The Use of APCI-MS with HPLC and Other Separation Techniques for Identification of Carotenoids and Related Compounds

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Abstract: The heat labile carotenoids cannot be analyzed by gas chromatography (GC) and GC–mass spectrometry (GC–MS). The only useful method appears to be high-performance liquid chromatography (HPLC) with UV/visible (UV/Vis) or mass spectrometric detection (LC–MS). Reversed-phase HPLC (RP-HPLC) is a preferred method, which is frequently used with C18 stationary phase, usually with gradient elution. In contrast to other ionization techniques, xanthophylls and carotenes form both molecular ions and protonated molecules during positive ion APCI (atmospheric pressure chemical ionization). APCI is an ideal method of ionization for low- to medium-polar compounds, which include also carotenoids and related compounds. Since their molecular mass does not exceed 2000 amu even in the case of glycosides or esters with fatty acids, this method is exceedingly suitable for their analysis. The review summarizes the data on its use for this group of compounds.

Keywords: Triacylglycerols, Atmospheric pressure chemical ionization, Mass spectrometry, High-performance liquid chromatography, Gas chromatography, Supercritical fluid chromatography, LC-MS/APCI.

1. INTRODUCTION

Carotenoids, typically bright yellow, orange, red, or purple polyisoprenoid compounds with absorption maxima in the range of 400–500 nm [1] are found predominantly in photosynthesizing organisms such as green plants, algae and some bacteria. The number of currently known carotenoids exceeds 700 [2] and it keeps on rising – more than 1800 papers on these compounds have been published in 2007 alone.

Carotenoids interact with chlorophyll during photosynthesis to absorb light and transfer energy [3]. Another result of their conjugated polyene chain is the ability of carotenoids to quench singlet oxygen and free radicals and thereby protect plants from photooxidative damage [4, 5]. In addition to their protective functions in plants, diverse biological functions in humans have been attributed to carotenoids [6, 7]. Approximately 50 carotenoids are precursors of vitamin A, which is essential for vision, cellular differentiation, and embryological development. It has been suggested that carotenoids can function in humans as antioxidants and immunoenhancers, as well as prevent age-related macular degeneration, cancer and cardiovascular disease [8]. Because of these clinical effects, the importance of the detection and identification of carotenoids in natural sources, biological tissues, and clinical samples has been increasing during the last 10 years.

Carotenoids have been classified into two groups, hydrocarbon carotenes and oxygenated xanthophylls [9], and the structures of carotenes and xanthophylls that are common in the human diet are shown in Fig. (**1**). Because of their extended system of conjugated double bonds, carotenoids are usually unstable in the presence of light, heat, or oxygen [9]. Therefore, the isolation, identification, and quantitation of these pigments can be challenging [10]. Complex biological

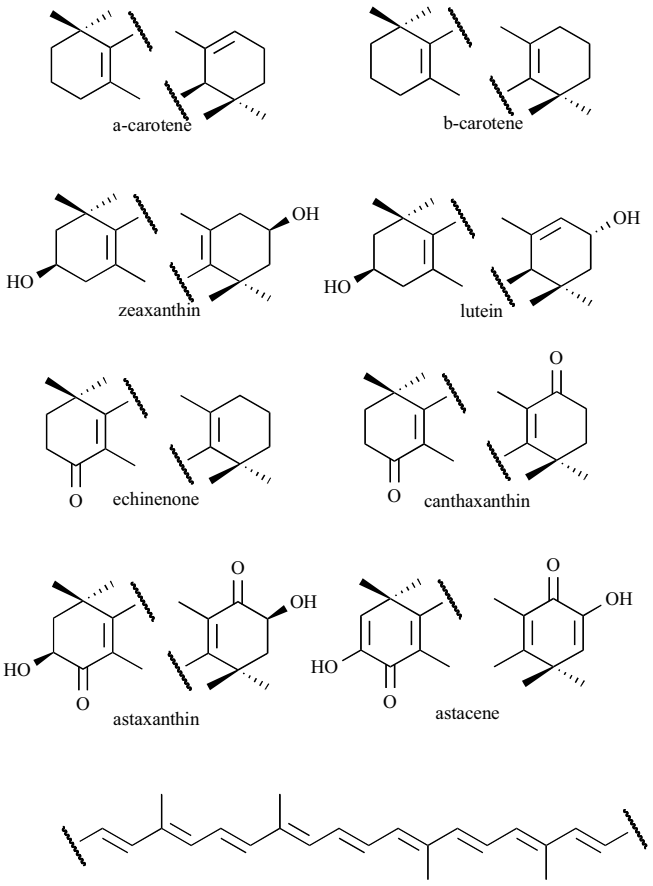

Fig. (1). Structures of representative carotenoids.

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samples such as human serum, tissues, and plant material often contain compounds than can interfere with carotenoids or mixtures of carotenoids with similar structures. The identification and quantitation of specific carotenoids therefore requires highly sensitive and selective analytical methods [10, 11].

The use of gas chromatography (GC) and GC–mass spectrometry (GC–MS) for their analysis is not suitable because carotenoids are heat labile. The only useful method appears to be high-performance liquid chromatography (HPLC) with UV/visible (UV/Vis) detection or, much more conveniently, with mass spectrometry detection (LC–MS). Reversed-phase HPLC (RP-HPLC) is a preferred method, which is frequently used with a C18 stationary phase, usually with gradient elution [11]. C30 columns are often used to enhance the separation of *cis* and *trans* isomers and provide efficient separation of xanthophylls and carotenes [12, 13]. Compared to C18 stationary phases, C30 phases afford more interactions between carotenoids and the solid phase and thereby greater chromatographic separation of carotenoids having only small structural differences. Although isocratic mobile phases may be used, gradient elution can shorten the separation time while providing superior resolution and sharper chromatographic peaks. Typically, the polar xanthophylls elute first during reversed-phase HPLC followed by the less polar carotenoids. Interestingly, lycopene elutes after β-carotene and α-carotene on a C30 column but elutes before them when using monomeric C18 columns [12, 13].

Since the levels of carotenoids in biological tissues are often low, highly sensitive analytical methods must be used for their analysis [14]. Xanthophylls and carotenes form both molecular ions and protonated molecules during positive-ion APCI (atmospheric pressure chemical ionization). APCI is an ideal method of ionization for low- to medium-polar compounds, which include also carotenoids and related compounds. Since their molecular mass does not exceed 2000 amu even in the case of glycosides or esters with fatty acids, this method is exceedingly suitable for their analysis. This review summarizes the data on its use for this group of compounds.

One of the numerous LC/MS techniques, electrospray (ESI), is both an ionization technique and an efficient solvent removal interface. During ESI, a fine mist of charged droplets is formed from the HPLC eluate at atmospheric pressure by spraying the solution through a capillary electrode at high potential (usually 2 kV). As the charged droplets are electrostatically attracted towards the opening of the mass spectrometer, they encounter a cross-flow of heated nitrogen curtain gas that increases solvent evaporation and prevents most of the solvent molecules from entering the mass spectrometer. When the droplets shrink in size until the electrostatic repulsion between ions in each droplet exceeds the combined energy of solvation and surface tension, ions are ejected from the droplets into the gas phase or else the droplet disintegrates and releases analyte ions. Because of the efficiency of this combined ionization and desolvation process, ESI is compatible with HPLC flow rates from a few μl/min up to 1 mL/min. Although ions produced by ESI are usually preformed in solution by acid/base reactions (i.e., $[M+H]^+$ or $[M-H]^-$), carotenoid ions are probably formed by a field desorption mechanism at the surface of the droplet. As a result of this unusual ionization process, ESI of carotenoids produces abundant molecular cations with little fragmentation, and no molecular anions. Advantages of ESI over previous LC/MS techniques for carotenoid analysis include lower limits of detection, abundant molecular ions of both xanthophylls and carotenes, substantially improved ease of operation, reduced maintenance, higher sample throughput, and compatibility with autosamplers for unattended operation. One of the potential limitations of electrospray is the relatively narrow range of linearity of response it shows for carotenoid quantitation.

Although ionization during both ESI and atmospheric pressure chemical ionization (APCI) takes place at atmospheric pressure, APCI uses a heated nebulizer instead of a strong electromagnetic field to facilitate solvent evaporation and obtain a fine spray of the HPLC mobile phase. Ionization takes place by spraying the analyte solution into a corona discharge, which produces numerous reactive species that can ionize the analyte including reagent gas ions formed from the mobile phase. Ions are then drawn into the aperture of the mass spectrometer. As in ESI, a cross-flow of a curtain or bath gas (usually heated nitrogen) prevents most uncharged solvent molecules from entering the low pressure mass spectrometer. Molecular ions and protonated molecules were observed in positive-ion APCI, and molecular ions and deprotonated molecules were detected during negative-ion APCI. The relative abundance of molecular ions and protonated or deprotonated molecules varies with the mobile phase composition. For instance, polar solvents such as alcohols lead to an increased abundance of protonated carotenoids (even protonated β-carotene), while less polar solvents such as methyl-*tert*-butyl ether facilitate the formation of more abundant molecular ions. The limit of detection, ease of use, compatibility with HPLC solvents and flow rates, and suitability of APCI for automated or unattended LC-MS carotenoid analysis are comparable to those of ESI. The main advantage of APCI compared to ESI is its higher linearity of detector response over a carotenoid concentration range of at least three orders of magnitude, which suggests that LC-MS/APCI might become the preferred mass spectrometric technique for carotenoid quantitation. Disadvantages of APCI include the multiplicity of molecular ion species, which might lead to ambiguous molecular weight determinations, and the abundant fragmentation, which tends to reduce the abundance of the molecular ions.

2. RESULTS

2.1. Separation and Identification of Carotenoids

Analysis and mass spectra of carotenoids have been the subject of several theoretical studies. However, a thorough theoretical study including the analysis of both free and esterified carotenoids is still missing, most papers providing only partial data. A survey of the carotenoid sources, analytical and identification methods and conditions including references is given in Table **1**.

As stressed in one of the fundamental reviews on liquid chromatography - mass spectrometry (LC/MS) of carotenoids [10], the sensitivity, specificity and selectivity of LC/MS and LC/MS/MS make these techniques essential

Table 1. Sources of Carotenoids, HPLC Techniques and Analytical Conditions Used for their Separation and Identification

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[12]	different phases (C18 a C30)	5-µm silica, RT	MTBE/water	1.0					only UV detection at 450 nm	Extract of a food matrix SRM[5], β-carotene dietary supplement
[15]	YMC C30	(250×4.6);3;RT	a) tocopherols: 30-min linear gradient acetone/water from (90:10) to (100:0 v/v) b) carotenoids: acetone/water (90:10) for 5 min, followed by a 10-min linear gradient to (100:0)	1.0 split to 0.05	ESI[8]	4.5		Full MS MS/MS	400-800	Carotenoids and tocopherols from tomato, carrot, and vegetable juices, a vitamin drink, and a commercial infant food product
[16]	ProntoSil C30	(250×2.0);3;25	MeOH/MTBE (75:25 v/v)	1.0	APCI	Not shown	400	Full MS	350-400	Crude rice bran oil
[18]	C30	(125×4.6);5;20	60 min gradient from (85:15) to (10:90 v/v) MeOH/MTBE with 1.0 mM $AcONH_4$[6]	1.0	ESI	5.1	350	Full MS	200-610	Standard mix-ture of both xanthophylls and carotenes
[19]	X-Terra C18	(50×2.1); RT	gradient elution (min/%A): 0/95; 7/95;8/100; 15/100 with (A) ACN/MeOH (85:15)% plus 0.1% HCOOH, (B) water plus 0.1% HCOOH	Not shown	ESI	4.9	Not shown	MRM[18]	252→152, 278→94, 318→258, 344→107, 370→164	Human eye
[20]	Vydac C18	(250×4.6); RT	gradient (min/%A/%B/%C): 0/98/2/0; 10/75/18/7; 15/68/25/7; 25/68/25/7; 30/98/2/0; 35/98/2/0 with (A) ACN+0.05% TEA, (B) MeOH+0.05% TEA+50 mM $AcONH_4$, (C) EtOAc+0.05% TEA	1.0	APCI	3	200	Full MS SIM	50-1050 not specified	Total-diet survey (TDS)
[22]	a) Vydac C18 b) Fusica-C microbore C18	a) (300×0.32);5;RT b) (250×2.1);5;RT	MeOH/ACN/EtOAc (30:50:20; v/v/v)	a) 0.1 b) 0.004	FAB[4]	6 (Xe)	RT	Full MS MS/MS	1-650	Carotenoids from tomato extract
[23]	Vydac TP218 C18	(150×2.1);5;35	ACN/MeOH/EtOAc (50:30:20)	0.1	FAB	6.0	Not shown	Full MS, MS/MS	1-700	Blueberries, carrots, tomatoes
[24]	ProntoSIL C30	(250×4.6);3;22	retina: acetone/water (86:14 v/v) spinach: acetone/water (86:14 v/v) 21 min, followed by a 4-min linear gradient to (97:3 v/v) and hold the line till 40 min	1.0	APCI	10	300	Full MS	200-800	Lutein and zeaxanthin in spinach and in retina
[25]	ProntoSIL C30	(250×4.6);3;RT	acetone/water isocratically (84:16) for 21 min, then linearly to (97:3) in 4-min	1.0	APCI	10	300	Full MS	200-800	Variety of spin-ach samples

(Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[26]	a) Xterra MS C18[10] b) YMC C30[11]	a) (100×2.1);3.5; RT[10] b) (250×4.6);3;RT[11]	a) ACN/MTBE (95:5 v/v)[10] b) 40-min linear gradient MeOH/MTBE from (50:50) to (40:60 v/v)[11]	a) 0.4[10] b) 0.65[11]	APCI	3.85	350	MS/MS	Not shown	Human plasma
[27]	YMC C30	(250×4.6);3;RT	MeOH/MTBE from (55:45) to (45:55) in 15 min	1.0	APCI	2.5	300	SIM	537	Human Serum and Prostate Tissue
[30]	a) Microsorb C18 b) silica-based CN	a) (250×4.6);5;RT b) (250×4.6);5;RT	a) ACN/MeOH/DCM/hexane (85:10:2.5:2.5 v/v/v/v) 10 min, followed by 40-min linear gradient to (45:10:22.5:22.5) b) hexane/DCM/MeOH/ DIPEA (74.65:25.00:0.25:0.10 v/v/v/v)	0.7	ECNI[7]	Not shown	45	Full MS	100-700	Carotenoids and their metabolites in human milk and serum
[31]	ProntoSIL C30	(250×4.6);3;RT	acetone/water isocratically (86:14) for 21 min, then linearly to (97:3) in 4-min	1.0	APCI	4.0	300	Full MS	400-700	Intrinsically labeled vegeta-bles (spinach, carrots)
[32]	Microsorb CN	(250×4.6);5;RT	hexane/DCM/MeOH/ DIPEA (80:19.2:0.7:0.1)	1.0	APCI	Not shown	500	SIM	569±0.5, 599±0.5	*Flavobacterium multivorum*
[33]	YMC C30	(150×4.6);3;RT	gradient elution (min/%A): 0/100; 1/100; 8/70; 13/70; 22/45; 24/45; 34/5; 38/5; 40/100; 50/100 with (A) MeOH/MTBE/water (83:15:2), (B) (8:90:2)	1.0	APCI	Not shown	300	Full MS	540-570	Collard, spinach
[34]	YMC C30	(250×4.6);3;RT	gradient elution (min/%B) 0/15; 12/30; 18/30; 18/100; 23.0/100 with (A) 1 mM $AcONH_4$ in MeOH, (B) MTBE	1.0	APCI	3.8	400	SIM	551, 591, 537, 577, 451, 482, 269, 276, 289, 545.	Kale (*Brassica oleracea*)
[35]	YMC C30	(250×4.6);3;RT	gradient elution (min/%B): 0/15; 12/30; 18/30; 18/100; 23/100; 23/15; 33/15 with (A) 1 mM $AcONH_4$ in MeOH, (B) MTBE	1.0	APCI	3.8	400	SIM	551, 591, 537, 545, 577, 269, 276, 289, 417	Kale (*Brassica oleracea* variety *acephala*)
[36]	YMC C30	(150×2.0);3;RT	gradient elution (min/%A): 0/15; 3/30; 37.5/30 with (A) MTBE, (B) MeOH+1mM $AcONH_4$	0.2	APCI	3.0	400	SIM	536, 546	Serum, feces
[37]	YMC C30	(100×4.6);5;35	gradient elution (min/%A) 0/100; 44/50; 46/0; 49/0; 54/100 with (A) MeOH/MTBE/water (81:15:4), (B) MeOH/MTBE (90:10)	1.0	APCI	6.0	450	Full MS	Not shown	Commercial lutein supple-ment
[38]	YMC C30	(150×3.0);3;25	gradient elution (min/%B): 0/0; 22/30; 32/51.3; 55/62.7; 60/100; 65/100; 70/0 with (A) MeOH/MTBE/water (81:15:4), (B) MeOH/MTBE/water (4:92:4)	0.42	APCI	2.7	400	Full MS	100-1100	Red pepper pods (*Capsicum annuum*)

(Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[39]	YMC C30	(250×4.6);5;35	gradient elution (min/%A): 0/99; 10/90; 20/70; 30/0; 35/99;40/99 with (A) MeOH/water (96:4 v/v), (B) MTBE/MeOH/water (90:6:4 v/v/v)	1.0	APCI	3.7	450	Full MS	80-1200	Marigold Flowers (*Tagetes erecta* L.) and Several Fruits
[40]	C30	(125×2.0); ;RT	50 min linear gradient from MTBE/ACN containing 1 mM of $AcONH_4$ (15:85) to (90:10)	0.7	APCI	Not shown	350	Full MS	500-1200	Marigold flower
[41]	YMC C30	(250×4.6);5;35	gradient elution (min/%A): 0/99; 10/90; 20/70; 30/0; 35/99;40/99 with mobile phases (A) MeOH/water (96:4 v/v), (B) MTBE/MeOH/water (90:6:4 v/v/v)	1.0	APCI	3.7	450	Full MS	80-1200	Multivitamin dietary supplements
[42]	YMC C30	(250×4.6);5;35	gradient elution (min/%A): 0/99; 10/90; 20/70; 30/0; 35/99;40/99 with (A) MeOH/water (96:4 v/v), (B) MTBE/MeOH/water (90:6:4)	1.0	APCI	3.7	450	Full MS	300-1200	Oranges, orange juice, minneola, tangerine, kumquat,tangelo, nectarine, avocado, canned corn (sweet yellow whole kernels, drained), and fresh carrot
[43]	YMC C30	(250×4.6);5;RT	30 min linear gradient of MeOH/MTBE from (95:5 v/v) to (5:95 v/v)	1.0	APCI	4.5	250	Full MS	Not shown	Mandarin essential oil
[44]	YMC C30	(250×4.6);5;35	gradient elution (min/% A): 0/99; 39/44; 45/0; 50/99; 55/99 with (A) MTBE/MeOH/water (90:6:4), (B) (15:81:4)	1.0	APCI	3.7	450	Full MS	80-1200	Fresh mango (*Mangifera indica*)
[45]	YMC C30	(250×4.6);5;35	gradient elution (min/%A): 0/99; 10/90; 20/70; 30/0; 35/99;40/99 with (A) MeOH/water (96:4 v/v), (B) MTBE/MeOH/water (90:6:4 v/v/v)	1.0	APCI	3.7	450	Full MS	40-1200	Wolfberries (*Lycium barbarum*), Chinese lanterns (*Physalis alkekengi*), orange pepper (*Capsicum annuum*), and sea buckthorn (*Hippophae rhamnoides*)
[46]	YMC C30	(250×4.6);5;35	a)[12] gradient (min/%A) 0/99; 8/99; 45/0; 50/99; 55/99 with (A) MeOH/water/TEA (90:10:0.1 v/v/v), (B) MeOH/MTBE/water/TEA (6:90:4:0.1); b)[13] gradient (min/%A) 0/99; 39/44; 45/0; 50/99; 55/99 with (A) MeOH/MTBE/water/TEA (81:15:4:0.1), (B) dtto (6:90:4:0.1)	1.0	APCI	3.7	400	Full MS	200-1200	Potatoes (*Solanum tuberosum*)

(Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[47]	YMC C30	(250×4.6);5;35	MeOH/MTBE/water A (81:15:4), (6:90:4), gradient (min/% A): 0/99; 39/44; 45/0; 50/99; 55/99	1.0	APCI	3.7	400	Full MS	80-1200	Shrimp (*Pandalus borealis*) and in a microalga (*Haematococcus pluvialis*)
[48]	ProntoSil C30	(250×4.6);3;RT	gradient elution (min/%A): 0/100; 20/100; 190/40 with (A) MeOH/MTBE/water (83:15:2), (B) (8:90:2)	1.0	APCI	3.5	250	Full MS	50-1400	Krill (*Euphausia superba Dana*)
[49]	Finesse Genesis C18	(150×2.1);4;RT	gradient (min/%A/%B/%C): 0/90/5/5; 5/90/5/5; 8/67/30/3; 9/57/40/3; 20/57/40/3 with (A) MeOH, (B) MTBE, (C) water, all contained HCOOH (0.1%, v/v), wash step 57/40/3 for 5 min	0.2	APCI	4.0	300	Full MS	100-650	Fish eggs
[51]	YMC C18	(250×4.6);5;20	60 min gradient from (83:17) to (98:2 v/v) acetone/water	0.8	APCI	3.5	400	Full MS	100-2200	Astaxanthin esters in *Haematococcus pluvialis*
[52]	Hichrom HIRPB-250AM	(250×2.1);5;RT	ACN/MeOH/DCM (50:25:25) linear from 8.3 min to (25:50:25) in 58.3 min held until 60 min	0.4	APCI	Not shown	400	Full MS	100-1700	Snow alga *Chlamydomonas nivalis*
[53]	YMC C30	(150×3.0);3;20	gradient from 100% A to 15% B within 55 min with (A) MeOH/MTBE/water (92:4:4), (B) MTBE/MeOH/water (90:6:4)	0.42	APCI	Not shown	400	Full MS	50-1000	Spinach (*Spinacia oleracea* L.) and sweet corn (*Zea mays* L.), dietary supplements
[54]	Spherisorb ODS2	(150×4.6);3;RT	gradient elution (min/%A) 0/100; 10/80; 20/50; 25/100 with (A) ACN/MeOH (9:1), (B) EtOAc	1.0	APCI	Not shown	Not shown	Full MS	50-2000	Spinach, kale and potato
[55]	Microsorb C18	(250×4.6);3;20	gradient (min/%A/%B/%C): 0/85/10/5; 10/85/10/5; 40/45/10/45 with (A) ACN, (B) MeOH, (C) hexane/DCM (50:50, with 0.1% diisobutylethylamine)	0.7	ECNI[16]	Not shown	45[17]	Full MS	100-700	Human serum
[56]	Mightsil RP-18	(75×4.6);5;25	isocratically with MeOH/ACN (70:30)	1.0	APCI	3.0	200	SIM	537, 411	Human serum
[57]	YMC C30	(100×2.0);3;20	MeOH/THF/water+0.1% TEA from (88:2:10) to (92.5:7.5:0) in 30 s, hold for 25 min	0.7	APCI	3.8	200	SIM	537, 547, 269, 274, 279	Feces

(Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[59]	Microsorb-CN	(250×4.6);5;RT[3]	hexane/DCM/MeOH/ DIPEA (80:19.2:0.7:0.1 v/v)	1.0	APCI	Not shown	500	SIM	551±0.7,569±0.7, 569±0.8, 567±0.7	Dietary carotenoids in the retina of the human eye
[60]	ProntoSil C30	(250×4.6);3;20	90 min linear gradient from MeOH/MTBE/water (1:15:4) to (6:90:4)	1.0	APCI	8.0	450	Full MS Full MS SIM	200-700 80-1000 537	Vegetable mixture juice of tomatoes, carrots, spinach and celery
[61]	C30	(125×2.0); RT	60 min linear gradient from ethanol/MTBE containing 1.0 mM $AcONH_4$ (85:15) to (10:90)	0.3	APCI	Not shown	Not shown	Full MS	200-610	Plant extract, human blood
[63]	YMC C30	(150×4.6);3;15	gradient: 4% A, 95.2% B, 0.8% C at 0 min, decreasing to 4% A, 55.3% B, 40.7% C with (A) water, (B) MeOH, (C) MTBE	0.75	APCI	3.0	350	Full MS	100-1200	Mango (*Mangifera indica* L.)
[64]	YMC C30	(250×4.6);5;25	gradient (min/%A/%B/%C): 0/5/90/5; 12/0/95/5; 20/0/86/14; 30/0/75/25; 50/0/50/50; 60/0/37.5/62.5; 73/0/5/95 with (A) water, (B) MeOH, (C) MTBE	1.0	APCI	Not shown	400	SIM	457	Citrus fruit
[67]	YMC C30	(250×4.6);3;22	gradient elution (min/%A) 0/95; 30/70; 50/50; 85/5 with (A) MeOH with 0.1% TEA, (B) MTBE	0.9	APCI	Not shown	350	Full MS MS/MS	100-700	Amazonian Fruits buriti (*Mauritia vinifera*), mamey (*Mammea americana*), marimari (*Geoffrola striata*), peach palm (*Bactrys gasipaes*), physalis (*Physalis angulata*), and tucuma (*Astrocaryum aculeatum*)
[68]	a) Symmetry C18[15] b) Atlantis dC18	a) (75×4.6);3.5;30 b) (150×2.0);5;30	MeOH/THF/ACN (60:30:10)	0.4	APCI	3.5	Not shown	SIM	537	Tomato, kiwi, mango
[69]	Nova-Pak Silica	(300×0.39); RT	dioxane/hexane (1:99)	1.0	APCI	3.0	300	Full MS	150-1500	Berry *Gou Qi Zi*
[70]	YMC C30	(100×2.1);3;25	gradient elution (min/%B): 0/3; 12/3; 13/38; 15/38;16/68; 21/68; 25/3 with (A) 3% water in MeOH with 0.05M $AcONH_4$, (B) MTBE, both (A) and (B) contained 0.1% (w/v) BHT and 0.05% TEA	0.5	APCI	2.8	Not shown	Full MS	Not shown	Leaf tissue of plant *Arabidopsis thaliana*

(Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[71]	Microsorb CN	(250×4.6);5;RT	hexane/DCM/MeOH/ DIPEA (80:19.2:0.7:0.1)	1.0	APCI	3.7	500	SIM	569±0.5, 599±0.5	Chlorophycean microalga *Chlorella prototheco*ides
[72]	Zorbax XDB-C18	(50×2.1);5;RT	gradient elution (min/%A): 0/80; 25/100; 55/100 with (A) MeOH, (B) water, both containing 1% of HCOOH	0.2	APCI	Not shown	335	Full MS	100-1500	*Spirulina platensis* microalga
[73]	Spherisorb ODS2	(150×4.6);3;RT	ACN, MeOH, 0.01M $AcONH_4$, EtOAc	0.7	APCI	Not shown	450	MS/MS	Not shown	Filamentous anoxygenic phototrophic bacteria *Chloronema*
[74]	LiChroCART Superspher 100 RP-18	(125×2.0);4;40	gradient from 50 to 100% acetone in 15 min	0.5	APCI	Not shown	450	Full MS	100-800	Fungi red yeasts B*asidiomycota*
[75]	LiChrospher 100 RP-18	(125×4.0);5;RT	gradient elution (min/%A): 0/100; 20/0 with (A) ACN/H_2O/HCOOH (86:10:4), (B) EtOAc/HCOOH (96:4)	1.0	APCI	Not shown	Not shown	Full MS	200-610	Fungus, *Puccinia distincta*
[76]	Discovery C18	(100×4.6);5;25	gradient elution (min/%A): 0/95; 10/95; 30/100 with (A) ACN (B) ACN/water (50:50)	0.8	APCI	2.5	500	Full MS	200-800	Red coral (*Corallium rubrum*)
[77]	VP-ODS	(150×2.0); ;25	15 min linear gradient MeOH/water from (50:50 v/v) to (0:100 v/v) and hold the line for 35 min	0.2	APCI	Not shown	Not shown	Not shown	Not shown	*Thraustochytrium* CHN-1 strain
[78]	Adsorbosphere C18	(250×4.6);5;RT	gradient elution (min/%A/%B%C): 0/100/0/0; 4/0/100/0; 38/0/25/75; 39/0/25/75; 43/0/100/0; 47/100/0/0 with (A) MeOH/0.5M $AcONH_4$ pH 7.2 (80:20), (B) (90:10) ACN/water, (C) 100% EtOAc	1.0	APCI	2	450	Full MS MS/MS	50-1400	Continental shelf (*Louisiana shelf)* sediments
[79]	ODS2 C18	(150×4.6);3;RT	ternary gradient with (A) ACN, (B) MeOH, (C) 0.5M $AcONH_4$ and (D) EtOAc	0.7	APCI	Not shown	450	Full MS, MS/MS	200-1400	*Emiliania huxleyi* (Prymnesiophycae)
[80]	YMC C30	(250×4.6);5;25	acetone/water (92:8 v/v) with 0.0125M $AcONH_4$	1.0	APCI	4	375	SIM	537, 543	Carrot Extract in Oil
[81]	YMC C30	(250×4.6);5;35	gradient elution (min/%A): 0/93.5; 34/0; 38/93.5; 43/93.5 with mobile phases: (A) MeOH/water/TEA (90:10:0.1 v/v/v), (B) MTBE/MeOH/water/TEA (90:6:4:0.1 v/v/v/v)	1.0	APCI	3.7	400	Full MS	80-1200	Carotenoid food additives (CFA).

(Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[82]	YMC C30	(250×4.6);5;35	gradient (%A/min): 99/0, 90/10, 70/20, 0/30, 99/35,99/40 with (A) MeOH, (B) MeOH/MTBE/water (6:90:4)	1.0	APCI	3.2	400	Full MS	300-700	Commercial egg yolks
[83]	Lichrospher 100RP-18	(250×4.6);3;30	water/acetone/AcOH (37:63:0.1)	1.0	APCI	5.0	300	Full MS	100-800	Paprika oleoresins
[84]	YMC C30	(250×4.6);5;25	gradient elution (min/%A/%B%C) 0/40/60/0; 5/20/80/0; 10/4/81/15; 60/4/11/85; 71/0/100/0 to 72 min with (A) water, (B) MeOH, (C) MTBE	1.0	Not shown	Not shown	Not shown	Not shown	Not shown	Citrus juice
[85]	YMC C30	(250×4.6);3;RT	12 min gradient MTBE/MeOH with 1 mM $AcONH_4$; from (15:85) to (30:70) followed by (30:70 v/v) for 13 min [9]	1.0	APCI	2.8	325	SIM	537, 547, 269, 274, 279	Human serum
[86]	Spherisorb ODS2	(150×4.6);3;RT	ACN, MeOH, 0.01M $AcONH_4$, EtOAc	0.7	APCI	Not shown	450	MS/MS	Not shown	Extracts from a sediment (Priest Pot, Cumbria, UK), a microbial mat (les Salines de la Trinital, South Catalonia, Spain) and a culture (*C. phaeobacteroides*)
[87]	YMC C30	(250×4.6);5;35	gradient elution (min/%A): 0/99; 10/90; 20/70; 30/0; 35/99;40/99 with mobile phases (A) MeOH/water (96:4 v/v), (B) MTBE/MeOH/water (90:6:4 v/v/v)	1.0	APCI	3.7	450	Full MS	80-1200	Papaya and tangerine
[88]	YMC C30	(250×4.6);5;35	gradient (min/%B) 0/0; 10/0, 40/50; 50/100; 55/0; 60/0 with (A) MeOH/MTBE/water (81:15:4), (B) dtto (6:90:4).	1.0	APCI	3.7	400	Full MS	200-1200	Paprika (*Capsicum annuum*)
[89]	RP-Amide C16	(250×4.6);5;35	MeOH	1.0	APCI	2.4	400	MID[14]	248, 249	Hot Chilli Spices,Oven-Baked Foods
[90]	Luna C18	(150×4.6);5;30	acidified water with 0.25% HCOOH/ACN from (80:20) for 5 min to (20:80) in 15 min	0.8	ESI	2.5	350	Full MS	100-1500	Fruits *Crocus sativus Stigmas, Gardenia jasminoides*
[91]	Zorbax RX-SIL	(150×2.1);5;RT	gradient elution (min/%A) 0/99; 5/90; 20/50; equilib. with 99% A for 10 min with (A) hexane, (B) 1% isopropanol in EtOAc	0.5	ESI	4.5	550	MRM[18]	431.4→165.2, 537.5→119.2 553.5→119.2, 551.5→119.2 569.5→119.2	Grain, vegetable, vegetable oils and fruits

(

Table 1) contd.....

Pub. No	Column Type	Column Characteristic[1]	Mobile Phase	Flow (mL/min)	Ion Source	Corona/ Electrospray Voltage (kV)	Vap. Temp (°C)	Scan Type	Mass Range[2] (*m/z*)	Analyzed Samples
[92]	YMC C30	(100×2.0);RT	gradient elution (min/%A):0/70; 30/10; or 0/80; 25/10 with (A) MeOH/water/AcOH (50:50:0.5), (B) MeOH/MTBE/AcOH (50:50:0.5)	0.25	APCI	3.8	200	Full MS SIM	100-500 269	Human serum

[1]lenght (mm); particle size (μm); temperature (ºC).
[2] in case of SIM single mass.
[3] room temperature.
[4] continuous-flow FAB LC/MS technique.
[5] Standard Reference Material.
[6] In order to enhance the formation of molecular ions, solution-phase carotenoid oxidation was carried out by means of postcolumn addition of a halogenated solvent to the HPLC effluent.
[7] (ECNI) Electron capture negative ionization, particle beam interface, operated at a desolvation temperature of 45°C.
[8] The ionization of carotenoids and tocopherols is enhanced by the addition of silver ions. The silver solution was added postcolumn through a liquid junction at a flow rate of 50 μL/min, which results in an overall concentration of silver ions of 5 μmol/mL.
[9] Analysis of *β*-carotene; HPLC conditions are modified for analysis of retinol, see literature [15].
[10] determination of total lycopene, *all-trans*-lycopene and lycopene *cis* isomers as a single chromatographic peak and separated from the internal standard [$_{13}C^6$]-*β*-carotene.
[11]Separation of *all trans*-lycopene from its various *cis* isomers.
[12]Carotenoids analysis.
[13] Carotenoid esters analysis.
[14] MID Multiple ion detection.
[15] two coupled columns.
[16] Electron capture negative ionization.
[17]desolvation temperature.
[18]MRM Multiple Reaction Monitoring
Acetic acid = AcOH, acetonitrile = ACN, ammonium acetate = $AcONH_4$, butylated hydroxytoluene = BHT, dichloromethane = DCM, dimethylformamide = DMF, *N*,*N*-diisopropylethylamine = DIPEA, ethyl acetate = EtOAc, formic acid = HCOOH, methanol = MeOH, methyl-*terc*-butyl ether = MTBE, tetrahydrofuran = THF, triethyl amine = TEA.

tools for the characterization and identification of carotenoids. LC/MS analysis is particularly important for studies of carotenoids obtained from natural sources, since these compounds are usually present in trace quantities and are often contaminated with biological matrices. Five LC/MS techniques have been used for carotenoid analysis including 1) moving belt; 2) particle beam; 3) continuous-flow fast atom bombardment; 4) electrospray (ESI); and 5) atmospheric pressure chemical ionization (APCI), the last two having retained their importance even today. These techniques provide comparable sensitivity (at the low pmol level) and produce abundant molecular ions. Whereas ESI forms primarily molecular ions showing little fragmentation, APCI produces molecular ions and/or protonated or deprotonated molecules, and possibly also other ions such as $[M+H-H_2O]^+$ or $[M+H-FA]^+$depending on the structure, especially for xanthophylls.

A variety of bonded phase parameters (endcapping, phase chemistry, ligand length, and substrate parameters) were studied for their effect on column retention and selectivity toward carotenoids [12]. Decisions were made on how each of these variables should be optimized based on the separation of carotenoids. A column was designed with the following properties: high absolute retention, enhanced shape recognition of structured solutes, and moderate silanol activity. These qualities were achieved by C-30 surface modification of a moderate pore size, moderate surface area silica, without subsequent endcapping. The effectiveness of this "carotenoid phase" was demonstrated for the separation of a mixture of structurally similar carotenoid standards, *i.e. trans*-α- and *trans*-β-carotene, *trans*-lycopene, echinenone, zeaxanthin, astaxanthin, δ-carotene, capsanthin, canthaxanthin, 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene, β-cryptoxanthin, and lutein.

In LC/MS, nonpolar substances in the majority of cases cannot be ionized by standard ESI because they obviously lack a site for protonation or deprotonation [15]. Addition of silver ions was therefore used; adducts thus formed render the carotenoids amenable to MS. All carotenoids were separated by C-30 RP-HPLC and could be identified by online MS/ESI. A mixture of different carotenoids was analyzed by scanning the mass range from *m/z* 500 to 800. The detection limit for canthaxanthin was near 500 fmol while that of β-carotene was below 300 fmol. An increase in sensitivity in the multiple reaction mode can be attained by monitoring ions formed by the loss of elemental silver from the adduct. Extracts of tomato, carrot, and vegetable juices, a vitamin drink, and a commercial infant food product were analyzed by LC/MS. After postcolumn argentation, the carotenoids present in the mass-selective extracts of the TIC (Total Ion Current) could be identified by their masses and their retention times.

Stoggl *et al.* [16] reported a comparison of the efficiency of C18 and C30 silica stationary phases in separating and detecting tocopherols, carotenoids, and γ-oryzanol in one single run; among carotenoids, the separation concerned only β-carotene and that only on C30 column.

2.2. Ionization Techniques Used for Identification of Carotenoids

Although APCI and ESI are very similar techniques, not very many papers have been devoted solely to ESI. An example of using this technie is, e.g., the study by Guaratini *et al.* [17] who investigated the balance between radical molecular ions and protonated molecules of xanthophylls (oxygen-containing carotenoids) with a conjugated Π-system (polyene) and oxygen as a heteroatom in ESI and HRESI mass spectrometry for different polyenes (with and without heteroatoms). Solution and gas-phase chemistry can occur during the ionization of xanthophylls such as neoxanthin, lutein, violaxanthin and zeaxanthin without an oxidative agent and can contribute to defining the chemical influences in the balance between $M^{+\bullet}$ or $[M+H]^+$.

One of the pioneering studies described ESI-LC-MS of carotenoids, which used a C-30 RP-HPLC column and a gradient solvent system containing methanol/methyl *tert*-butyl ether/ammonium acetate at a flow rate of 1.0 mL/min [18]. The entire HPLC column effluent passed through a photodiode array absorbance detector and then into the ESI-LC-MS interface without solvent splitting. Maximum sensitivity was achieved for both the photodiode array detector, which records the UV/Vis spectra of each carotenoid, and the MS, which measures the molecular ions of each carotenoid. Molecular ions, $[M]^+$, without evidence of any fragmentation, were observed in the electrospray mass spectra of both xanthophylls and carotenes. In order to enhance the formation of molecular ions, solution-phase carotenoid oxidation was carried out by means of postcolumn addition of a halogenated solvent to the HPLC effluent. The limits of detection for lutein and beta-carotene were between 1 and 2 pmol each, which was 100-fold lower than the detection limit of the photodiode array detector signal.

ESI-MS/MS was developed for identification of oxidized carotenoids in biological samples [19]. Upon collision-induced dissociation, protonated molecules $[M+H]^+$ of carotenoids produced a number of structurally characteristic product ions, see Fig. (**2**). A series of complicated clusters of product ions differing in 14 (CH_2) and 26 (C_2H_2) Da was characteristic of the polyene chain of intact carotenoids. All carotenoid ethyl oximes of zeaxanthin (synthesized from O-ethyl hydroxylamine and oxidation products of zeaxanthin) cleavage products were characterized by the losses of 60 and 61 Da in their MS/MS spectra. Through the application of the LC/MS/MS method, the authors identified two oxime derivatives of 3-hydroxy-β-ionone and 3-hydroxy-14'-apocarotenal with protonated molecules at *m/z* 252 and *m/z* 370 respectively, in a human eye sample.

Around the same time, Clarke *et al.* [20] reported the use of LC-MS/APCI for the analysis of nine carotenoids (neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene) from United Kingdom total-diet survey samples. Seven of the compounds identified in this study produced protonated molecular ions as base peaks, while neoxanthin and lutein produced fragments resulting from the loss of water as base peaks. All of the xanthophylls produced substantial $[M+H-H_2O]^+$ fragment ions, which were at the same time the base peaks for neoxanthin and lutein. These two compounds also showed substantial $[M+H-2H_2O]^+$ fragments; nonprotonated molecular ions were not observed under APCI conditions but were observed using ESI with high cone voltages. Hydroxy-containing carotenoids were detected with

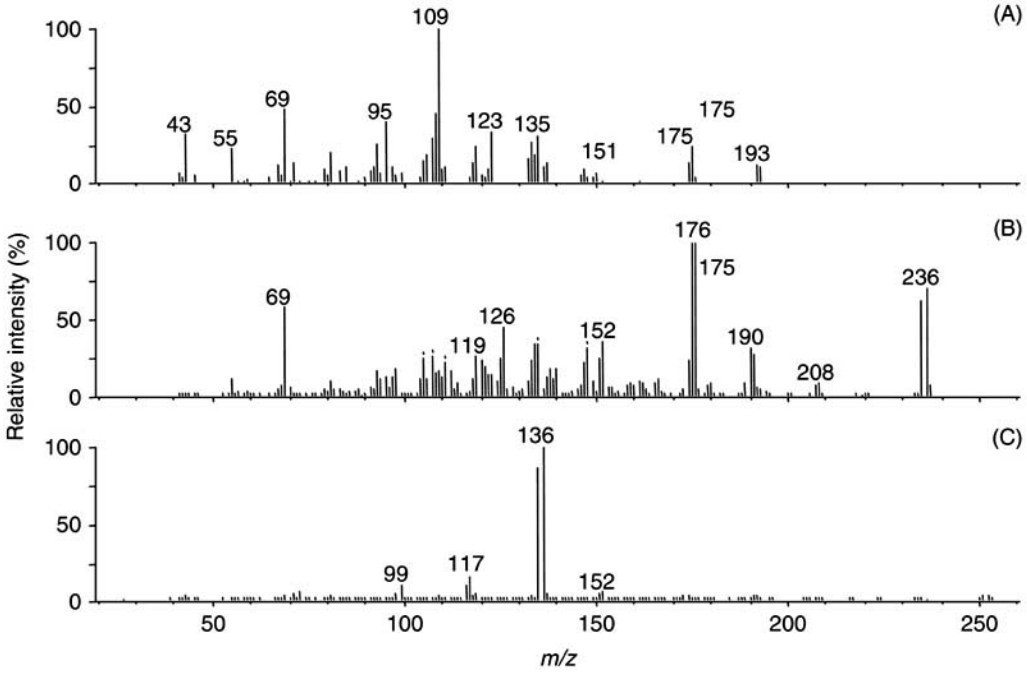

Fig. (2). Product ion spectra of the protonated molecule of β-ionone at *m/z* 193 (A), β-ionone ethyl oxime at *m/z* 236 (B) and 3-hydroxy-β-ionone ethyl oxime at *m/z* 252 (C) [19].

greater sensitivity than nonhydroxy components. Limits of detection, comparison of HPLC-UV and LC-MS/APCI, SIM chromatograms and calibration curves for α-, β-carotenes were also given.

Another technique, the FAB (fast-atom bombardment), has been employed only rarely and is no longer in use. Even so we present several papers using this technique in order to illustrate the broad possibilities of the connection of HPLC with mass spectrometer.

Very few works describe thoroughly the use of MS for identification of carotenoids; one of these studies [21] makes use of FAB spectra. Positive ion FAB MS-MS and high-energy collisionally activated dissociation (CAD) was used to differentiate 17 different carotenoids, including β-apo-8'carotenal, astaxanthin, α-carotene, β-carotene, γ-carotene, ζ-carotene, canthaxanthin, β-cryptoxanthin, isozeaxanthin bis(pelargonate), neoxanthin, neurosporene, nonaprene, lutein, lycopene, phytoene, phytofluene, and zeaxanthin. The carotenoids were either synthetic or isolated from plant tissues. Instead of protonated molecules, both polar xanthophylls and nonpolar carotenes formed molecular ions, $[M]^+$, during FAB ionization. Following collisionally activated dissociation, fragment ions of selected molecular ion precursors showed structural features indicative of the presence of hydroxyl groups, ring systems, ester and aldehyde groups and the extent of aliphatic polyene conjugation. The fragmentation patterns observed in the mass spectra may be used as a reference for the structural determination of carotenoids isolated from plant and animal tissues.

Continuous-flow FAB-LC/MS with coaxial flow addition of the FAB matrix 3-nitrobenzyl alcohol was used for analysis and determination of isomeric carotenoids, α-carotene, β-carotene, and lycopene in a tomato extract [22]. Carotenoid molecular ions were detected at *m/z* 536, 542, 544, and 540 corresponding to lycopene and β-carotene (both weighing 536), phytofluene, phytoene, and two isomers of ζ-carotene, respectively.

Schmitz *et al.* [23] described fast-atom-bombardment and continuous-flow fast-atom-bombardment mass spectrometry in carotenoid analysis of blueberry, tomato and carrot extract. The FAB matrix (3-nitrobenzyl alcohol) was introduced into the mobile phase by postcolumn addition.

2.3. Separation and Identification of Geometrical Isomers (Stereoisomers)

As mentioned in the Introduction, carotenoids are polyene compounds; this makes the existence of *cis*/*trans* (E/Z) isomerism possible. Natural materials often contain, beside all-E, also mono- and di-Z geometrical isomers (stereoisomers). Their physical properties, especially the wavelength shift in the UV/Vis spectrum (shift of ~-4 až -5 nm towards shorter wavelengths) allow for their detection by

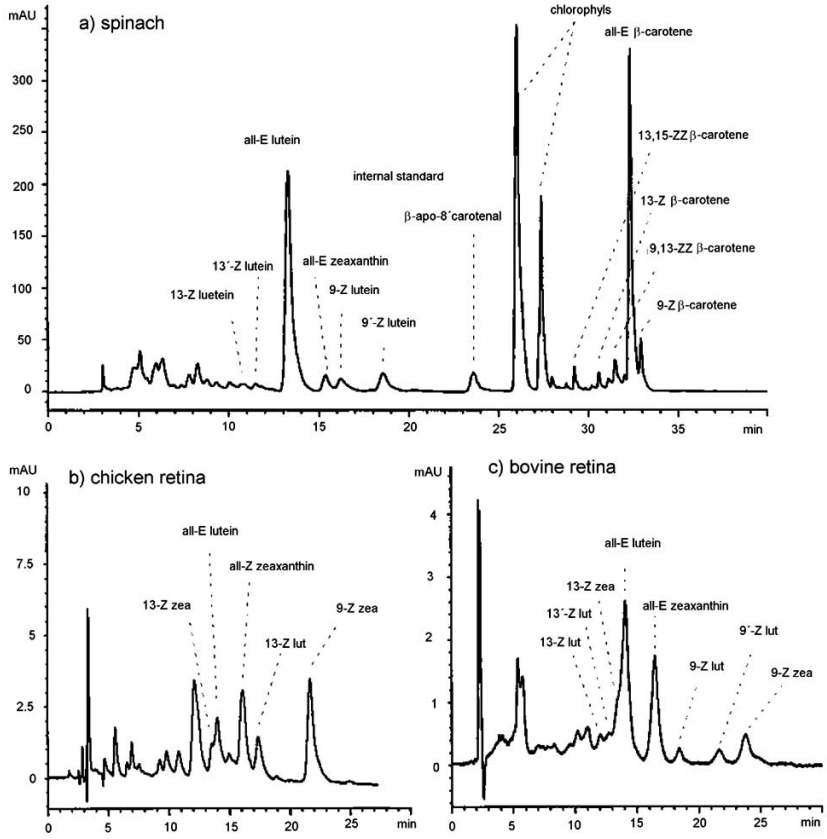

Fig. (3). Comparison of the HPLC separations of carotenoid stereoisomers from different biological samples on a Bischoff ProntoSIL (particle size 3 μm, pore diameter 200 Å) C30 column: (a) spinach extract, (b) chicken retina, and (c) bovine retina. Conditions: flow rate 1 mL/min, 22 °C, UV absorbance detection at 450 nm (in milliabsorbance units, mAU) [24].

HPLC with UV detector. Their ^{1}H, and ^{13}C-NMR spectra naturally differ but an on-line setup, *i.e.* LC-NMR, has only rarely been published for carotenoids. The mass spectra of all-E as well as mono- and di-Z geometrical isomers are identical and these stereoisomers differ only in their chromatograhic behavior, as illustrated in Fig. (**3**).

An analytical method was published for the determination of all main stereoisomers of β-carotene, lutein, and zeaxanthin (Fig. **4**) in the upper picogram range from food spinach as well as in chicken and bovine retina using C30 stationary phases [24]. The identification of the carotenoid stereoisomers was achieved with on-line coupled LC-MS/APCI and HPLC-NMR experiments. HPLC separations were performed with C-30 phases due to their enhanced shape selectivity compared to C-18 phases. On-line coupling is suitable for the differentiation and identification of carotenoids, especially the very similar carotenoids lutein and zeaxanthin, within one chromatographic run. To get an unequivocal structural elucidation of carotenoid stereoisomers, HPLC-NMR coupling is the method of choice. Identification was based mostly on the protonated molecule $[M+H]^+$, although $[M+H-H_2O]^+$ and $[M+H-92]^+$ (loss of toluene) were also detected.

Spinach, the popular source of carotenoids, served in another study for identification of lutein, β-carotene, and zeaxanthin by means of LC-MS/APCI, and HPLC-NMR on-line coupling was used for unequivocal structural elucidation [25]. Separation and structure determination of the main carotenoid stereoisomers (13-Z-lutein, 13'-Z-lutein, all-E-lutein, 9-Z-lutein, 9'-Z-lutein, all-E-zeaxanthin, 13,15-Z,Z-β-carotene, 13-Z-β-carotene, 9,13-Z,Z-β-carotene, all-E-β-carotene, and 9-Z-β-carotene) was achieved by the use of hyphenated analytical techniques and exclusion of light and oxygen. HPLC analysis with highly selective C-30 columns was used for quantitative determination of the main Z/E carotenoid stereoisomers, while LC-MS/APCI and HPLC-NMR on-line coupling was used for structural elucidation. Whereas LC-MS/APCI can distinguish between the carotenoids lutein and zeaxanthin, mainly on the basis of $[M+H]^+$ and $[M+H-H_2O]^+$ ions, HPLC-NMR enables identification of all the main Z/E stereoisomers.

Two studies described the presence and detection of geometrical isomers of lycopene [26, 27]. The total lycopene and its *cis* and all-*trans* isomers in human plasma were identified using LC-MS-MS. This method was based on the observation that during $APCI^-$ with collision-induced dissociation, a unique fragment of *m/z* 467 was formed from the molecular ion of *m/z* 536 by elimination of a terminal isoprene group. Men with clinical stage prostate adenocarcinoma consumed tomato sauce and HPLC with a C-30 column and LC-MS/$APCI^+$ was used for measuring the ratio of lycopene *cis*/*trans* isomers; a total of 10 lycopene isomers were determined (one all-*trans* and 9-*cis*-isomers).

The influence of thermal treatment and light exposure on degradation and isomerization of the predominant carotenoids (lutein and β-carotene) was assessed in green leafy vegetables [28]. Detection and identification was performed by RP-HPLC (C30 column) and was also based on UV spectra of the degradation products, *e.g.* 9-*cis*-β-carotene and 13-*cis*-β-carotene.

A C-30 stationary phase was used also for violaxanthin detection in a fraction of orange juice (*Citrus sinensis*) to identify the different geometrical isomers occurring in it [29]. Under the HPLC conditions described, several violaxanthin isomers ((15Z)-violaxanthin, (13Z)-violaxanthin, (9Z)-violaxanthin, (all-E)-violaxanthin and some (di-Z)-violaxanthins) were detected, of which (all-E)-violaxanthin and, above all, (9Z)-violaxanthin, happened to be the most important in quantitative terms. The structure was identified by the not-too-suitable EI-MS.

A total of 34 carotenoids including geometrical isomers and eight metabolites have been isolated from human breast milk and serum by LC-photodiode array (PDA) and MS [30]. The compounds identified by their UV spectra and $[M]^+$ and $[M-H_2O]^+$ included, *e.g.*, lycopene, (Z)-lycopene, neurosporene, ξ-carotene, γ-carotene, α-carotene, (all-E)-β-carotene, (9Z)-β-carotene, (13Z)-β-carotene, phytofluene, and phytoene.

2.4. Separation and Identification of Labeled Carotenoids

Several studies describe the preparation of compounds labeled by stable isotopes, *i.e.* deuterium and ^{13}C. The preparation was performed with the use of various photosynthesizing organisms, either algae or higher plants. The cultivation was carried out in heavy water or in an atmosphere enriched in isotopically labeled carbon dioxide ($^{13}CO_2$).

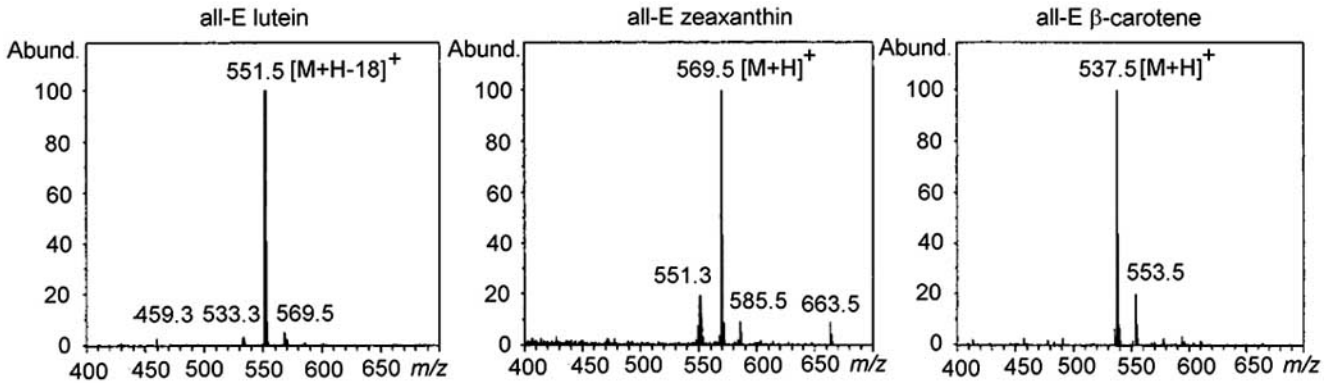

Fig. (4). Mass spectra of (all-*E*)-lutein, (all-*E*)-zeaxanthin, and (all-*E*)-β-carotene from spinach [24].

Putzbach [31] determined the deuterium distribution of partially deuterated carotenoids in intrinsically labeled vegetables, using LC-MS/APCI and ^{1}H NMR. The structures of biosynthetic deuterated carotenoids were identified as (all-E)-lutein and (all-E)-β-carotene from spinach, and (all-E)-β-carotene and (all-E)-α-carotene from carrots. The undeuterated (*all-E*)-lutein shows a peak at *m/z* 551 $[M+H-H_2O]^+$. The deuterated (*all-E*)-lutein has an isotopomer distribution from *m/z* 552 to 562 with the most abundant at *m/z* 556 $[M+(^2H5)+H-H_2O]^+$. The undeuterated (*all-E*)-*β*-carotene shows a peak at *m/z* 537 $[M+H]^+$, and the deuterated (*all-E*)-β-carotene has an isotopomer distribution from *m/z* 538 to 549 with the most abundant at *m/z* 542 $[M+(^2H_5)+H]^+$. (all-E)-Lutein and (all-E)-β-carotene showed partial deuteration from ^{2}H1 to ^{2}H12, with the abundance maximum at ^{2}H5. (all-E)-β-carotene and (all-E)-α-carotene from carrots showed partial deuteration from ^{2}H1 to ^{2}H17, with the abundance maximum at ^{2}H11. The deuteration was distributed randomly throughout the carotenoid molecules.

Flavobacterium multivorum, a zeaxanthin-producing organism, was grown aerobically in a medium prepared with deuterated water [32]. MS/APCI and resonance Raman spectroscopy (RRS) analysis revealed an about 75% replacement of hydrogen by deuterium atoms as indicated by the molecular mass cluster at around *m/z* 600. LC-MS/APCI with limits of detection of 2.5 pg was 20 000 times more sensitive than HPLC/RRS.

Some studies dealing with labeled compounds concern their administration to humans and monitoring of their metabolism in the human body by LC-MS/APCI.

The relative bioavailability of food-derived lutein in humans was studied by Lienau *et al.* [33]. The plant lutein did not form the molecular ion, but rather the $(M+H-H_2O)^+$ ion at *m/z* 551. The most abundant isotopomer of labeled lutein was *m/z* 559 ^{2}H8-$(M+H-H_2O)^+$. The extracted ion chromatograms were in the *m/z* regions 551 to 554 and 556 to 562. The *m/z* area 551 to 554 was assigned as the predominant natural abundance isotopomers of lutein $(M+H-H_2O)^+$ while the region of *m/z* 556 to 562 was the labeled lutein with its different degrees of deuteration. The quantitation of the labeled lutein in serum samples enabled the calculation of the enrichment for each time point after the dose. Area-under-the-curve analyses of four different subjects (integrated over 29 days) yielded serum lutein responses of 128, 145, 149, and 262 μg-day/mg dietary lutein, following an acute dose of spinach containing 15.4, 18.8, 18.8 and 9.8 mg labeled lutein, respectively.

A method was described for studying the bioavailability of nutrients from kale (*Brassica oleracea* var. *Acephala*) by labeling the nutrients in the plant tissue with ^{13}C, administering the labeled vegetables to an adult volunteer, and analyzing plasma samples for lutein, β-carotene, retinol, and phylloquinone using LC-MS/APCI [34]. Both labeled and unlabeled compounds were identified based on $[M+H]^+$ and $[M+H-H_2O]^+$ ions, *e.g.* for β-carotene (*m/z* 537 representing $[M+H]^+$) and ^{13}C40-β-carotene (*m/z* 577).

Another study [35] described the feeding of the kale (*Brassica oleracea* variety *Acephala* cultivar Vates) to seven adult volunteers and analyzing serial plasma samples for labeled [^{13}C] lutein, β-carotene, and retinol; no data on LC-MS/APCI were given.

The application of the method with labeled β-carotene [^{13}C] was tested in a study concerned with the bioavailability and bioconversion of β-carotene into retinol in human serum and feces samples [36]. Analysis was performed by means of LC/MS assay based on the detection of molecular anions in SIM mode of β-carotene using negative ion APCI with a reversed-phase C-30 column for HPLC separation. The identification involved using negative ion APCI mass spectra of β-carotene and [^{13}C10]-β-carotene. The M^+ ions were detected as the base peaks at *m/z* 536 and 546, respectively, saponification being used to improve the selectivity of analyses by eliminating interfering triglycerides and diglycerides. LC/MS analysis of retinol in a hexane extract of serum made use of SIM of *m/z* 269 (all-*trans*-retinol), *m/z* 274 (all-*trans*-[^{13}C5]-retinol), and *m/z* 279 (all-*trans*-[^{13}C10]-retinol).

2.5. Separation and Identification of Carotenoid Esters

Carotenoids in natural environments, in both plants and animals, are predominantly esterified by fatty acids. The degree of esterification differs for individual carotenoids and is related to the number of hydroxyl groups present in the xanthophylls. Identification of peaks in chromatograms is thus complicated. Monohydroxy-carotenoids, such as β-cryptoxanthin, could be found free or esterified by one fatty acid (monoester); dihydroxycarotenoids, such as lutein and zeaxanthin, could be found free or esterified by one (monoester) or two (diester) fatty acids. In order to simplify the separation, most methods for quantitative carotenoid analysis employ a previous saponification; however, this treatment leads to the loss of a large proportion of the information about the structure of the native compounds. Alkaline saponification usually includes using aqueous-ethanolic or methanolic solutions of potassium hydroxide, however, significant degradation and losses of carotenoids have been observed due to saponification. Simultaneous analysis of free and esterified carotenoids has been suggested, thus avoiding saponification.

The ester composition of plants and animals is not constant but depends on variety, age of the organisms, environmental conditions, etc. For example, in some fruits during ripening, the esterification degree of carotenoids increases. In addition, a high stability against possible thermo-, photo- and enzymatic oxidation reactions has been related to the esterification degree. In recent years, the methods for carotenoid determination without saponification have increased in use. The use of ESI or APCI has facilitated the identification of the esterified forms of carotenoids. The esters have a wide range of apolarity and the chromatographic methods include mostly RP-HPLC on C18 columns. Both isocratic and gradient mobile phase systems are used, which are based on mobile phases containing predominantly MeOH, acetone, 2-propanol and acetonitrile.

LC-MS analysis at approximately *m/z* 300-1300 of the samples by APCI showed $[M+H]^+$ (pseudomolecular ions) for the peaks characteristic for xanthophyll diesters and allowed the attribution of their molecular weights. The data of the APCI mass spectra of the astaxanthin diesters include assignments of fragmentation pathways for each compound

analyzed. Some fragmentation of the diacyl-xanthophyll was evident. The parent ion $[M+H]^+$ dissociates to ions $[M+H-FA_1]^+$ and/or $[M+H-FA_2]^+$, and further ions $[M+H-FA_1-H_2O]^+$, $[M+H-FA_2-H_2O]^+$, and ions common to all diesters $[M+H-FA_1-FA_2]^+$; $[M+H-FA_1-FA_2-H_2O]^+$ and $[M+H-FA_1-FA_2-2xH_2O]^+$.

The first successful chromatographic separation of mixed fatty acid lutein diesters was described in study [37]. Synthetic mixtures of 24 mono- and diesters of the asymmetric hydroxylated carotenoid lutein with lauric, myristic, palmitic, and stearic acids were analyzed by LC-UV/Vis and LC-MS and characterized by NMR. Preferential MS loss of fatty acids or water occurred initially at the 3'-hydroxy position in the ε -ionone ring and subsequently at the 3-hydroxy position in the β-ionone ring. This selective fragmentation led to facile assignment of the specific fatty acids to the appropriate regioisomeric ionone ring. A commercial lutein supplement contained low levels of two pairs of regioisomeric monoesters and nearly equal levels of three homogeneous diesters and five pairs of mixed diesters. Palmitic acid was the predominant fatty acid, with lower amounts of myristic, stearic, and lauric acids. The synthesized compounds were then used for identifying the composition of a commercial lutein supplement.

Carotenoids and carotenoid esters were extracted from red pepper pods (*Capsicum annuum* L.) and a total of 42 compounds were identified including 4 non-esterified, 11 mono- and 17 diesters [38]. They were characterized by collision-induced dissociation experiments in MS/APCI. 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 β-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 study gave a detailed description of fragmentation of free carotenoids as well as carotenoid esters and carotenoid diesters in MS. For instance, capsanthin-laurate-palmitate produced $[M+H]^+$ ion at *m/z* 1005, the fragmentation of which produced ions at *m/z* 805, 749 and 549, indicating the loss of a lauric and palmitic acid moiety. Fragmentation of zeaxanthin-laurate-myristate was similar, the compound exhibiting 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. The results obtained from LC-DAD-MS/APCI (diode array detector) experiments demonstrated that the carotenoid profile of red pepper pods is considerably more complex than hitherto considered.

An important study described in great detail the separation and identification of regioisomers of lutein monoesters [39]. In this study LC-MS/APCI was employed for the identification of eight lutein monoesters, formed by incomplete enzymatic saponification of lutein diesters of marigold (*Tagetes erecta*) by *Candida rugosa* lipase. Additionally, the main lutein diesters naturally occurring in marigold oleoresin were chromatographically separated and identified. The LC-MS method allows for characterization of lutein diesters occurring as minor components in several fruits; this was demonstrated by analysis of extracts of cape gooseberry (*Physalis peruviana*), kiwano (*Cucumis metuliferus*), and pumpkin (*Cucurbita pepo*). The assignment of the regioisomers of lutein monoesters was based on the characteristic fragmentation pattern: the most intense daughter ion generally results from the loss of the substituent (fatty acid or hydroxyl group) bound to the ε-ionone ring, yielding an allylic cation. The mass spectra are generally characterized by three signals. Two of them are generated by the neutral loss of the fatty acid or water from the respective quasimolecular ions, whereas the third signal, which is found in each mass spectrum, represents the lutein backbone $([M+H-H_2O-FA]^+$. The diesters have been found to undergo fragmentation, yielding as major ions $[M+H-FA_1]^+$, $[M+H-FA_2]^+$, $[M+H-FA_1-FA_2]^+$. It was not possible to chromatographically separate regioisomers of mixed lutein diesters (*i.e.* myristoylpalmitoyl-lutein and palmitoylmyristoyl-lutein); in contrast, 3´-O-palmitoyl-lutein and/or 3-O-palmitoyl-lutein could be separated.

MS/APCI and collisionally induced dissociation (CID) were employed to obtain structural information of lutein esters from marigold extract [40]. Both molecular ions and structurally significant fragments ($[M+H-FA_1]^+$, $[M+H-FA_2]^+$, $[M+H-FA_1-FA_2]^+$) corresponding to the loss of fatty acids were observed in high abundance in the study. Six lutein diesters including lauroylmyristoyl-lutein, dimyristoyl-lutein, myristoylpalmitoyl-lutein, dipalmitoyl-lutein, palmitoylstearoyl-lutein and distearoyl-lutein were characterized in a marigold flower extract. Separation of the two mixed esters, *i.e.* 3 or 3´, was not successful.

HPLC/DAD and LC-MS/APCI analysis of several fat-soluble vitamins {vitamins A and E, vitamin esters (E-acetate, A-acetate, A-palmitate) and coenzyme Q10} including lutein diesters from *Tagetes erecta,* lutein, zeaxanthin, all-*trans*-β-carotene and 9-*cis*-β-carotene on a C30-column was described in study [41].

Considerable attention was also paid to commercially available plants, *e.g.* citrus fruits. *β*-Cryptoxanthin and zeinoxanthin were identified on the basis of their quasimolecular ion $[M+H]^+$ in saponified citrus fruit extracts by comparison to reference compounds extracted from corn and by their typical fragmentation pattern in LC-MS/APCI analyses [42]. The tendency for fragmentation was low in both cases and only ion $[M+H-H_2O]^+$ was formed. To identify esters of structural cryptoxanthin isomers in native orange juice extracts, four saturated acyl esters were synthesized. LC-MS/APCI studies revealed for the first time that the dominant acylation partners of both xanthophylls were 12:0, 14:0, and 16:0 in nearly equal amounts of roughly one-third, whereas 10:0 and 18:1 were present in lower extents; other acylation partners were not identified. Mass spectrum was dominated by the loss of the respective fatty acid from the quasimolecular ion, resulting in a common backbone ion $[M+H-FA]^+$.

LC-MS/APCI was used to identify carotenoid esters in mandarin essential oil [43]. A gradient of methanol and methyl-*t*-butyl ether on a C-30 reversed-phase column was

employed. The polymeric C-30 stationary phase demonstrated superior resolution for carotenoid separation in comparison to the traditional C-18 column. Carotenoid esters such as β-cryptoxanthin laurate, β-cryptoxanthin myristate, and β-cryptoxanthin palmitate were present in the non-volatile residue of the oil. These compounds were identified on the basis of $[M+H]^+$, $[M+H-H_2O]^+$, and $[M+H-FA]^+$ ions. The authors showed a major fragmentation pathway of protonated β-cryptoxanthin and β-cryptoxanthin monoesters acylated at the β-ionone ring. These analytical techniques have been used also with other plants, with no substantial innovations.

The carotenoid pattern of mango (*Mangifera indica*) was investigated by LC-MS/APCI analyses [44]. A carotenoid ester from the mesocarp was identified as violaxanthin dibutyrate. Additionally, further carotenoid peaks were tentatively assigned to 9-*cis*-violaxanthin and neochrom or luteoxanthin, respectively, by their UV/Vis and MS data of the saponified extracts. The fragmentation pattern of violaxanthin dibutyrate is characterized by two paths, affording different daughter ions. The loss of water from the quasi-molecular ion (*m/z* 741) yields *m/z* 723. Subsequent elimination of butyric acid (88 Da) as a neutral molecule results in *m/z* 635. Alternatively, removal of a fatty acid residue (88 Da) gives *m/z* 653 and subsequent elimination of water from the resulting daughter ion (*m/z* 635) occurs.

LC-MS/APCI was used to unequivocally identify zeaxanthin esters of a standard mixture and in dried wolfberries (*Lycium barbarum*), Chinese lanterns (*Physalis alkekengi*), orange pepper (*Capsicum annuum*), and sea buckthorn (*Hippophae rhamnoides*) [45]. Zeaxanthin monoesters, *i.e.,* laurate, myristate, palmitate, stearate and zeaxanthin diesters (dilaurate, dimyristate, dipalmitate, and distearate) were identified. Identification was based on the quasimolecular ion $[M+H]^+$ and the fragmentation pathway, which was dominated by the loss of one $[M+H-FA]^+$ or two $[M+H-2FA]^+$ fatty acids. The paper gives the fragmentation ions for zeaxanthin mono- and diesters, which is a highly useful piece of information.

LC-MS/APCI was used for identification and quantification of carotenoids in potato cultivars (*Solanum tuberosum*) [46]. In each case, the carotenoid pattern was dominated by violaxanthin, antheraxanthin, lutein, and zeaxanthin, which were present in different ratios, whereas neoxanthin, β-cryptoxanthin, and β-carotene generally were only minor constituents. A two-stage, cleanup procedure involving column chromatography on silica gel and enzymatic cleavage of residual triacylglycerides by lipases was developed for LC-MS/APCI analyses of carotenoid esters. This facilitated the direct identification of several potato carotenoid esters without previous isolation of the compounds. Carotenoids and carotenoid esters were identified by DAD and on the basis of the quasimolecular ion $[M+H]^+$, esters being also identified by means of ions $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-H_2O-FA_1]^+$, $[M+H-H_2O-FA_1-FA_2]^+$, $[M+H-FA_1]^+$, and $[M+H-FA_1-FA_2]^+$.

Astaxanthin naturally occurs in a wide variety of marine and aquatic organisms that have bright red to pink coloring due to the accumulation of astaxanthin (**1**). Because animals are not capable of de novo synthesis of astaxanthin, microalgae serve as its natural dietary source. They are used worldwide as a feed supplement in fish farming to grow healthy and well-colored individuals. Astaxanthin is the most commonly used carotenoid in salmonid fish farming and is deposited in the supplied, unesterified form in fish muscle. Natural sources of astaxanthin such as krill or algae supply it in the esterified form. After hydrolysis of the esters it is deposited in the flesh of salmonids in the free form. Considerable attention has therefore been devoted to the analysis of astaxanthin esters, which is complicated by the existence of a) *cis*/*trans* isomers, b) optical isomers, *i.e.*, 3*S*,3′*S;* 3*R*,3′*S*; 3*R*,3′*R*, and c) mono- an/or di-esters. As a result, the number of individual isomers rapidly increases, making the analysis highly exacting.

Negative-ion LC-MS/APCI was used for the identification of astaxanthin esters in extracts of commercial shrimp (*Pandalus borealis*) and dried microalga (*Haematococcus pluvialis*) samples [47]. The assignment of astaxanthin esters was based on the detection of the molecular ion (M-) and the formation of characteristic fragment ions, resulting from the loss of one or two fatty acids. Detection limits, based on the intensity of M-, a signal-to-noise ratio of 3:1 were estimated to be 0.05 μg/mL (free astaxanthin), 0.28 μg/mL (astaxanthin esters), and 0.78 μg/mL (astaxanthin-diesters), respectively. This LC-MS/APCI method has allowed for the first time the characterization of native astaxanthin esters in *P. borealis* and *H. pluvialis* without using time-consuming isolation steps with subsequent gas chromatographic analyses of fatty acid methyl esters. The results suggest that the pattern of astaxanthin-bound polyunsaturated fatty acids of *P. borealis* does not reflect the respective fatty acid pattern found in triacylglycerides.

Isolation and identification of astaxanthin fatty acid esters in krill (*Euphausia superba*) was the subject of another study [48]. Astaxanthin, geometrical isomers of astaxanthin and esters were identified by LC-MS/APCI; however, the mass spectra were not described, the description concerning only conditions of chromatographic analyses. Astaxanthin, the geometrical isomers, and its fatty acid monoesters and fatty acid diesters were identified by SIM (selected ion monitoring).

Carotenoids and retinoids were determined in eggs from Chinook salmon (*Oncorhynchus tshawytscha*) [49] using liquid-liquid partitioning to minimize analyte degradation, and fraction analysis using HPLC-electrospray (positive)-quadrupole mass spectrometry (LC-MS/ESI^+; SIM or MRM modes). The eggs contained β-carotene, lutein, zeaxanthin, β-cryptoxanthin, canthaxanthin, astaxanthin, and all-*trans*-retinol. Astaxanthin, all-*trans*-retinol, lutein and canthaxanthin were identified in the eggs and determined at concentrations of 4.12, 1.06, 0.12 and 0.45 μg/g (egg wet weight), respectively. The authors describe the MS identification in considerable detail.

Preparative HPLC with a normal-phase Lichrosorb silica column has been developed to isolate and characterize some minor impurities such as astacene, dehydro astacene and apoastaxanthinal of astaxanthin using n-hexane-acetone-tetrahydrofuran as mobile phase and detection at 475 nm [50]. The compounds were characterized by UV/Vis, ESI-MS, H-1 and C-13 NMR spectroscopy. The impurities collected using the developed conditions were over 98% pure.

Carotenoids of green alga *Haematococcus pluvialis* were identified by LC-MS/APCI [51]. Identification of each compound was based on the characteristic fragment ions of the positive ion mode, negative ion mode, and MS-MS, i.e. $[M+H]^+$ and $[MH-18]^+$ for free carotenoids (4 compounds), $[M+H]^+$, $[M+H-H_2O]+$, and $[M+H-FA]^+$ for monoesters and $[M+H]^+$ $[M+H-FA_1]^+$, $[M+H-FA_2]^+$ and/or MS^2 for diesters. The basic peaks of other astaxanthin diesters showed characteristic fragment ions of losing one fatty acid, but their fragment ions of losing the second fatty acid had weaker relative intensity.

The development of analytical techniques and exploration of less common organisms from extreme environment brought, among other things, the finding that the snow alga *Chlamydomonas nivalis* contains astaxanthin glucoside esters, which were identified by means of LC-MS/APCI [52]. The analysis was based on the use of preparative HPLC and subsequent identification of astaxanthin diglucoside diesters by microbore LC-MS/APCI (Fig. **5**). The combination of these two techniques was used to identify more than 100 molecular species. The astaxanthin diglucoside diester, i.e. (all-E)-[di-(6-O-oleoyl-β-D-glucopyranosyloxy)]-astaxanthin, was also synthesized to unambiguously confirm its structure. The mass spectra of individual compounds were described in detail (Fig. **6**). The parent ion $[M+H]^+$ dissociates to six ions, i.e. $[M+H-FA_1]^+$, $[M+H-FA_2]^+$, $[M+H-FA_1-H_2O]^+$, $[M+H-FA_2-H_2O]^+$, $[M+H-Glc-FA_1]^+$, $[M+H-Glc-FA_2]^+$, $[M+H-Glc-FA_1-H_2O]^+$, $[M+H-Glc-FA_2-H_2O]^+$, and ions common to all astaxanthin diglycoside diesters $[M+H-FA_1-FA_2]^+$; $[M+H-FA_1-FA_2-H_2O]^+$; $[M+H-FA_1-FA_2-2xH_2O]^+$; $[M+H-FA_1-FA_2-Glc]^+$; $[M+H-FA_1-FA_2-Glc-H_2O]^+$; $[M+H-FA_1-FA_2-2xGlc]^+$; $[M+H-FA_1-FA_2-2xGlc-H_2O]^+$; $[M+H-FA_1-FA_2-2xGlc-2xH_2O]^+$. Semiquantitation was based on the abundance of $[M+H-FA_1]^+$ and/or $[M+H-FA_2]^+$ ions for determining peaks inseparable by HPLC.

2.6. Identification of Carotenoids After Separation by Counter-Current Chromatography

Xanthophylls such as (all-E)-lutein and (all-E)-zeaxanthin were isolated by high-speed counter-current chromatography (HSCCC) from spinach and sweet corn. The concomitant pigments violaxanthin, neoxanthin, β-carotene and chlorophylls *a* and *b* of spinach were also included [53]. HSCCC yielded a semi-purified extract which contained >97% zeaxanthin and lutein (ratio 1:4). In the separation of pigments obtained from 200 mg of crude spinach extract by HSCCC the area of the lutein peak comprised 93% of the total peak area as determined by HPLC at 445 nm. Concomitant small side peaks represented (Z)-isomers of (all-E)-lutein. All in all, the HSCCC method did not seem to bring any advantages as compared to preparative HPLC.

In another paper about the HSCCC method, a two-phase solvent system consisting of hexane-ethanol-water was developed for the purification of lutein from the saponification mixture of marigold flower extract [54]. The structure of lutein was confirmed by comparing the retention time, UV/Vis spectrum and LC-MS/APCI with the standard.

2.7. Carotenoids from Humans

Numerous papers have been devoted to the analysis of carotenoids and related compounds in human organs and body fluids. The analyses are most often carried out after administration of labeled compounds (see above) or foods rich in carotenoids. The determinations concern mostly the levels of individual carotenoids in, e.g., human serum, or

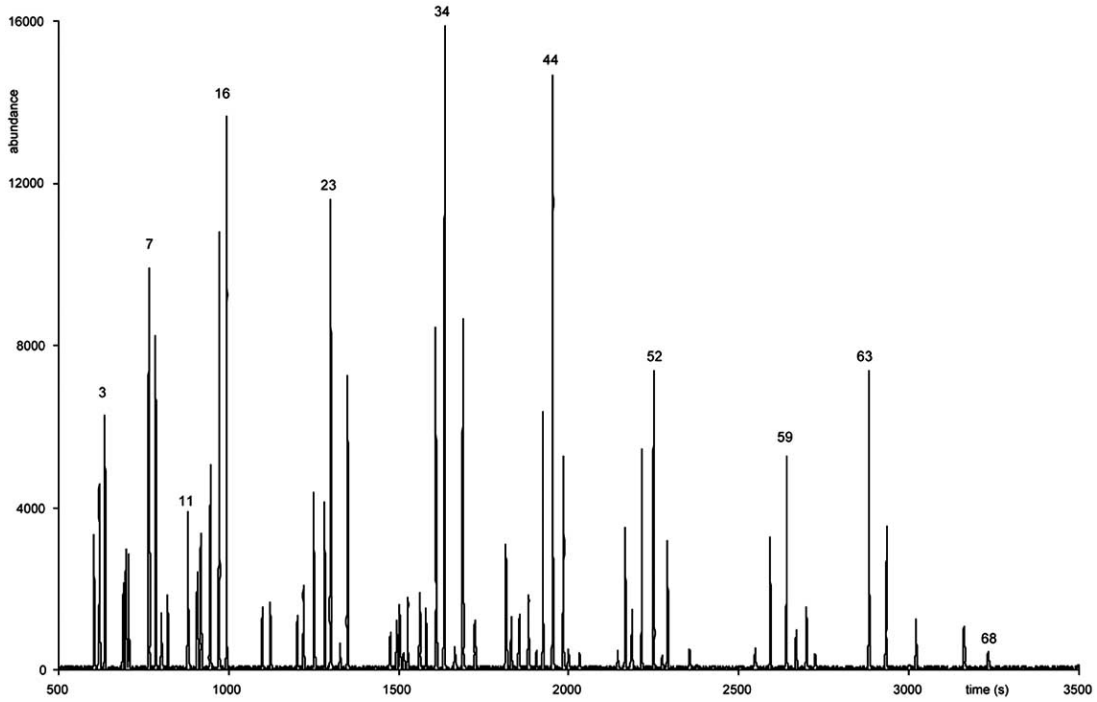

Fig. (5). Separation of astaxanthin glucoside esters from snow alga *Chlamydomonas nivalis* by means of LC-MS/APCI. For peak assignment, see [52].

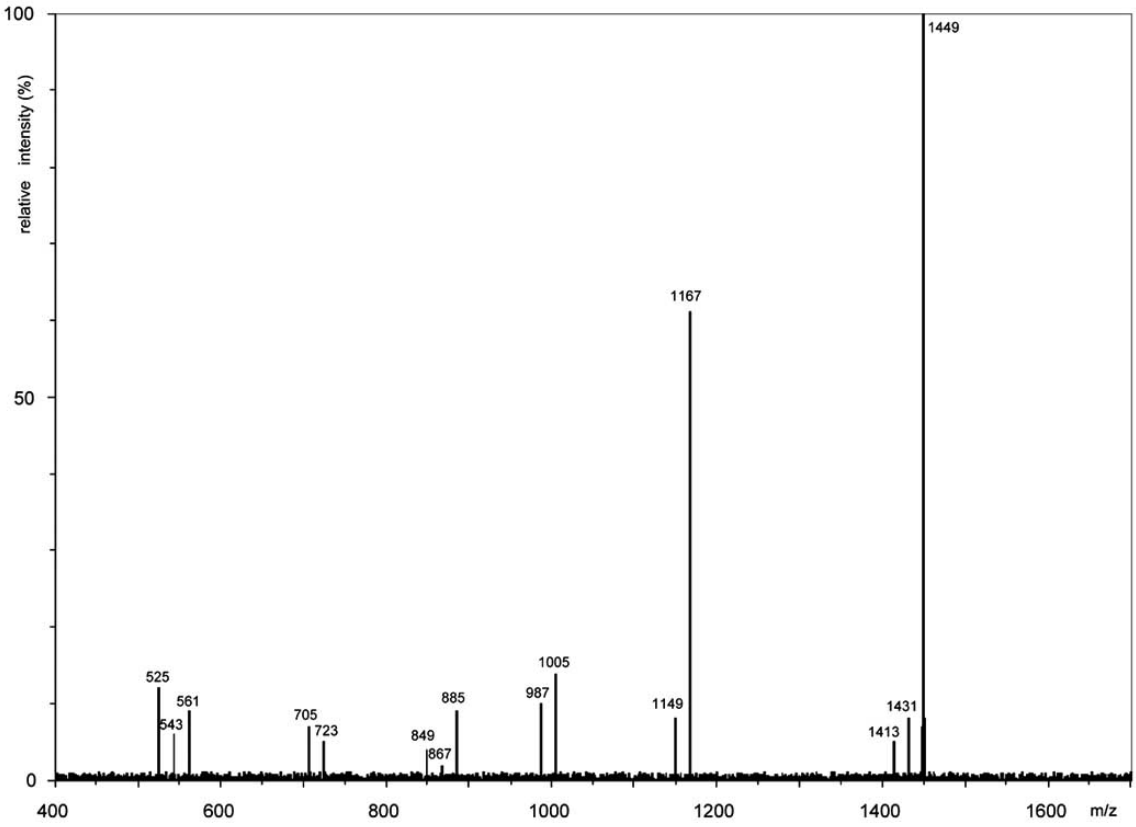

Fig. (6). APCI spectrum of astaxanthin glucoside ester in peak 63 (di-[(6-O-oleoyl-β-D-glucopyranosyl)oxy]-astaxanthin, *i.e.* 18:1-G-A-G-18:1) in Fig. (**5**) [52].

some degradation (oxidation) products formed in the human body and its organs, e.g. eyes.

Different anhydroluteins have been isolated and characterized from extracts of human plasma using semipreparative RP-HPLC column [55]. The identification of anhydroluteins was accomplished by comparison of the UV/Vis and mass spectral data as well as HPLC-UV/Vis-MS spiking experiments using fully characterized synthetic compounds, *i.e.* anhydrolutein, 2',3'-anhydrolutein, and 3',4'-anhydrolutein. The presence of anhydroluteins in human plasma was postulated to be due to acid-catalyzed dehydration of the dietary lutein as it passes through the stomach.

A SIM determination was developed of lycopene, α-carotene and β-carotene by MS/APCI in serum [56]. Separation of carotenoids was performed using a solution of methanol and acetonitrile as the mobile phase on a C-18 column. In the positive mode of APCI/MS, three carotenoids were monitored at *m/z* 537. This method was linear for all analytes in the range of ~10-50 ng carotenes. The detection limit of LC-MS/APCI-SIM for carotenoids was about 3 ng per 1 mL of serum. An important feature of this study was the identification of the three carotenoids under conditions of a vast excess of cholesterol and its derivatives.

A similar work [57] used the same method for comparing the relative bioavailability of β-carotene from pumpkin, which was found to be 1.8 times greater than that from spinach, based on data from feces and serum.

Eighteen carotenoids, as well as vitamin A, have been separated from extracts of human plasma by RP-HPLC and silica-based nitrile-bonded columns [58]. Individual carotenoids were identified based on their UV and MS spectra. The use of two columns with different phases was necessary because the polar carotenoids which eluted in the vicinity of lutein were unresolved on the C18 column. Several geometrical isomers of lutein and zeaxanthin were also identified.

Measurement of 3'-oxolutein, which is the major oxidative metabolite of dietary carotenoids in the retina of the human eye, was published [59]. Normal-phase LC-MS/APCI allowed for quantitative analysis of 3'-oxolutein from central and peripheral retinas obtained from individual human donors. The limit of quantitation for 3'-oxolutein in human retina at a signal-to-noise ratio of 10 was 6 pg. The method used was again SIM $[M]^+$ and $[M+H]^+$.

2.8. Carotenoids from Plants

Many studies concern the analysis of carotenoids in plant materials. Even a cursory assessment of individual papers, however, brings to the fore the total lack of a systematic and conceptual approach. As mentioned several times in this review, a study presenting an overall view of carotenoid analysis as a whole is sorely missing. The authors active in this field can only envy workers analyzing, e.g., intact triacylglycerols by LC-MS/APCI, who can make use of several review articles excellently summarizing not only the chromatography and mass spectrometry, but also the contents of

triacylglycerols in all known and common kinds of oils and also in many not-so-common ones.

One of the first studies [60] describing very thoroughly the use of APCI reported on HPLC-UV-MS/APCI separation and determination of different carotenoids as well as *cis*/*trans* isomers of β-carotene (Fig. **7**) in different vegetables such as tomatoes, carrots, spinach and celery. All HPLC separations were carried out on RP C-30 phases. The analysis of a carotenoid mixture containing astaxanthin, canthaxanthin, zeaxanthin, echinenone and β-carotene was achieved by scanning the mass range from *m/z* 200 to 700. The detection limit for β-carotene in positive-ion MS/APCI was determined to be 1 pmol and isomers (9, 13-di-*cis*, 9-*cis*, 13-*cis*, 15-*cis* and all-*trans* β-carotene) were separated. In addition, an extract of non-polar substances in vegetable juice has been analyzed by LC-MS/APCI.

Further studies dealt extensively with APCI of carotenoids contained in sweet potatoes, carrots and tomatoes. The compounds were analyzed using LC-MS/APCI with a narrow-bore C-30 RP-HPLC column and a gradient solvent system containing methanol-methyl *tert*-butyl ether-ammonium acetate [61]. Also UV/Vis detection was used for additional carotenoid characterization. Positive-ion APCI produced protonated molecules and molecular ions for both xanthophylls and, unexpectedly, hydrocarbon carotenes, and $[M]^-$ and $[M-H]^-$ ions were observed during negative-ion APCI. In order to investigate the origin of the unexpected $[M+H]^+$ ions, positive-ion APCI of β-carotene was investigated using deuterochloroform as the only solvent. The hydroxylated xanthophyll lutein fragmented during positive-ion APCI to eliminate water from the protonated molecule and form the base peak $[M-H_2O]^+$. Using CID in the ion source, additional fragmentation pathways with characteristic tandem mass spectra of carotenoids were observed such as retro-Diels-Alder fragmentation, $[M-56]^+$, for α-carotene and loss of toluene from the molecular ion, $[M-92]^+$ for lutein, α- and β-carotene. The limits of detection for protonated molecules of α-carotene and lutein were approximately 3 and 13 pmol, respectively. In negative-ion APCI, the limits of detection were approximately 3 and 1 pmol for $[M]^-$ ions of α-carotene and lutein, respectively.

In order to verify the possible existence of carotenoid profiles in *Cucurbita* species, study [62] determined the carotenoid concentrations in squashes and pumpkins currently marketed in Brazil by HPLC-DAD, complemented by HPLC-MS for identification. The compounds so identified included neoxanthin, violaxanthin, lutein, zeaxanthin, α-cryptoxanthin, β-carotene-5,6-epoxide, α-, β-, ζ-carotenes and (13Z)-β-carotene. Their structure was determined on the basis of fragmentation in MS; for neoxanthin the visible absorption spectrum (λ_{max} and spectral fine structure) reflected the chromophore with eight conjugated double bonds and an allene group in the polyene chain. The presence of three hydroxyl groups and one 5,6-epoxide, indicated initially by the chromatographic behavior, was confirmed by the positive response to acetylation and the 5,6-epoxide test (hypsochromic shift of 20 nm, corresponding to one epoxide at the 5,6-position). The mass spectrum exhibited a molecular ion and characteristic fragments at *m/z* 520 $[M-80]^+$, representing the loss of one epoxide group, at *m/z* 419 $[M-181]^+$, and at *m/z* 172, corresponding to a β-ring with a 5,6-epoxy and a hydroxyl substituent. Those at *m/z* 352, 221, and 181 referred to fragments with the β-ring containing epoxy and hydroxyl substituents with part of the lateral polyene chain resulting from cleavage at the C-12,13, C-10′,11′, and C-8′,9′ bonds, respectively.

Carotenoids were also identified and quantitated in the mango cultivars grown in Mexico by LC-MS with C-30 stationary phase and diode array, fluorescence, and mass (time-of-flight) detectors [63]. All-*trans*-β-carotene and dibutyrates of all-*trans*-violaxanthin and 9-*cis*-violaxanthin were the most abundant. In addition, more than 10 peaks were detected but remained identified, although they were denoted as esters (e.g. all-*trans*-violaxanthin ester or 9-*cis*-violaxanthin ester).

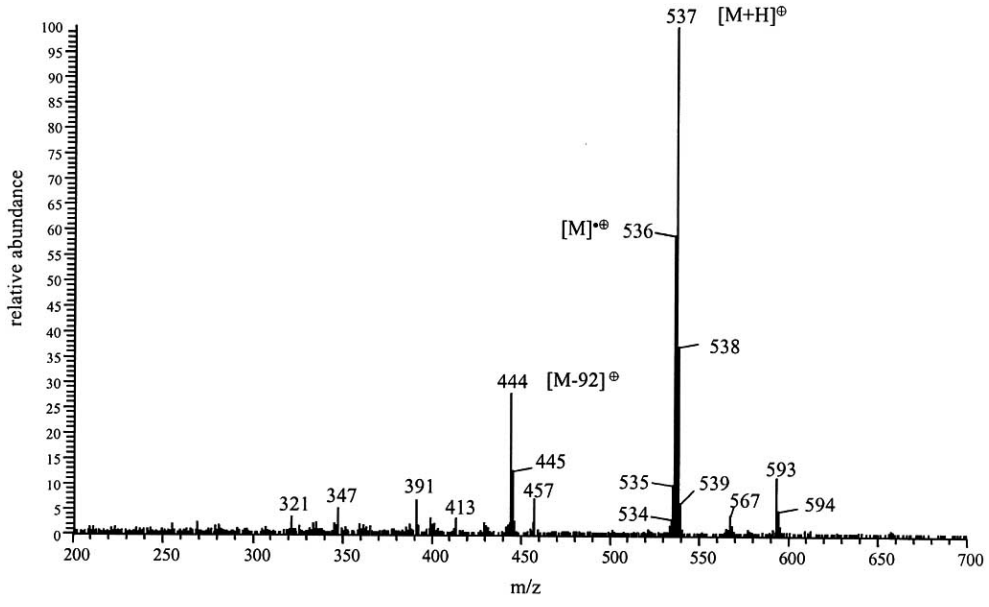

Fig. (7). Positive ion APCI mass spectrum of β-carotene [60].

A total of 18 carotenoids in the flavedo and juice sacs of 39 citrus varieties [64] were identified by LC-MS/APCI. The varieties were classified into 5 clusters, in which the carotenoid profiles were carotenoid-poor, phytoene-abundant, violaxanthin-abundant, violaxanthin- and β-cryptoxanthin-abundant, and phytoene-, violaxanthin-, and β-cryptoxanthin-abundant, respectively. The identification was carried out using LC-DAD-MS on the basis of the comparison of the retention time, mass and UV/Vis absorption spectra, predominantly by monitoring single ions such as $[M+H]^+$ and $[M-H_2O+H]^+$, and co-chromatography with authentic or tentatively identified standards.

Hagiwara *et al.* [65] described the quantitative analysis of carotenoids lycopene, α-carotene, and β-carotene in vegetable juice by RP-LC-MS/APCI on a C18 column using selected ion monitoring (SIM) with cholesteroyl benzoate as internal standard. With methanol as the mobile phase, mass spectra exhibited strong protonated molecular ions with little fragmentation, which were used for quantitative analysis.

A rapid and sensitive method was described for the simultaneous quantitation of β-carotene, β-cryptoxanthin, lutein and zeaxanthin in olive oil, carrot juice, and wheat bran [66]. The samples were identified by normal phase liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry (NP-LC-MS/MS/APCI). The molecular ions $[M]^{\bullet+}$ (electron-loss) in ESI and $[M+1]^{\bullet+}$ in APCI were used for detection of natural samples. The detection using APCI mode was 100 times more sensitive than that with an ESI mode.

The major and minor carotenoids from six fruits, buriti (*Mauritia vinifera*), mamey (*Mammea americana*), marimari (*Geoffrola striata*), peach palm (*Bactrys gasipaes*), physalis (*Physalis angulata*), and tucuma (*Astrocaryum aculeatum*), all native to the Amazonia region, were determined by HPLC-PDA-MS/MS [67]. A total of 60 different carotenoids were separated on a C-30 column, all-*trans*-beta-carotene being the major carotenoid found in all fruits. The presence of apo-10'-β-carotenol, found in mamey was not previously reported in foods. In addition, this was the first time that the identification of β-zeacarotene in natural sources was supported by MS data. MS/APCI spectra of zeinoxanthin and α-cryptoxanthin have been shown. The compounds given in the study were identified by means of their fragmentation in MS, *e.g.* $[M+H]^+$, $[M+H-18]^+$ and $[M+H-18-18]^+$. In addition, the MS/MS showed the presence of fragments at *m/z* 477 and 463, resulting from the respective losses of toluene $[M+H-92]^+$ and xylene $[M+H-106]^+$ or bis-allylic cleavage of the single bonds between the C-7 and C-8 $[M+H-137]^+$ and the C-11 and C-12 $[M+H-205]^+$.

LC-MS/APCI has been established to determine ascorbic acid, lycopene and β-carotene in vegetables and fruits [68]. The detection was based on $(M+H)^+$ ion; lycopene and β-carotene are structural isomers and showed similar mass spectra. The paper contains no MS data.

Gou Qi Zi, i.e. Chinese wolfberry, is the common name for the fruit of two very closely related species *Lycium barbarum* and *L. chinense.* The carotenoid contained in it was identified by APCI [69]. The molecular ions of carotenoids were detected by APCI and three compounds were identified as dipalmityl zeaxanthin, monopalmityl zeaxanthin and zeaxanthin, respectively. Interestingly, the plant material was found to contain only a single xanthophyll in the form of an ester with a single fatty acid.

A reversed-phase C-30 column was used for the baseline separation of eight of the major carotenoids (*trans*-neoxanthin, *trans*-violaxanthin, *cis*-neoxanthin, *trans*-antheraxanthin, astaxanthin, *trans*-lutein, β-apo-caroten-8-al, *trans*-zeaxanthin, *trans*-β-carotene, and 9-*cis*-β-carotene) and the two chlorophylls (a and b) within 18 min from *Arabidopsis thaliana* leaf tissue [70]. These compounds were identified *via* the use of authentic standards, UV/Vis spectral characteristics and LC-MS/APCI$^-$. The details of the APCI analysis were not given; some of the compounds are characterized by their *m/z* values that correspond probably to $[M-H]^+$, as can be deduced from these values and molecular weight.

2.9. Carotenoids from Exotic Sources

In conclusion, it is worthwhile to mention the analysis of carotenoids from exotic sources, live or inanimate, i.e., those that are not commonly accessible. One of these groups includes lower photosynthesizing organisms, especially algae and cyanobacteria. Analysis of esters of xanthophylls is described in a different place.

As indicated by the LC-MS/APCI, aerobic cultivation of *Chlorella protothecoides* in a mineral medium with 70% deuterated water yielded lutein with 58% replacement of hydrogen by deuterium atoms [71]. The study gives the mass spectra as dependent on the time of growth in heavy water.

Blue-green alga *Spirulina platensis* has been analyzed by LC coupled to MS with two different interfaces, APCI and ESI, which allowed the authors to perform tandem MS by using an ion trap analyzer [72]. The compounds identified in the extracts included carotenoids, chlorophylls and their degradation products: siphonein, zeaxanthin, myxoxanthophyll fucoside, astaxanthin, unidentified carotenoids, pheophytin and *o*-allomer, chlorophyll A, pheophytin A, β-carotene and pyropheophytin A. The identification was based on UV spectra, and also on the values of [M+H]+ and main fragments obtained by LC-MS and LC-MS/MS.

Apart from many bacteriochlorophylls (C and D) differing in the degree of alkylation of the macrocycle at C-8 and/or C-12 and the alcohol moiety esterified to the propionic acid group at C-17, the phototrophic bacterium *Chloronema* was found to contain also β- and γ-carotenes [73].

Another group of microorganisms comprises fungi, either yeast that is used for industrial production of carotenoids, or molds. Carotenoid pigments are produced by yeasts belonging to the Basidiomycota, which are of special interest to carotenoid researchers because they are easily grown and manipulated in industry. They also serve as producers of carotenoids of commercial value, predominantly astaxanthin. Red yeast carotenoids belonging to an oxidative series were isolated from frozen wet cells of red yeasts (*Rhodotorula glutinis var. dairenensis, Dioszegia takashimae, Sporobolomyces coprosmae, Sporobolomyces roseus and Xanthophyllomyces dendrorhous*) by RP-HPLC using a C18 column and

a water/acetone solvent system. Nine different carotenoids, *i.e.* astaxanthin, β-carotene, γ-carotene, 2-hydroxytorularhodin, 2-hydroxytorularhodin methyl ester, 2-hydroxytorulene, plectaniaxanthin, torulene, and torularhodin) were separated from the monocyclic β-carotene to 2-hydroxytorularhodin and from the bicyclic β-carotene to astaxanthin [74]. Pigment identity was confirmed by LC–MS/APCI.

The yellow-orange color of aeciospores of the daisy rust fungus, *Puccinia distincta*, was found to be due to the carotenoid pigments γ-carotene and β-carotene, which were identified by means of LC-MS/APCI [75]. The combined concentration of β-and γ-carotene in the aeciospores was $3.3x10^{-15}$ mol $spore^{-1}$, *i.e.* about 2 pg of the two carotenoids, with γ-carotene accounting for 66%. The pigments are likely to play an important role as antioxidants also in these organisms, especially in the protection of sensitive biological molecules from reactive oxygen species, such as free radicals.

Carotenoids were also identified in marine invertebrates such as the precious red coral, *C. rubrum* (Octocorallia: Gorgonacea), one of the most widely known coral species, famous since the ancient time for its beauty and the occult powers supposedly played by its skeleton. By LC/MS, the authors [76] determined the presence of *trans*-canthaxanthin (4,4′-diketo-β-carotene) as the major carotenoid in all samples (in demineralized spicules 4000 μg/g) and probably *cis*-canthaxanthin as the minor carotenoid in tissues and spicules. Another source has been revealed to include protozoans and sediments formed from dead plankton.

The orange-pigmented *Thraustochytrium* was found to contain astaxanthin as the main carotenoid pigment [77]. Echinenone, canthaxanthin, phoenicoxanthin and β-carotene were also identified by HPLC-MS. The total extractable carotenoid level was found to increase with culture age.

Class-specific biomarkers of zooplankton grazing on phytoplankton based on novel esters of carotenols and chlorins (carotenoid chlorin esters, CCEs), which are highly specific grazing markers, were identified in surface and deep sediments from the Louisiana shelf [78]. The biomarkers used included esterification products such as fucoxanthin pheophorbide-α ester, which were partially biodegraded either enzymatically or nonenzymatically. The main reactions taking place in the process were found to be demethoxycarbonylation, carotenoid 3' deacetylation, carotenoid 5'-dehydration or dehydration of fucoxanthinol. A list of the more than 30 identified substances including the graphical depiction of mass spectra is given. The analysis was performed by the HPLC-MS/MS method.

We should not forget to mention a well-known marine microorganism that is a common component of sediments and, because of its unusual content of lipids, has been analyzed many times. The coccolithophorid *Emiliania huxleyi* was found to contain a wide range of acyloxyfucoxanthins with substituents of between four and eight carbons at the C-19' position [79]. Identification by means of LC-MS/APCI and LC/MS^n (n=1-3) was based on $[M+Na-FA]^+$, $[M+Na-FA]^+$, $[M+Na-FA-H_2O]^+$, [M+Na-FA-epoxidated endocyclic ring]$^+$ as major ions.

2.10. Carotenoids as Food Additives

Three studies were concerned with the analysis of carotenoids in samples serving as food additives. Carrot extract in oil was used as the source of carotenoids and other compounds [80]. Unfortunately, the paper does not contain any mention of the use of APCI except the statement that two ions at *m/z* 537 were used for β-carotene and *m/z* 543 for β-carotene-d_6.

Carotenoid food additives such as norbixin, bixin or carotenoids as capsanthin, lutein, canthaxanthin β-apo-8'-carotenal, β-apo-8-carotenoic acid ethyl ester, β-carotene, and lycopene were analyzed on a RP C30 column [81], see Fig. (**8**). The mass spectra were recorded using LC-MS/APCI. Limits of quantitation ranged from 0.53-0.79 mg/l. Individual compounds were identified by means of ions $[M+H]^+$ and $[M+H-H_2O]^+$ and UV/Vis data.

Egg yolk has served as an important dietary source of lutein and zeaxanthin [82]. HPLC-DAD method was developed allowing for simultaneous separation of eight xanthophylls used to fortify poultry feed. Peak identification was carried out by LC-MS/APCI. Lutein and zeaxanthin were the predominant xanthophylls in egg yolk produced in accordance with ecological husbandry because the concentrations of these xanthophylls ranged from 1274 to 2478 μg/100 g and from 775 to 1288 μg/100 g, respectively. Canthaxanthin, β-apo-8'-carotenoic acid ethyl ester, and citranaxanthin were also identified. Experiments with boiled eggs proved that beta-apo-8'-carotenoic acid ethyl ester was the xanthophyll with the highest stability, whereas lutein was degraded to the largest extent. The compounds form intensive $[M+H]^+$, and $[M+H-H_2O]^+$ and also a sodium adduct ion ($[M+Na]^+$ amounting to 40% of base peak.

2.11. Degradation Products of Carotenoids

As a topic related to the analysis of carotenoids in plant material, we present here isolated applications of LC-MS to the analysis of carotenoids in plant material that has been partially modified either thermally or oxidatively. The high-temperature treatment of paprika (*Capsicum annuum* L.) oleoresins yielded oxidation and degradation products, which were isolated semipreparatively and their structure elucidated by EI-MS [83]) and also by LC-MS/APCI.

The thermal and oxidative degradation of vitamin C, two carotenoids (β-carotene and β-cryptoxanthin), and hesperidin, as a function of temperature, were determined for citrus juice (*Citrus sinensis* and *Citrus clementina*) [84]. The HPLC-DAD of degradation products showed that the isomerization of the epoxide function in position 5,6 into a furanoxide function in position 5,8 was a common reaction for several xanthophylls, i.e. auroxanthin, violaxanthin, mutatoxanthin, zeaxanthin, antheraxanthin, zeinoxanthin, β-cryptoxanthin, β- and ζ-carotene.

CONCLUSION

The future of carotenoid analysis can be seen in two areas. The first is the use of new ionization techniques such as, e.g., atmospheric pressure photoionization mass spectrometry, MS/MS combination, and also the use of ultra performance liquid chromatography. The other area offers the possi-

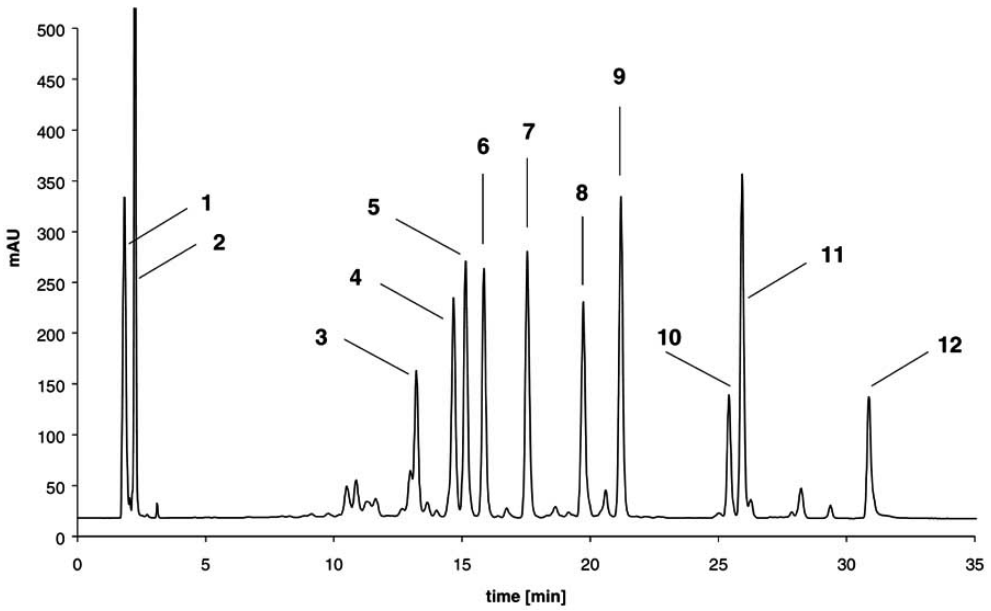

Fig. (8). HPLC chromatogram (DAD, 450 nm) of a CFA standard mixture (spiked plant extract) [81]. For peak assignment, see below. 1-norbixin; 2-bixin; 3-capsanthin; 4-lutein; 5-zeaxanthin; 6-canthaxanthin; 7-β-*apo*-8′-carotenal; 8-β-*apo*-8′-carotenoic acid ethyl ester; 9-echinenon (I.S.); 10-α-carotene; 11-β-carotene; 12-lycopene.

bility of analyzing a much larger spectrum of samples. To our knowledge, LC-MS/APCI has been used for analyzing only carotenoids from common sources that are conspicuous at first sight by being red-colored; many other sources are still unexplored, e.g. fishes other than salmonid fish, milk of most mammals including cows, or various plants, especially those that are not commonly used as food ingredients. Practically completely unexplored are lower organisms (microorganisms) including those that have in their Latin names the word red (ruddy-colored) such as various algae, yeasts, fungi, etc. We foresee explosive development in this area in the next decade.

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ABBREVIATIONS

APCI	=	Atmospheric pressure chemical ionization
CAD	=	Collisionally activated dissociation
CCEs	=	Carotenoid chlorin esters
DAD	=	Diode array detector
EI-MS	=	Electron impact mass spectrometry
ESI	=	Electrospray ionization
FAB	=	Fast-atom bombardment
GC	=	Gas chromatography
GC-MS	=	Gas chromatography with mass spectrometric detection
HPLC	=	High-performance liquid chromatography
HRESI	=	High resolution electrospray ionization
HSCCC	=	High-speed counter-current chromatography
LC-MS	=	Liquid chromatography with mass spectrometric detection
LC-MS-APCI	=	Liquid chromatography-mass spectrometric detection with atmospheric pressure chemical ionization
LC-MS-ESI	=	Liquid chromatography-mass spectrometry with electrospray ionization
LC-MS-FAB	=	Liquid chromatography-mass spectrometry with fast-atom bombardment ionization
LC-NMR	=	Liquid chromatography with NMR detection
MS-MS-ESI	=	Tandem mass spectrometry with electrospray ionization
NP-LC-MS-MS-APCI	=	Normal phase liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization
PDA	=	Photodiode array
RP-HPLC	=	Reversed-phase HPLC
SIM	=	Selected ion monitoring
TIC	=	Total ion current
UV/Vis	=	UV/visible detection

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