Rapid Identification of Carotenoids in a Combination of Liquid Chromatography / UV-Visible Absorption Spectrometry by Photodiode-Array Detector and Atomospheric Pressure Chemical Ionization Mass Spectrometry (LC/PAD/APCI-MS)

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Abstract: The combination method of high performance liquid chromatography / UV-Visible (Vis) absorption spectrometry by photodiode-array detector and atmospheric pressure chemical ionization mass spectrometry (LC/PAD/APCI-MS) were applied for the rapid identification of carotenoids obtained from several natural sources. APCI-MS spectra provided not only information of the molecule weight but also the presence of functional groups such as hydroxy, acetyl and glycosyl groups in carotenoids. On the other hand, UV-Vis spectra gave the information of chromophores which consist of the conjugated double bonds and end groups in carotenoids. This combination method allows the carotenoids to be characterized by retention time in HPLC, APCI-MS and UV-Vis spectral data so that it offers rapid and accurate method for the identification of carotenoids extracted from various natual sources.
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1 Introduction

Carotenoids are the pigments responsible for yellow, orange, red and purple colors distributed throughout nature. More than 600 different structures of carotenoids have been identified from biological sources (1,2), which are very labile and closely related compounds. Therefore, a rapid, careful and reliable method for analysis of carotenoids is needed (2).

High performance liquid chromatography / atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) has been found to be one of the most powerful techniques for the identification of natural products, especially thermally labile compounds (3). Recently, Clarke *et al.* (4) reported the performance of positive-ion LC/APCI-MS for the determination of nine carotenoids. Subsequently, van Breemen *et al.* (5) demonstrated the advantage of LC/APCI-MS with a narrow bore C_{30} reverse phase HPLC column, including a collision-induced dissociation (CID) experiment for the determination of some carotenoids. However, it is difficult to identify the carotenoid only with APCI-MS spectral data.

On the other hand, UV-Visible (Vis) spectra including the absorption maxima gave the chromophore information in carotenoids which can not be provided from APCI-MS data. Therefore, we have applied the combination method of high performance liquid chromatography / UV-Visible (Vis) absorption spectrometry by photodiode-array detector and atmospheric pressure chemical ionization mass spectrometry (LC/PAD/APCI-MS)

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for the rapid identification of carotenoids. This paper reports the performance of LC/PAD/APCI-MS for rapid and accurate identification of carotenoids obtained from various natural sources.

2 Experimental

2·1 LC/PAD/APCI-MS

A Hitachi M-1000 APCI mass spectrometer was connected to a Hitachi L-6200/L-6000 HPLC system (Hitachi, Ltd., Tokyo, Japan) equipped with a Shimadzu SPD-M10AVP photodiode-array detector (Shimadzu corporation, Kyoto, Japan). The UV-Vis absorption spectra were recorded from 250 to 600 nm using a photodiode-array detector (PAD) located in-line between the HPLC column and the APCI-MS. The positive-ion APCI mass spectra were acquired by scanning from m/z 200 to 800 using a Hitachi M-1000 quadrupole mass spectrometer. The APCI interface section was set at a drift voltage of 15-50 V, a multiplier voltage of 1800 V, a needle voltage of 3000 V and a nebulizer temperature of 200-250℃.

2·2 HPLC Systems

HPLC systems used for the separation of carotenoids in this study were as follows: System 1 (stationary phase HPLC); column: YMC-Pack SIL-06 (particle size 5 μm, YMC, Inc. Wilmington, USA) 250 mm L. × 4.6 mm I.D., mobile phase: tetrahydrofuran (THF): hexane (35:65), flow rate: 1.0 ml/min; System 2 (reversed phase HPLC); column: YMC-ODS A-302 (particle size 10 μm, YMC, Inc. Wilmington, USA) 250 mm L. × 4.6 mm I.D., mobile phase: methanol (MeOH) -water (9:1), flow rate: 1.0 ml/min.

2·3 Standard Carotenoid Samples

The standard carotenoids used in this study are shown in **Fig. 1.** They were obtained from the following sources: α-Carotene, β-carotene, β-cryptoxanthin, zeaxanthin, lutein, antheraxanthin, mutatoxanthin, luteoxanthin, cucurbitaxanthin A, violaxanthin, luteoxanthin, auroxanthin, and neoxanthin from pumpkin (*Cucurbita maxima*), γ-carotene, and lycopene from tomato (*Lycopersicon esculentum*), lactucaxanthin from lettuce (*Lactuca sativa*), rhodoxanthin from the fruit of yew (*Taxus cuspidata*), cryptocapsin, capsanthin, capsanthin 3,6-epoxide, and capsorubin from paprika (*Capsicum annuum*), fucoxanthin from brown alga (*Undaria pinnatitida*), peridinine from fresh water red tide (*Peridinium bipes*), myxoxanthophyll, and echinenone from blue-green alga (*Mycrocystis sp.*), and loroxanthin from chlorella (*Chlorella vulgaris*).

2·4 Extraction of Carotenoids from Biological Materials

The carotenoids were extracted with MeOH from plants according to our routine procedures. Because of the presence of esterified carotenoids in the case of pumpkin, paprika, lettuce and tomato, these MeOH extracts were saponified with 5% KOH/MeOH (weight/vol) at room temperature for 2 hrs in a usual manner. Each extract was dissolved in a mobile phase and filtered through a 0.45 μm polytetrafluoroethylene membrane filter and then analyzed by LC/PAD/APCI-MS.

3 Results and Discussion

3·1 Selection of Optimum Condition for LC/APCI-MS of Carotenoids

In order to determine the suitable APCI-MS parameters flow injection of zeaxanthin was carried out in positive-ion mode. The drift voltage range for observation of the protonated molecule $[M+H]^+$ at m/z 569 of zeaxanthin was 15-50 V, while the most effective voltage for generation of significant fragment ions was 40-50 V. The optimum nebulizer temperature was found to be 200-250℃.

About 100 ng of each carotenoid is needed to record the accurate full scan mass spectrum.

3·2 Positive-ion APCI Mass Spectra of Some Carotenoids

Table 1 shows the typical ions resulting from positive-ion APCI mass spectra of carotenoids obtained by LC/APCI-MS. Protonated molecules $[M+H]^+$ were observed for all carotenoids investigated.

APCI mass spectra of carotenes (hydrocarbons) provided only protonated molecule $[M+H]^+$ without significant fragment ions having structural information. The LC/APCI mass spectra of α-carotene, β-carotene, γ-carotene and lycopene, which possess the same molecular formula $C_{40}H_{56}$, showed prominent protonated molecules at the same m/z 537 and their spectra were very similar to each other. Therefore, these four carotenes could not be distinguished by only APCI

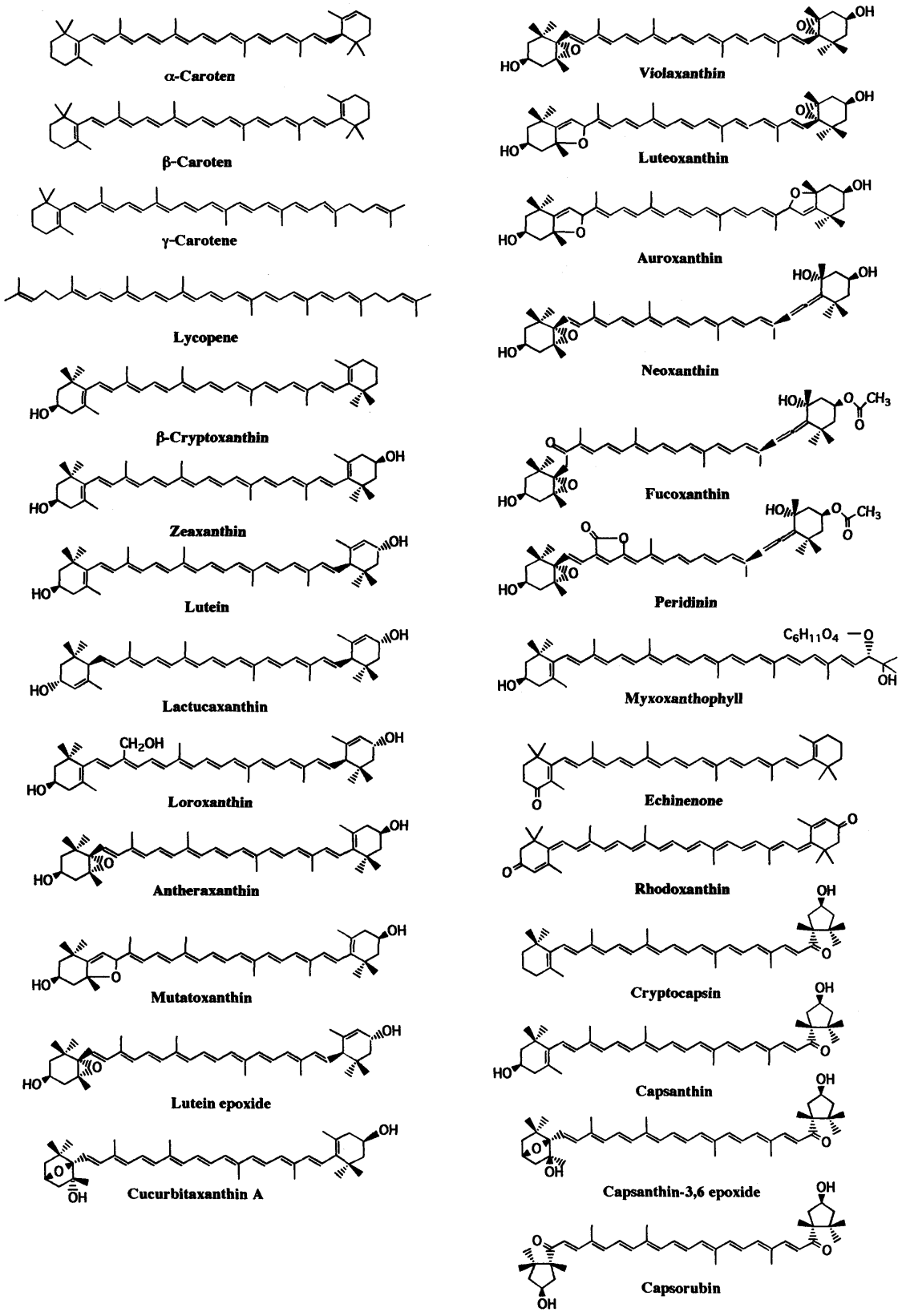

Fig. 1 Structures of Carotenoids Used in This Study.

Table 1 Characteristic Ions Resulting from LC/APCI-MS and UV-Vis Absorption Maxima Obtained by PAD of Carotenoids.

Compound	Formula	Major ions	Identity	Abundance (%)	Absorption maxima (nm)
Carotens					
α-Carotene	$C_{40}H_{56}$	537	$[M+H]^+$	100	423, 448, 476[a]
β-Carotene	$C_{40}H_{56}$	537	$[M+H]^+$	100	450, 476[a]
γ-Carotene	$C_{40}H_{56}$	537	$[M+H]^+$	100	440, 460, 490[a]
Lycopene	$C_{40}H_{56}$	537	$[M+H]^+$	100	445, 472, 503[a]
Xanthophylls					
β-Cryptoxanthin	$C_{40}H_{56}O$	553	$[M+H]^+$	100	450, 476[b]
		535	$[M+H-H_2O]^+$	10	
Zeaxanthin	$C_{40}H_{56}O_2$	569	$[M+H]^+$	100	450, 476[b]
		551	$[M+H-H_2O]^+$	26	
Lutein	$C_{40}H_{56}O_2$	569	$[M+H]^+$	21	423, 446, 476[b]
		551	$[M+H-H_2O]^+$	100	
		533	$[M+H-2H_2O]^+$	5	
Lactucaxanthin	$C_{40}H_{56}O_2$	569	$[M+H]^+$	5	423, 438, 468[b]
		551	$[M+H-H_2O]^+$	100	
		533	$[M+H-2H_2O]^+$	10	
Loroxanthin	$C_{40}H_{56}O_3$	585	$[M+H]^+$	40	423, 446, 476[b]
		551	$[M+H-H_2O]^+$	100	
		533	$[M+H-2H_2O]^+$	5	
Antheraxanthin	$C_{40}H_{56}O_3$	585	$[M+H]^+$	100	423, 446, 476[b]
		567	$[M+H-H_2O]^+$	20	
Mutatoxanthin	$C_{40}H_{56}O_3$	585	$[M+H]^+$	100	408, 426, 456[b]
		567	$[M+H-H_2O]^+$	18	
Lutein epoxide	$C_{40}H_{56}O_3$	585	$[M+H]^+$	30	423, 438, 468[b]
		567	$[M+H-H_2O]^+$	100	
Cucurbitaxanthin A	$C_{40}H_{56}O_3$	585	$[M+H]^+$	100	423, 446, 476[b]
		567	$[M+H-H_2O]^+$	14	
Violaxanthin	$C_{40}H_{56}O_4$	601	$[M+H]^+$	100	423, 438, 468[b]
		583	$[M+H-H_2O]^+$	20	
Luteoxanthin	$C_{40}H_{56}O_4$	601	$[M+H]^+$	100	403, 420, 448[b]
		583	$[M+H-H_2O]^+$	20	
Auroxanthin	$C_{40}H_{56}O_4$	601	$[M+H]^+$	100	381, 402, 428[b]
		583	$[M+H-H_2O]^+$	20	
Neoxanthin	$C_{40}H_{56}O_4$	601	$[M+H]^+$	100	423, 438, 468[b]
		583	$[M+H-H_2O]^+$	40	
		565	$[M+H-2H_2O]^+$	5	
Fucoxanthin	$C_{42}H_{56}O_6$	659	$[M+H]^+$	42	446, 476[b]
		641	$[M+H-H_2O]^+$	100	
		623	$[M+H-2H_2O]^+$	6	
		581	$[M+H-H_2O-AcOH]^+$	70	
		563	$[M+H-2H_2O-AcOH]^+$	6	
Peridinin	$C_{39}H_{50}O_7$	631	$[M+H]^+$	10	455, 485[b]
		613	$[M+H-H_2O]^+$	34	
		596	$[M+H-2H_2O]^+$	6	
		553	$[M+H-H_2O-AcOH]^+$	100	
		535	$[M+H-2H_2O-AcOH]^+$	18	
Myxoxanthophyll	$C_{46}H_{66}O_7$	731	$[M+H]^+$	10	450, 478, 510[b]
		713	$[M+H-H_2O]^+$	14	
		567	$[M+H-Ram]^+$	100	

Table 1 continued

Compound	Formula	Major ions	Identity	Abundance (%)	Absorption maxima (nm)
		549	$[M+H-H_2O-Ram]^+$	10	
Echinenone	$C_{40}H_{54}O$	551	$[M+H]^+$	100	458[b]
Rhodoxanthin	$C_{40}H_{50}O_2$	563	$[M+H]^+$	100	455, 487, 521[b]
Cryptocapsin	$C_{40}H_{56}O_2$	569	$[M+H]^+$	100	450, 475, 505[b]
		551	$[M+H-H_2O]^+$	10	
Capsanthin	$C_{40}H_{56}O_3$	585	$[M+H]^+$	100	450, 475, 505[b]
		567	$[M+H-H_2O]^+$	20	
Capsanthin-3, 6 epoxide	$C_{40}H_{56}O_4$	601	$[M+H]^+$	100	445, 470, 498[b]
		583	$[M+H-H_2O]^+$	25	
Capsorubin	$C_{40}H_{56}O_4$	601	$[M+H]^+$	100	445, 479, 510[b]
		583	$[M+H-H_2O]^+$	10	

Rham: Rhamnose ($C_6H_{12}O_5$).

[a]recorded in MeOH : H_2O (9 : 1), [b]recorded in THF-hexane (35 : 65).

mass spectra.

On the other hand, xanthophylls showed not only $[M+H]^+$ but also the same characteristic fragment ions such as $[M+H-nH_2O]^+$, $[M+H-AcOH]^+$ and $[M+H-sugar]^+$. Dehydrated fragment ions from protonated molecules $[M+H-nH_2O]^+$ were observed for all hydroxylated carotenoids investigated as shown in **Table 1.** Furthermore, intensities of these dehydrated fragment ions reflect the structural characteristics of the hydroxylated end group in carotenoids. For example, zeaxanthin (β,β-carotene-3,3′-diol), lutein (β,ε-carotene-3,3′-diol) and lactucaxanthin (ε,ε-carotene-3,3′-diol), which possess the same molecular formula $C_{40}H_{56}O_2$, showed significant differences in the intensities of ion peaks at m/z 569 $[M+H]^+$, m/z 551 $[M+H-H_2O]^+$ and m/z 533 $[M+H-2H_2O]^+$ in LC/APCI mass spectra as shown in **Fig. 2.** Zeaxanthin showed a base peak at m/z 569 $[M+H]^+$ with a weak dehydrated ion at m/z 551 $[M+H-H_2O]^+$. In contrast, both lutein and lactucaxanthin possessing a 3-hydroxy-ε-end group showed m/z 551 $[M+H-H_2O]^+$ as the most abundant ion and also showed a fragment ion at m/z 533 $[M+H-2H_2O]^+$, which was hardly observed in zeaxanthin as shown in **Fig. 2.** These fragmentation patterns were in good agreement with EI-MS data (6) and provided structural information of hydroxylated carotenoids. Dehydrated ions were also obtained in expoxy carotenoids possessing hydroxy groups in their molecule such as violaxanthin, antheraxanthin and neoxanthin.

Fucoxanthin and peridinin showed characteristic fragment ion peaks $[M+H-H_2O-AcOH]^+$ and $[M+H-2H_2O-AcOH]^+$ indicating the presence of an acetylenic moiety in their molecule. In the case of glycoside carotenoids such as myxoxanthophyll, elimination of a sugar moiety from the protonated molecule was detected as a base peak ion. In APCI MS of myxoxanthophyll, the most abundant ion at m/z 567 corresponded to the elimination of rhamnose from the protonated molecule $[M+H-C_6H_{12}O_5]^+$.

On the other hand, keto carotenoids such as echinenone and rhodoxanthin provided only protonated molecules without significant fragment ions in APCI-MS.

3·3 UV-Vis Spectra of Some Carotenoids

The UV-Vis absorption maxima obtained by on line LC/PAD analysis of each carotenoid are shown in **Table 1.** These UV-Vis spectra provide the chromophore information in carotenoids which can not be provided from APCI-MS data. Therefore, α-carotene, β-carotene, γ-carotene and lycopene, which could not be distinguished by only APCI-MS, were characterized by a combination of UV-Vis spectral data with APCI-MS. Similarly, UV-Vis spectra could differentiate between 5,6-epoxy and 5,8-epoxy carotenoids. Thus, violaxanthin (5,6;5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol), luteoxanthin (5,8;5′,6′-diepoxy-5,8;5′,6′-tetrahydro-β,β-carotene-3,3′-diol), auroxanthin (5,8;5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene-3,3′-diol) possessing the same molecular formula $C_{40}H_{56}O_4$, could be characterized by a combination of UV-Vis spectra data with APCI-MS.

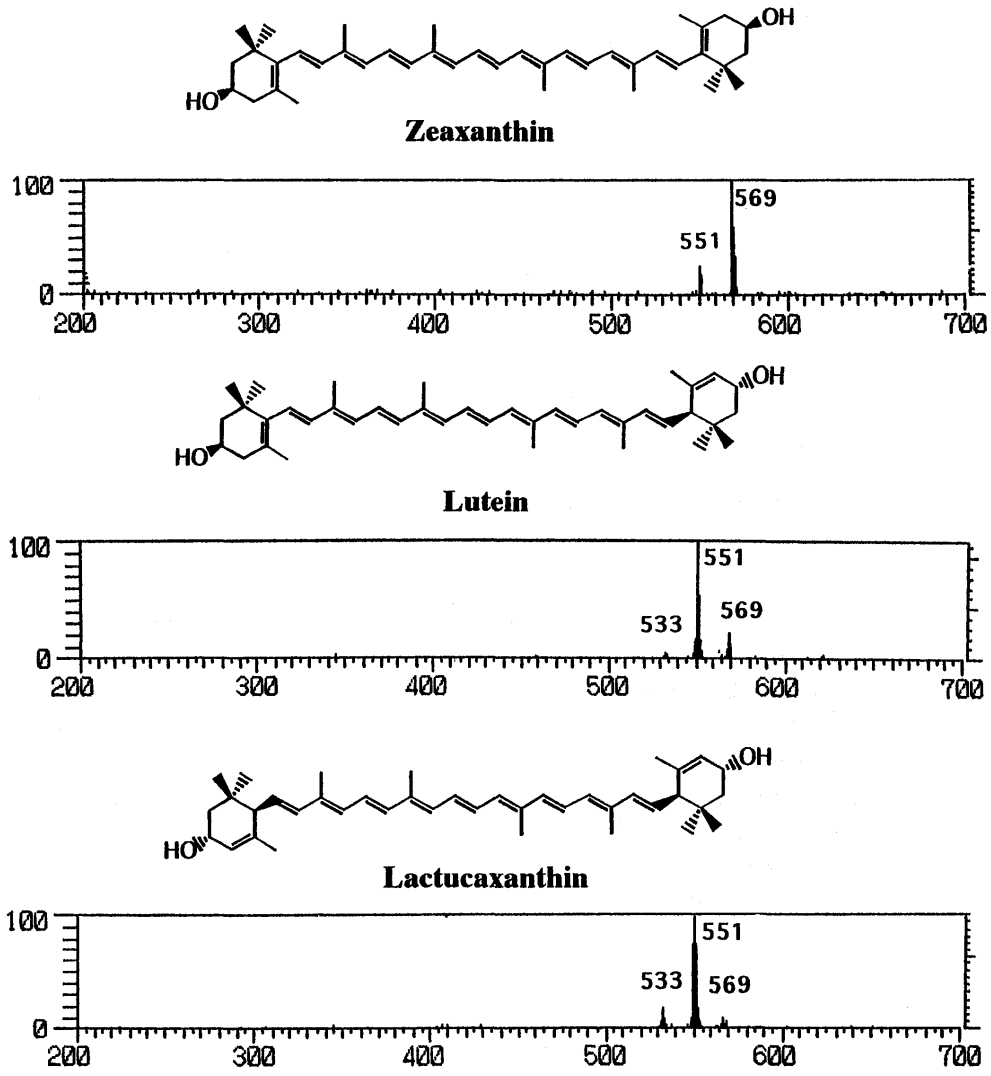

Fig. 2 Positive-ion APCI-MS Spectra of Zeaxanthin, Lutein and Lactucaxanthin.

Moreover, UV-Vis spectra distinguished the *cis* isomer from the corresponding *trans* carotenoid (7). **Fig. 3** shows the examples of the identification of geometrical isomers of lycopene in tomato by the LC/PAD/APCI-MS method.

3·4 Application of LC/PAD /APCI-MS for Identification of Natural Carotenoids

The LC/PAD/APCI MS was successfully applied to the identification of carotenoids extracted from various plants. The examples show the analysis of carotenoids in tomato by using a reversed phase HPLC system (**Fig. 3**), and paprika (**Fig. 4**) by using a stationary phase HPLC system. In tomato extract, peaks 4 to 8 showed the same protonated molecule at m/z 567 in APCI-MS, indicating a molecular formula of $C_{40}H_{56}$, therefore, the accurate identification of these carotenoids including geometrical isomers could be achieved by combination of UV-Vis spectral data and retention times in HPLC with APCI-MS as shown in **Fig. 3.** In a similar manner, eleven carotenoids in paprika extract were identified by this method as shown in **Fig. 4.**

The following carotenoids were identified by this method: α-carotene, β-carotene, β-cryptoxanthin, zeaxanthin, lutein, antheraxanthin, mutatoxanthin, cucurbitaxanthin A, violaxanthin, luteoxanthin, auroxanthin and neoxanthin from pumpkin; α-carotene, β-carotene, zeaxanthin, lutein, lactucaxanthin, antheraxanthin,

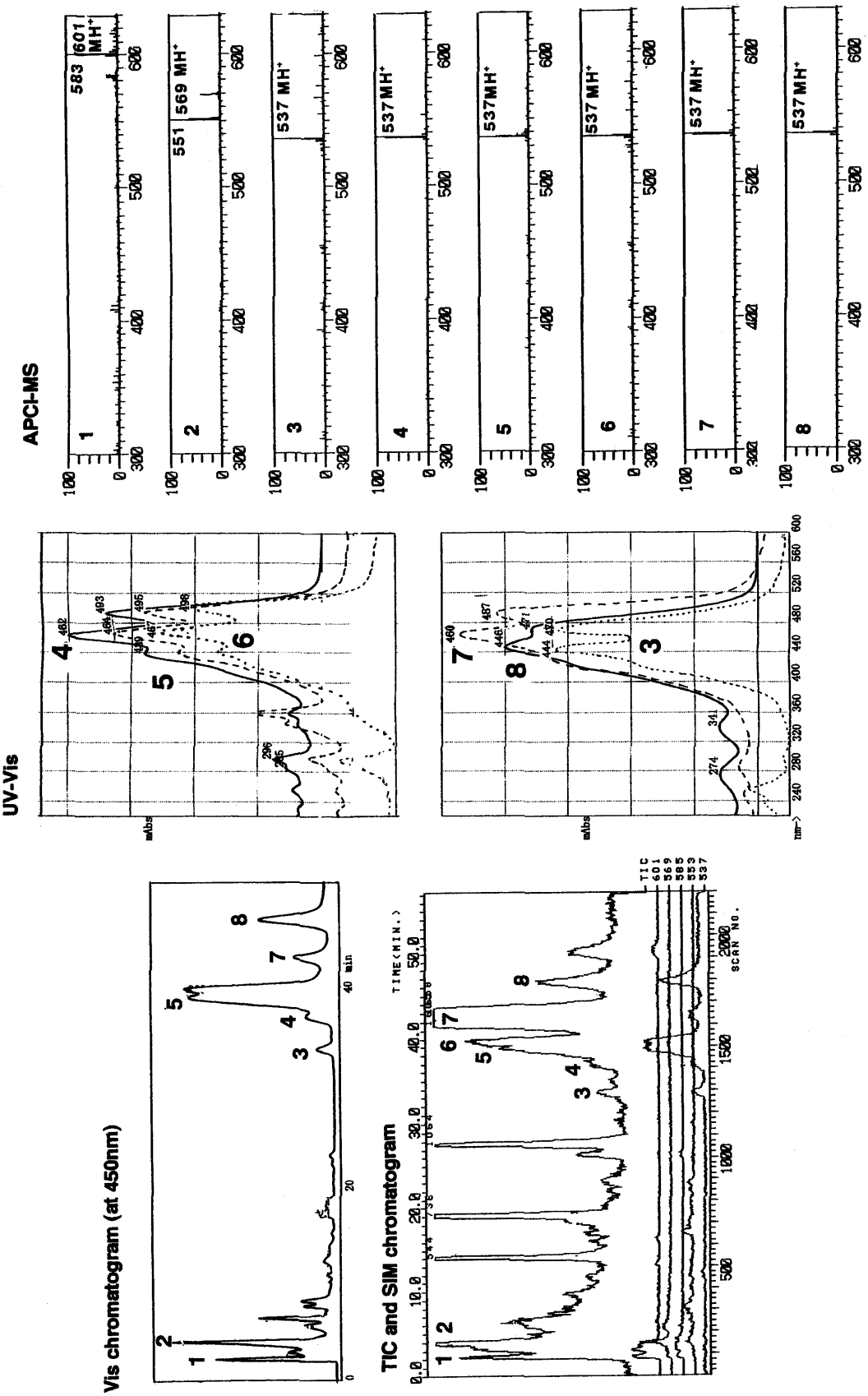

Fig. 3 Vis (450 nm), Total Ion and Selected Ion Chromatograms and UV-Vis and Positive-ion APCI-MS Spectra of Carotenoids in Tomato.
HPLC; column: YMC-ODS A-302 (particle size 10 μm) 250 mm L. $\times$ 4.6 mm I.D., mobile phase: MeOH-water (9:1), flow rate: 1.0 ml/min.
peak 1: violaxanthin, 2: lutein, 3: unidentified carotene, 4: 9-*cis*-lycopene, 5: 13-*cis*-lycopene, 6: lycopene, 7: γ-carotene, 8: β-carotene.

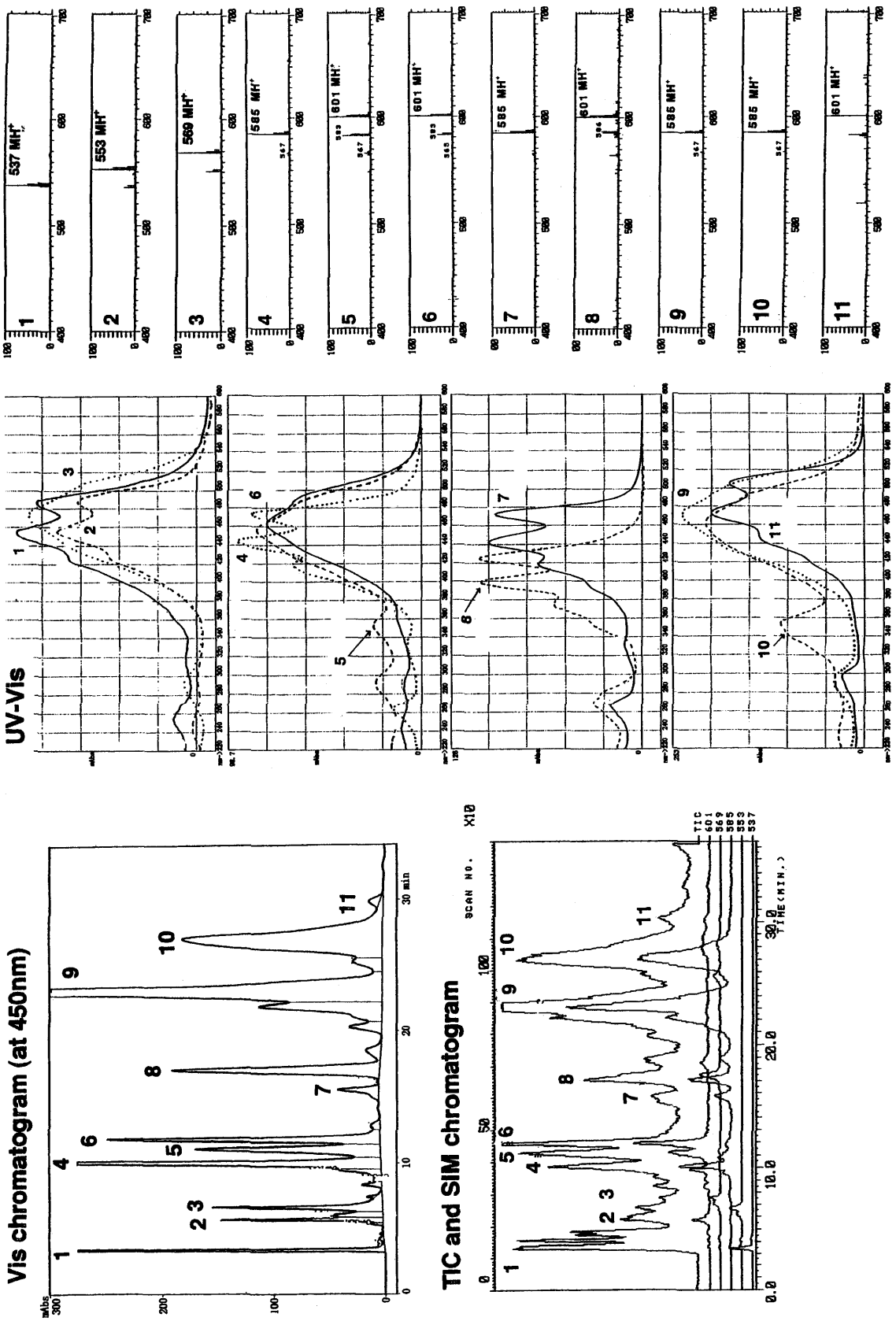

Fig. 4 Vis (450 nm), Total Ion and Selected Ion Chromatograms and UV-Vis and Positive-ion APCI-MS Spectra of Carotenoids in Paprika.
HPLC; column: YMC-Pack SIL-06 (particle size 5 μm) 250 mmL. × 4.6 mm I.D., mobile phase: THF : hexane (35:65), flow rate: 1.0 ml/min.
peak 1: β-carotene, 2: β-cryptoxanthin, 3: cryptocapsin, 4: cucurbitaxanthin A, 5: 9-*cis*-capsanthin 3,6-epoxide, 6: capsanthin 3,6-epoxide, 7: mutatoxanthin, 8: auroxanthin, 9: capsanthin, 10: 13-*cis*-capsanthin, 11: capsorubin.

lutein epoxide, auroxanthin and neoxanthin from lettuce (data not shown).

4 Conclusion

In conclusion, the LC/PAD/APCI-MS allows the carotenoids to be characterized by retention time in HPLC and APCI-MS spectral and UV-Vis spectral informations so that it offers a rapid and accurate method for the identification of natural carotenoids.

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