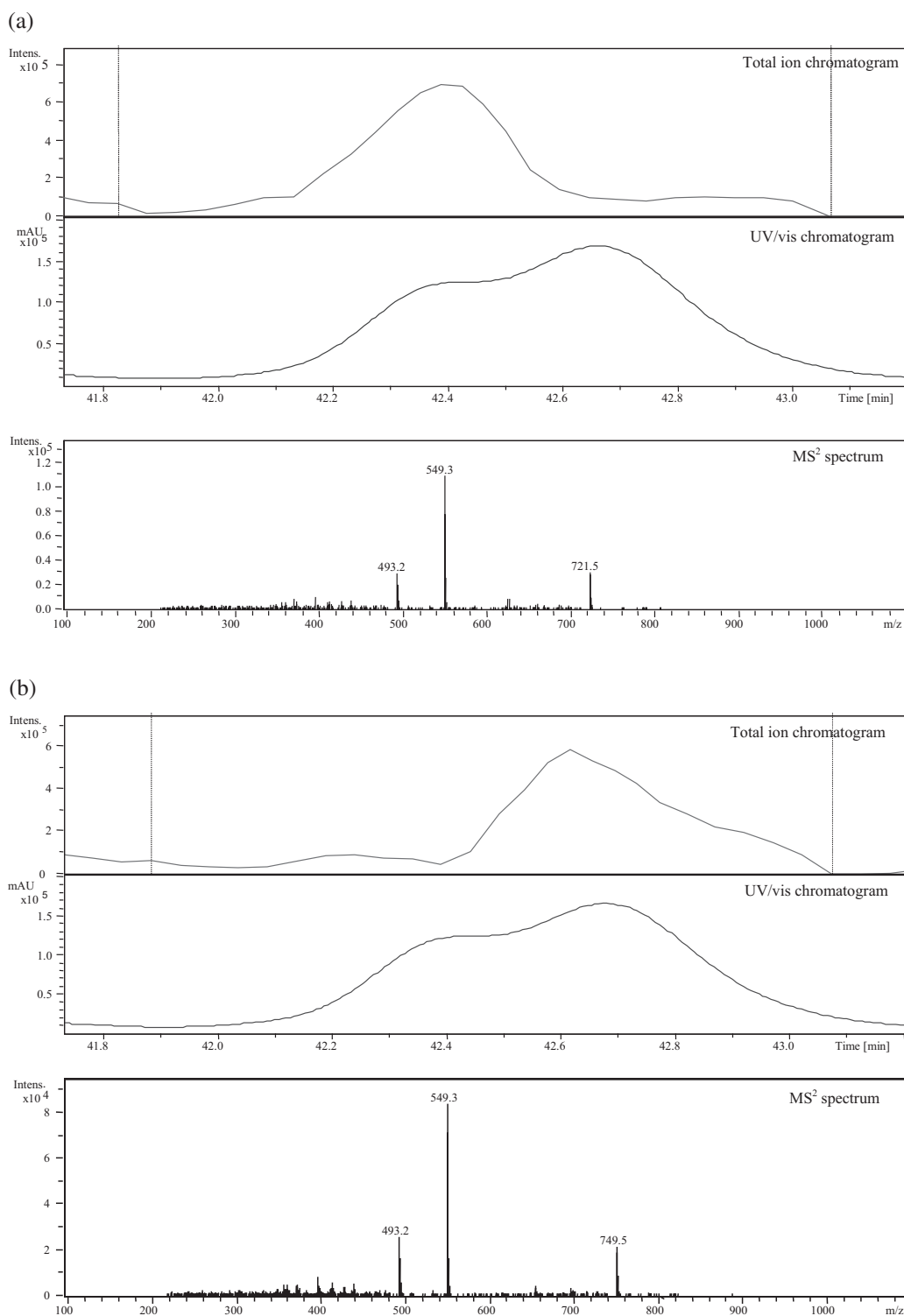
Finally, several zeaxanthin diesters were found in red pepper pods, which is in agreement with previous findings.<sup>7,16</sup> Compounds 37 and 42 were homogeneously esterified with myristic and palmitic acid. Isolation and fragmentation of the molecular ions at *m/z* 989 and 1045 displayed a loss of one myristic and one palmitic acid, respectively. Further fragmentation of *m/z* 761 and 789 produced a base peak at *m/z* 533, thus revealing the zeaxanthin backbone and the loss of another myristic and palmitic acid moiety. Another compound (38) showed an  $[M+H]^+$  ion at *m/z* 989, but a fragmentation pattern different from that of zeaxanthin-di-myristate. Since it exhibited a product ion at *m/z* 533, owing to the loss of 200 and 256 Da in the MS<sup>2</sup> experiment, compound 38 was characterized as zeaxanthin-laurate-palmitate.

Compound 34 exhibited an  $[M+H]^+$  ion at *m/z* 961. CID of this ion led to predominant product ions at *m/z* 761 and 733, indicating the loss of one lauric and one myristic acid moiety. Further fragmentation yielded a major product ion at *m/z* 533.

Therefore, compound 34 was tentatively identified as zeaxanthin-laurate-myristate. Compound 41, showing a similar fragmentation behavior, exhibited an  $[M+H]^+$  ion at *m/z* 1017. CID of this pseudomolecular ion led to predominant product ions at *m/z* 789 and 761, indicating the loss of one myristic and one palmitic acid moiety. Further fragmentation yielded a major product ion at *m/z* 533. Therefore, compound 41 was tentatively identified as zeaxanthin-myristate-palmitate.

## CONCLUSIONS

More than 30 carotenoid compounds were characterized in red pepper pods by liquid chromatography coupled to mass spectrometry. In view of the obvious lack of commercially available reference compounds of carotenoid esters, elucidation of such complex mixtures is only possible using mass spectrometric detection. Additionally, differences in the mass spectrometric behavior of esterified carotenoids in positive and negative ion mode measurements were observed in the present study. Furthermore, although the occurrence of zeaxanthin and  $\beta$ -cryptoxanthin monoesters in some *Capsicum* species has already been reported in the literature, their presence in red pepper pods has been confirmed by LC/MS for the first time. In-source fragmentation behavior and CID of the  $[M+H]^+$  ions of some capsanthin diesters allowed the differentiation of individual regioisomers by their specific fragmentation patterns.



**Figure 5.** (a) HPLC separation of capsanthin diester (35 and 36) detected at 450 nm (middle column), total ion chromatogram of isolated product ion at  $m/z$  777 (upper column), and MS<sup>2</sup> spectrum of isolated product ion at  $m/z$  777 (lower column). (b) HPLC separation of capsanthin diester (35 and 36) detected at 450 nm (middle column), total ion chromatogram of isolated product ion at  $m/z$  805 (upper column), and MS<sup>2</sup> spectrum of isolated product ion at  $m/z$  805 (lower column).

In agreement with an earlier report,<sup>16</sup> it could be shown that carotenoids in fully ripe red pepper pods are largely esterified with saturated lauric, myristic and palmitic acids. Unsaturated fatty acids as described by other authors<sup>7,16,35</sup> were not found as acyl compounds. The health-promoting potential of several free carotenoids has frequently been

reported; however, *in vivo* studies on bioavailability, conversion and physiological effects of carotenoid esters are scarce.<sup>36–39</sup> Since carotenoid esters were shown to have a higher stability than free carotenoids during processing of red pepper products depending on the esterified fatty acids, they may also contribute to the color stability.<sup>40,41</sup> Both

technological and physiological aspects support the need for further studies on carotenoid ester composition in spices and vegetables.

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