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Review

Liquid chromatographic separation of terpenoid pigments in foods and food products

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Abstract

The newest achievements in the use of various liquid chromatographic techniques such as adsorption and reversed-phase thin-layer chromatography and HPLC employed for the separation and quantitative determination of terpenoid-based color substances in foods and food products are reviewed. The techniques applied for the analysis of individual pigments and pigments classes are surveyed and critically evaluated. Future trends in the separation and identification of pigments in foods and food products are delineated.

Keywords: Reviews; Food analysis; Terpenoids; Pigments; Carotenoids

Contents

1. Introduction

Natural pigments such as carotenoids, anthocyanins, chlorophylls and chlorophyll derivatives, etc., are abundant in foods and food products. The concentration of pigments in foods can be measured by various spectroscopic methods [1,2]. These techniques are suitable for the exact determination of the total amount of pigments [3,4]. Information obtained by spectroscopy is generally not sufficient for the quantitative determination of the individual pigment fractions. The exact knowledge of the composition of a pigment mixture may improve the reliability of the prediction of the shelf life of the product and may help the development of adequate technologies preserving pigment composition. As pigments are characteristics for a given food their accurate de-

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termination may contribute to the authenticity control of the product [5].

Liquid chromatographic methods have been extensively employed for the analysis of pigments in foods [6]. A short review has been recently published on the new achievements in the liquid chromatographic analysis of pigments, however, its reduced length did not allow the discussion of the problem in detail [7]. The low operation cost, simplicity, numerous possibilities of detection and simultaneous analysis of a considerable number of samples made thin-layer chromatography (TLC) a frequently used technique in pigment analysis. Earlier results have been previously reviewed [8]. However, the high reproducibility and low detection limit made high-performance liquid chromatography (HPLC) a method of choice in pigment analysis. Multistep gradient elution in TLC [9] and gradient elution in HPLC [10] have increased the separation capacity of the liquid chromatographic analysis of pigments.

As pigments can be strongly bonded to the other constituents of foods and food products many methods have been employed for their extraction. Thus the application of both liquid–liquid extraction [11] and solid-phase microextraction [12] have been reported.

The objectives of this paper are to collect the newest achievements on the field of the chromatographic separation of terpenoid pigments in foods and food products, to describe and critically evaluate the techniques, to compare the benefits and shortcomings of the various chromatographic methods, and to outline the future perspectives in this rapidly evolving field of chromatographic analysis. An excellent review has been published on the chromatographic determination of carotenoids [13]. The aforementioned review did not concentrate on the determination of this class of pigments in foods, therefore, it has been assumed than the publication of this review dealing in detail with the newest results is justified.

2. Carotenoid analysis

Carotenoids are tetraterpenes present in a variety of foods and food products showing many advantageous biological and nutritional effects [14]. Because of the highly conjugated system of double bonds they are effective protectors against photooxidation [15–17] and are precursors of provitamin A [18,19]. Furthermore, it has been suggested that carotenoids play a preventive role against cancer [20,21] and health disease [22]. As it has been established that the isomers of carotenoid show different biological activities [23] chromatographic methods have been developed for the isomeric separation of this class of compounds too. The majority of carotenoids occurs in the nature as esters of several fatty acids [24].

There are a lot of different methods for the extraction of pigments for HPLC analysis. Pigments are generally extracted with acetone and/or methanol avoiding elevated temperature, exposure to light and acidity. Because of the strong binding of pigments to the other components of foods and food products, the extraction step has to be repeated several times to obtain total recovery of pigments. The combined extracts can be further purified by liquid–liquid extraction and preconcentrated by evaporation when it is necessary. To the best of our knowledge modern extraction methods such as supercritical fluid extraction, sonification, etc., have not been employed for sample preparation only the application of microwave-assisted extraction of pigments from paprika (*Capsicum annuum*) powders has been reported [25].

2.1. Thin-layer chromatography

Earlier TLC methods used silica stationary phase and organic mobile phase for the separation of carotenoids [26,27], alumina and diatomaceous earth did not find application in the analysis of such pigments. The development and commercialization of new TLC stationary phases (octyl-, octadecyl-, cyano-, diol-, and aminopropyl silica) highly increased the separation capacity of the TLC technique. The separation of color pigments of paprika has been performed on a considerable number of TLC stationary phases and the results have been compared [28]. Alumina, silica, silica–diatomaceous earth (1:1, m/m), diatomaceous earth, cellulose, polyamide, cyano, diol and amino silica stationary phases have been included in the experiments. Inorganic stationary phases have been also used in

Table 1
Quantitative distribution of *Capsicum annuum* cv. Belrubi carotenoids determined by normal-phase HPLC

Carotenoid	Concentration (g/g fresh mass$\times 10^{-4}$)	Proportion (%)
β-Carotene	2.75	6.72
Cryptocapsin	8.14	19.90
Cryptoflavin	1.09	2.66
β-Cryptoxanthin	0.79	1.93
Antheraxanthin	0.38	0.93
Capsolutein	2.13	5.21
Luteoxanthin	0.85	2.07
Zeaxanthin	1.25	3.06
Mutatoxanthin	1.64	4.01
Capsanthin	12.07	29.51
Capsanthin 5,6-epoxide	2.16	5.28
Violaxanthin	0.98	2.40
Capsorubin	1.79	4.38
Capsorubin isomer	1.38	3.37
Neoxanthin	1.74	4.25
Unidentified pigments (as β-carotene)	1.77	4.32
Total	40.91	100.00
Red pigments	25.54	62.43
Yellow pigments	15.37	37.57

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the reversed-phase (RP) separation mode after impregnating by overnight predevelopment in *n*-hexane–paraffin oil (95:5, v/v). Best separations have been achieved in adsorption TLC on alumina stationary phase with *n*-hexane–chloroform mixtures and in reversed-phase TLC on impregnated diatomaceous earth using water mixed with acetone or tetrahydrofuran.

The retention of 26 carotenoid standards has been measured on silica stationary phase using three different mobile phases [29]. The data clearly showed that carotenoids – even in unesterified form – cannot be separated using isocratic separation modes. The color pigments of chili (*C. fluorescence*) powders have also been separated [30] by using the same TLC techniques as in Ref. [28], and the powders have been tentatively classified according to the distribution of pigment fractions by principal component analysis (PCA) [31]. PCA suggested that the differences in the pigment profiles of chili powders of different origin can be employed for the facilitation of the identification of their origin.

As described by many authors, TLC is mainly used for preliminary examinations to give an indication of the number and variety of carotenoids present and to help in the selection of a suitable separation and purification procedure for the given mixture.

2.2. High-performance liquid chromatography

A wide variety of HPLC methods have found application in the analysis of free and esterified carotenoids. The effective separation of carotenoids can be obtained only by gradient elution. Because of the different absorption maxima of various pigment fractions and the difficulty of identification the application of diode array detection (DAD) [32–34] or mass-selective detection (MS) [35–37] have been extensively applied.

2.2.1. Normal-phase high-performance liquid chromatography

The majority of adsorption HPLC techniques used for carotenoid analysis employed silica stationary phase. Separation of saponified carotenoids was carried out on a silica column (250×4.6 mm I.D., particle size 5 μm) using gradient elution from 95% of light petroleum to 95% of acetone [38]. The

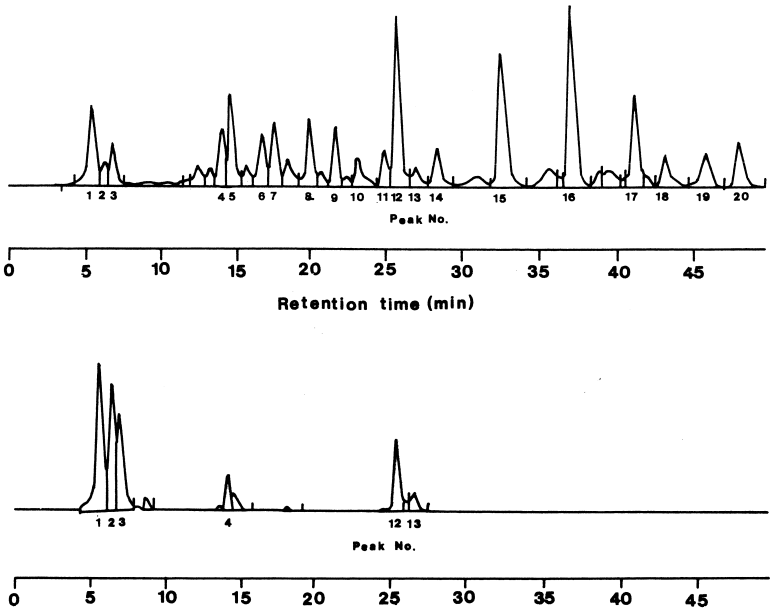

Fig. 1. Reversed-phase HPLC chromatogram of unsaponified (a, top) and saponified (b, bottom) extracts ofcarotenoid pigments from mature fruits of the paprika cultivar Lehava. 1=Capsanthin+capsorubin; 2=unidentified ketocarotenoids; 3=zeaxanthin+lutein; 4=β-cryptoxanthin; 5–7=monoesters of capsanthin and capsorubin; 8–10=monoesters of zeaxanthin and lutein; 11=unidentified; 12=all-*trans*-β-carotene; 13=13-*cis*-β-carotene; 14–17, 19, 20=diesters of capsanthin and capsorubin; 18=diester of lutein. Reprinted with permission from Ref. [44].

concentrations of the individual carotenoid fractions are compiled in Table 1. The pigment composition of various cultivars of *C. annuum* has been measured using the same method [39]. It was suggested that the pigment composition can be used for the identification of cultivars.

An alumina stationary phase has also been applied in the HPLC analysis of color pigments of *C. annuum* [40]. Separation was carried out in an alumina column (250×4 mm I.D., particle size 5 μm) using gradient elution. It was established that the separation capacity of alumina is similar to that of silica stationary phase (similar number of separated fractions), however, the pigment profiles were different on alumina and silica stationary phases indicating different retention mechanism.

Adsorption HPLC has also been employed for the separation of 15-*cis*-β-carotene, 13-*cis*-β-carotene, all-*trans*-β-carotene, and 9-*cis*-β-carotene [41]. Isomers were separated on a column packed with $Ca(OH)_2$ with an isocratic mobile phase consisting of hexane–acetone (99.2:0.8, v/v).

The separation of the color pigments of *C. annuum* have been performed on silica and octadecylsilica (C_{18}) columns under optimal conditions and the number of separated fractions was compared. It was found that silica and C_{18} stationary phases separated 25 and 34 pigment fractions, respectively, proving the superiority of reversed-phase separation mode [42].

The possibility of the application of preparative normal-phase HPLC for the separation of carotenoids from palm oil has been studied in detail [43]. Glass columns of 42±1 cm length were filled with silica (70–230 mesh), carotenoids were eluted with *n*-hexane while other components were eluted with ethyl acetate. It was found that the first fraction containing about 20% (m/m) of carotenoids can be used in commercial concentrates while the quality of palm oil is upgraded.

2.2.2. Reversed-phase high-performance liquid chromatography

The overwhelming majority of RP-HPLC sepa-

rations has been performed on C_{18} stationary phases using water and one or more organic solvents miscible with water.

RP-HPLC has been employed for the analysis of carotenoid pigments in paprika fruits with different genotypes [44]. Unsaponified pigments were extracted in the dark by shaking 50 mg powder with 50 ml of acetone for 10 min. Saponification was carried out by mixing 500 mg of paprika powder with 7.5 ml of absolute ethanol containing 2% 2,6-bis(1,1-dimethylethyl)-*n*-methyl-phenol (BHT) and with 1.25 ml of KOH (60%). Suspension was thermostated at 60°C for 25 min under continuous nitrogen flow. Separations were performed on an end-capped C_{18} column (250×3.4 mm I.D.) and on a C_{18} column (125×3.4 mm I.D.) coupled in series (particle size for both columns 5 μm). The constituents of mobile phase used for the gradient elution were acetonitrile–2-propanol (40:60, v/v, A) and water (B). Typical chromatograms of esterified and free carotenoids are shown in Fig. 1. The RP-HPLC method separated more than 30 pigment fractions and the fractions were chromatographically pure according to the peak purity test proving the good separation efficiency of the method. The concentrations of major carotenoids are compiled in Table 2. The data indicate that the carotenoid content of species and lines of *Capsicum* show considerable differences, however, the ratio of pigment fractions is similar.

Another study used C_{18} stationary phase and various mobile phases for the separation of the color pigments of paprika after saponification [45]. It was established that the saponification process did not exert a significant influence on the concentration of pigments the loss being lower than 5%. Separation were performed on a C_{18} column (250×4 mm I.D., particle size 5 μm) using gradient elution. The chromatogram of pigments obtained under optimal

Table 2
Carotenoid content (micrograms per gram of dry mass) in the fruits of various species and lines of *Capsicum*

Line No.	Species	A	B	C	D	E	F	G
4002	*C. baccatum*	1420	60	40	126	35	296	275
4005	*C. chinense*	4000	144	68	288	108	960	1172
4015	*C. frutescens*	1310	37	14	117	45	225	327
4017	*C. chacocense*	430	21	8	26	12	83	123
4018	*C. chacocense*	1000	52	17	81	31	195	227
4101	*C. annuum*	3250	117	55	254	88	672	862
4105	*C. annuum*	8790	659	281	580	211	1881	1881
4107	*C. annuum*	8070	331	152	581	234	1614	2212
4108	*C. annuum*	2950	103	50	145	62	589	856
4113	*C. annuum*	3570	182	75	136	68	700	996
4115	*C. annuum*	7900	561	213	419	166	1596	1968
4116	*C. annuum*	7460	216	104	455	209	1492	2373
4117	*C. annuum*	5480	137	77	345	170	1008	1557
4119	*C. annuum*	8850	566	212	681	257	2106	2125
4123	*C. annuum*	10 710	396	193	728	311	2334	2828
4126	*C. annuum*	16 600	714	299	1643	614	3518	3403
4128	*C. annuum*	10 370	581	218	840	332	2178	2374
4135	*C. annuum*	8040	249	113	410	193	1719	2420
4136	*C. annuum*	7360	132	66	493	177	1486	2421
4140	*C. annuum*	5670	187	108	323	85	1203	1916
	Mean	5886.4	260.0	112.8	414.2	162.9	1234.5	1543.6
	Standard deviation	4140.8	227.0	90.3	368.2	141.5	892.7	988.4
	Range	390–1660	14–714	5–299	26–1643	12–614	70–3518	100–3403
	RSD (%)	70.3	87.3	80.0	88.9	86.8	72.3	64.0

A=Total carotenoids; B=free capsanthin+capsorubin; C=free zeaxanthin+lutein; D=*trans*-β-carotene; E=*cis*-β-carotene; F=monoesters; G=diesters. Reprinted with permission from Ref. [44].

conditions is shown in Fig. 2. It was stated that the RP-HPLC method combined with the evaluation of data by PCA may help the characterization of paprika and the detection of adulteration (presence of exogene colorants).

A different RP-HPLC method has been employed for the study of the stability of the color pigments of paprika powder during various processing steps [46]. Separation was performed on a C_{18} column (250×4 mm I.D., particle size 5 μm) using DAD between 340 and 560 nm. It was established that the technological steps exert a negligible influence on the composition of the color pigments of paprika powders.

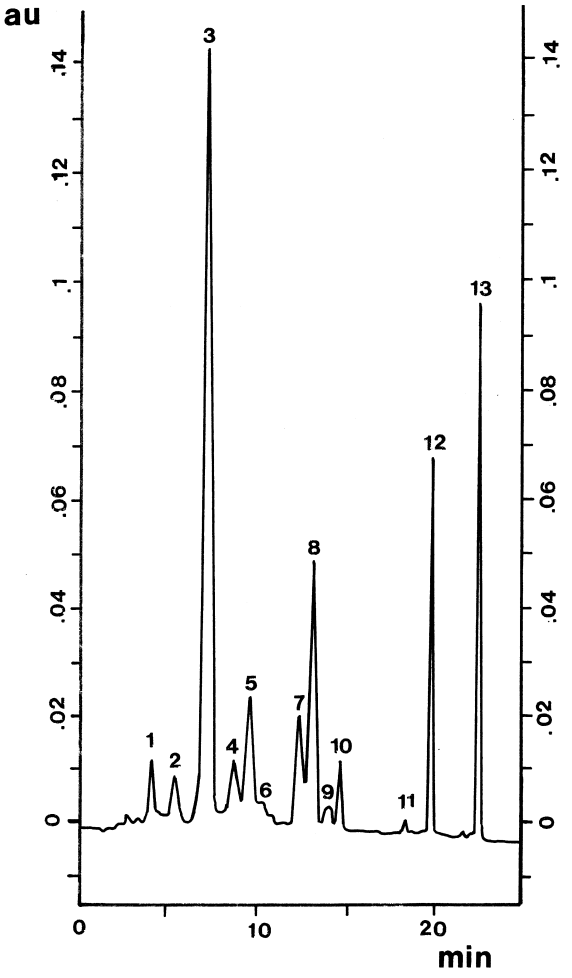

Fig. 2. Separation of color pigments of paprika under optimal RP-HPLC conditions. Identification of peaks: 1=capsorubin; 2=violaxanthin; 3=capsanthin+capsanthin epoxide; 4=antheraxanthin; 5=*cis*-capsanthin; 6=mutatoxanthin; 7=lutein; 8=zeaxanthin; 9=*cis*-zeaxanthin; 10=unknown; 11=cryptoxanthin; 12=β-cryptoxanthin; 13=β-carotene+*cis*-β-carotene. Reprinted with permission from Ref. [45].

RP-HPLC combined with a spectral mapping technique [47] has also been used for the study of the effect of storage conditions (time of storage in light or in dark) and the addition of seeds on the stability of the color pigments of paprika [48]. Separation of pigments was performed on an end-capped C_{18} column (250×4 mm I.D., particle size 10 μm) using gradient elution. The amount of each pigment fraction decreased during the experiment. Calculations proven that the length of storage exerted the highest effect on the decomposition rate of pigments, and the concentration of seeds added to the paprika powder influenced only the selectivity of decomposition. It was further established that the lipophilicity of pigments did not influence the decomposition rate. The same RP-HPLC and calculation method have been employed for the comparison of the pigment profile of paprika powders of various origin (two species from Hungary and South America, one species from Spain, South Africa, and Portugal) [49]. The composition of pigments showed considerable variations according to the origin of the product. It was suggested that the calculation of the similarities and dissimilarities among the pigment composition of paprika powders using the 15 most characteristic pigment fractions may increase the reliability of the authenticity test of commercial paprika powders.

RP-HPLC–DAD has been employed for the separation of the color pigments of a chili powder [34]. Separations were carried out on a C_{18} column (150×4 mm I.D., particle size 5 μm). Mobile phase consisted of various mixtures of methanol–acetonitrile (80:20, v/v) and water. The chromatograms indicated that the pigments of chili powder can be well separated under the RP-HPLC conditions applied. It has been further concluded that chili powder – in contrast to paprika powder – contains more yellow than red pigments.

Not only the pigments of *Capsicum* species but also the carotenoid composition of other food products has been measured by RP-HPLC. The results of a collaborative study including 17 laboratories for the RP-HPLC determination of carotene and retinol in 10 food products have been reported [50]. Analyses were performed on a C_{18} column (250×4 mm I.D., particle size 10 μm). Retinol was eluted by

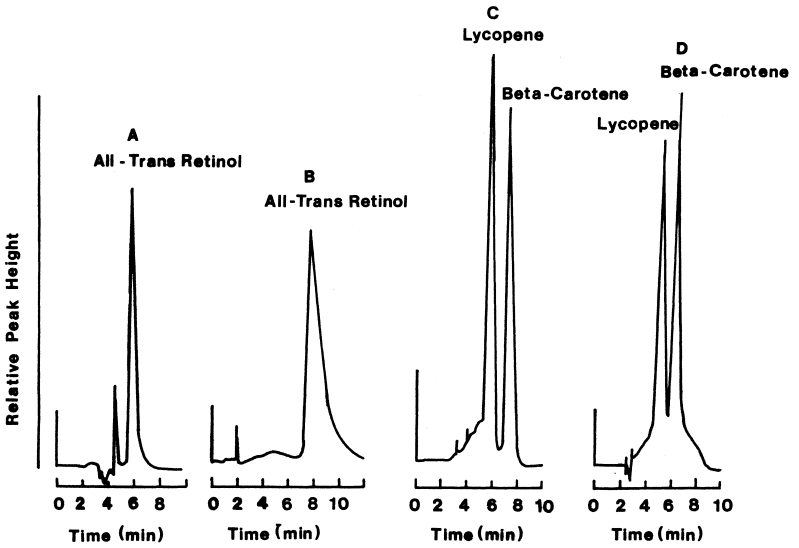

Fig. 3. Chromatograms of all-*trans*-retinol, β-carotene and lycopene. (A) All-*trans*-retinol in hexane at 325 nm. (B) All-*trans*-retinol in methanol at 325 nm. (C) Lycopene and β-carotene in hexane at 436 nm. (D) Lycopene and β-carotene in hexane at 450 nm. Reprinted with permission from Ref. [50].

methanol–water (90:10, v/v), detection wavelengths 325 or 313 nm. Mobile phase for the measurement of carotene consisted of acetonitrile–methylene chloride–methanol–water (70:20:8:2, v/v). Carotene was detected at 450 or 436 nm. Some typical chromatograms are collected in Fig. 3. The mean values and standard deviations of non-outliers are compiled in Table 3. The data indicated that the results of the official AOAC method are higher than those measured by RP-HPLC. The repeatability (within laboratory) of the RP-HPLC method was 3.46–15.65%, and the reproducibility ranged between 5.34 and 15.77%.

The loss of carotenoids during the saponification process has been studied in detail [51]. Pigments were separated on a C_{18} column (250×3.9 mm I. D., particle size 5 μm) using gradient elution. Chromatograms of unsaponified and saponified carotenoids are shown in Fig. 4. The chromatograms illustrate the good separation capacity of the RP-HPLC method

Table 3
Total activity (IU/g)[a] by HPLC and by AOAC chemical method

Commodity	HPLC	AOAC	RD[b]
Cereal	185.314±17.445	242.20	−23.49
Powdered infant formula	19.374±0.670	20.140	−3.81
Chicken feed	2.427±0.222	2.320	+4.61
Pureed carrots	74.388±9.81	120.00	−38.01
V-8 juice	6.987±0.534	12.57	−44.42
Low fat milk	2.688±0.230	3.540	−24.07
Baby food squash A	16.163±2.517	17.610	−8.22
Baby food squash B	17.53±2.763	17.120	−2.39
β-Carotene capsule	11 035±1138	11 867	−7.01
Retinol capsule	11 017±1199	11 796	−6.60

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[a] Sum of retinol and carotene.
[b] RD (relative difference)=100×(method value−AOAC value)/AOAC value.

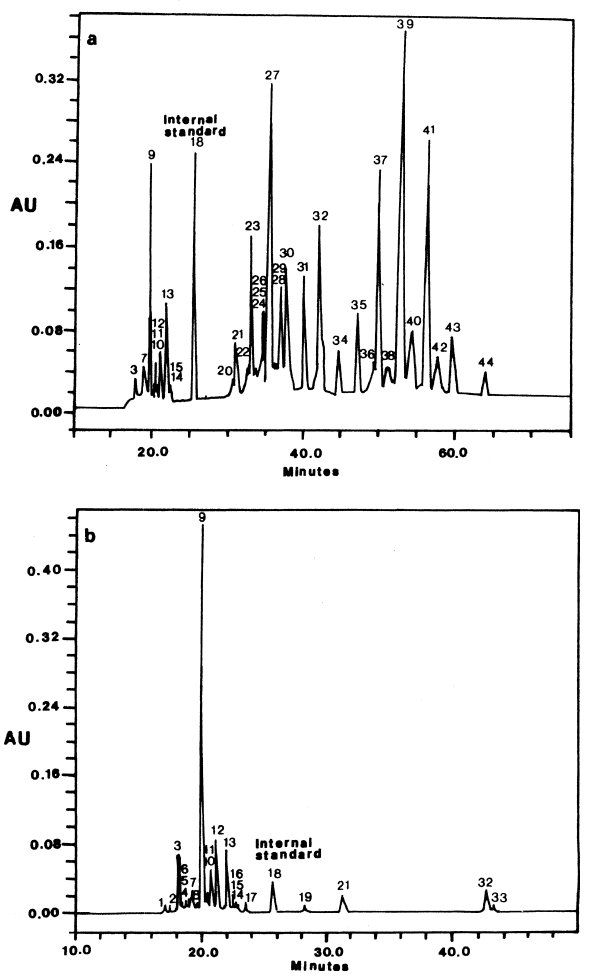

Fig. 4. Reversed-phase HPLC separation of (a) Sobrasada extract and (b) saponified Sobrasada extract in a Spherisorb C_{18} column, at maximum absorbances at each point in time. For peak numbers see Table 4. Reprinted with permission from Ref. [51].

and prove again that the majority of carotenoids are in esterified form under natural conditions. The concentration of free and esterified carotenoids are compiled in Table 4. The data indicated that saponification led to a considerable underestimation of some pigments in the fatty Sobrasada sausage.

A C_{18} column (150×4.6 mm I.D., particle size, 3 μm) has been employed for the qualitative analysis of retinoids in galenicals [46]. Analytes were detected by DAD and by fluorescence and the sensitivity of the detection method has been compared. Solutes were detected at 350 nm by DAD (β-carotene: 450 nm), emission wavelength for fluorescence detection was 520 nm, excitation wavelength was 350 nm for retinoic acid isomers and vitamin A palmitate and 450 nm for β-carotene. Chromatograms obtained with DAD and fluorescence detection are shown in Fig. 5. The chromatograms clearly show that the detection methods influences considerably the sensitivity of the analysis. The relative standard deviation (RSD) of the method was low (1.2–3.2%) showing the good reproducibility of the

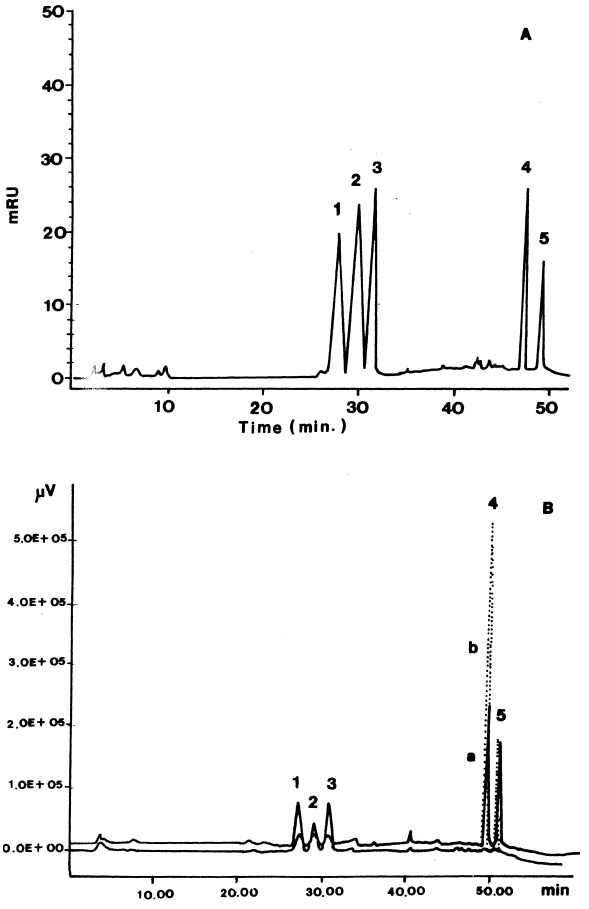

Fig. 5. Gradient LC separation of the ''retinoid solution'' components and retinoic acid isomers by (A) UV–DAD (350 nm) and (B) fluorescence detection with on-line photoreactor switched (a) off and (b) on with irradiation at 366 nm. LC conditions: 3 μm C_{18} column (150×4.6 mm I.D.), with a mixture of A–B, where A is methanol–10 m*M* ammonium acetate (75:25, v/v) and B is methanol–tetrahydrofuran (84:16, v/v) as mobile phase; flow-rate, 0.8 ml/min. Peaks: 1=13-*cis*-retinoic acid; 2=9-*cis*-retinoic acid; 3=all-*trans*-retinoic acid; 4=vitamin A palmitate; 5=β-carotene. Reprinted with permission from Ref. [46].

Table 4
Carotenoid composition of non-saponified and saponified Sobrasada extract and retention time for each pigment resolved

Peak pigment No.	t_R[a] (min)	Carotenoid composition (μg/g dry mass)[b]	
		Non-saponified extract	Saponified extract
1 Unidentified free 1	17.1	n.d.	0.26±0.02
2 Unidentified free 2	17.4	n.d.	0.25±0.02
3 Capsorubin	18.1	0.29±0.04	7.4±0.4
4 Unidentified free 3	18.4	n.d.	0.49±0.06
5 Unidentified free 4	18.6	n.d.	0.65±0.04
6 Unidentified free 5	18.9	n.d.	0.67±0.03
7 Violaxanthin	19.2	0.41±0.09	2.2±0.1
8 Unidentified free 6	19.6	n.d.	0.36±0.03
9 Capsanthin	19.9	6.8±0.3	60±3
10 Anteraxanthin	20.4	0.13±0.02	1.5±0.1
11 *cis*-Capsanthin	20.8	1.1±0.02	7.7±0.5
12 Unidentified free 7	21.2	0.74±0.06	5.7±0.3
13 Lutein and zeaxanthin	22.0	1.7±0.1	5.0±0.3
14 Unidentified free 8	22.5	0.12±0.03	0.44±0.04
15 Unidentified free 9	22.7	0.12±0.03	0.48±0.05
16 Unidentified free 10	22.9	n.d.	0.18±0.07
17 Unidentified free 11	23.5	n.d.	1.1±0.1
18 Cantaxanthin, I.S.	25.8		
19 Cryptocapsin	28.4	n.d.	0.65±0.02
20 Unidentified monoester 1	30.8	0.15±0.07	n.d.
21 β-Cryptoxanthin	31.5	1.3±0.1	1.8±0.1
22 Capsorubin monoester	32.6	0.44±0.12	n.d.
23 Capsanthin monoester 1	33.0	10±1	n.d.
24 Unidentified monoester 2	33.9	0.24±0.10	n.d.
25 Unidentified monoester 3	34.3	0.33±0.10	n.d.
26 Capsanthin monoester 2	34.6	4.9±0.4	n.d.
27 Capsanthin monoester 3	35.5	22±1	n.d.
28 Unidentified monoester 4	36.5	0.30±0.13	n.d.
29 Capsanthin monoester 4	37.1	8.1±0.4	n.d.
30 Lutein-zeaxanthin monoester 1	37.7	4.1±0.3	n.d.
31 Lutein-zeaxanthin monoester 2	40.3	2.3±0.1	n.d.
32 β-Carotene	42.8	5.1±0.2	2.3±0.1
33 *cis*-β-Carotene	43.4	n.d.	0.31±0.03
34 Capsanthin diester 1	45.0	2.2±0.1	n.d.
35 Capsorubin diester	47.4	3.6±0.1	n.d.
36 Unidentified diester 1	49.5	0.72±0.11	n.d.
37 Capsanthin diester 2	50.2	15±0.3	n.d.
38 Unidentified diester 2	51.5	0.74±0.33	n.d.
39 Capsanthin diester 3	53.3	30±1	n.d.
40 Unidentified diester 3	54.8	2.7±0.2	n.d.
41 Capsanthin diester 4	56.7	20±0.3	n.d.
42 Unidentified diester 4	58.4	1.4±0.1	n.d.
43 Capsanthin diester 5	60.2	4.6±0.2	n.d.
44 Unidentified diester 5	64.6	0.54±0.06	n.d.

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[a] Retention time of each peak.
[b] Data are given as mean±standard error of the mean (n=5; n.d.: not detected).

RP-HPLC system. It was concluded from the data that the method is selective, sensitive and the validation parameters are acceptable. It was proposed for the routine analysis of these drugs.

An octadecylsilica stationary phase has also found application in the study on the intestinal absorption of epoxy-β-carotenes in humans [52]. Analyses were carried out on a C_{18} column (100×3.6 mm I. D., particle size 3 μm). Analytes were detected by DAD. Typical chromatograms illustrating the change of concentration of carotenoids in human plasma are shown in Fig. 6. The results indicated that epoxy-β-carotenes are absorbed readily by humans, therefore, they possibly have some beneficial effect in reducing risk of cancer.

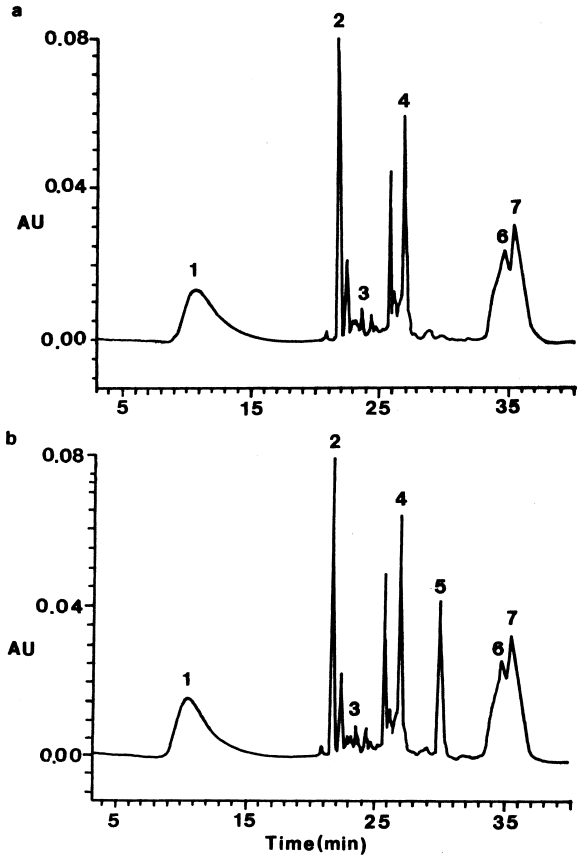

Fig. 6. Reversed-phase gradient HPLC profiles of carotenoids in human plasma. A human volunteer was given an oral dose of 5,6-epoxy-β-carotene (9.1 μmol). Plasma was analyzed for carotenoids before (a) and 6 h after (b) the oral dose. Peak identification: 1, bilirubin; 2, lutein; 3, zeaxanthin; 4, β-cryptoxanthin; 5, lycopene; 6, β-carotene. The detection wavelength was 445 nm. AU, absorbance units. Reprinted with permission from Ref. [52].

Not only octadecyl but also a C_{30} stationary phase has found application in the separation and quantitative determination of carotenoids. Thus, the RP-HPLC separation of orange juice carotenoids in a C_{30} stationary phase with DAD has been reported [53]. Carotenoids were separated on a polymeric C_{30} column (250×4.6 mm I.D.) with a mobile phase gradient. The chromatograms of saponified carotenoids detected at 350, 430 and 485 nm are shown in Fig. 7. It was found that this RP-HPLC method is suitable for the separation of 39 carotenoids in orange juice. The validation parameters of the method were good, the RSD of retention times and peak areas varied between 0.09 and 2.29% and 5.5–7.7%, respectively.

The combination of adsorption and RP separation modes have been frequently used for the separation and quantitative determination of carotenoids in various foods and food products. They employ typical RP stationary phases such as C_{18} and C_{30} but the mobile phases do not contain water which is the prerequisite of RP separation. Thus, an isocratic non-aqueous technique has been employed for the separation of capsanthin and capsorubin in red peppers and oleoresin [54]. Carotenoids were separated on a C_{18} column (250×4 mm I.D., particle size 5 μm). Isocratic mobile phase consisted of acetonitrile–2-propanol–ethyl acetate (80:10:10, v/v). The flow-rate was 0.8 ml/min, pigments were detected at 450 nm. Good linear relationships were found between the peak area and the concentration of capsorubin (10–100 ng range) and capsanthin (10–80 ng range). It has been established that good recoveries can be obtained by the method. The reproducibility was 3.0 and 2.2% for capsorubin and capsanthin, respectively. It was found that the method is rapid, suitable for the quantitative determination of capsanthin and capsorubin in plants and other food products.

The isomers of β-carotene in tomato juice have been determined on a C_{18} stationary phase using organic mobile phase [55]. Isomer separation was performed on a C_{18} column (250×4.6 mm I.D., particle size 5 μm). The mobile phase consisted of methanol–acetonitrile–2-propanol (54:44:2, m/m/m). Separations were carried out at different column

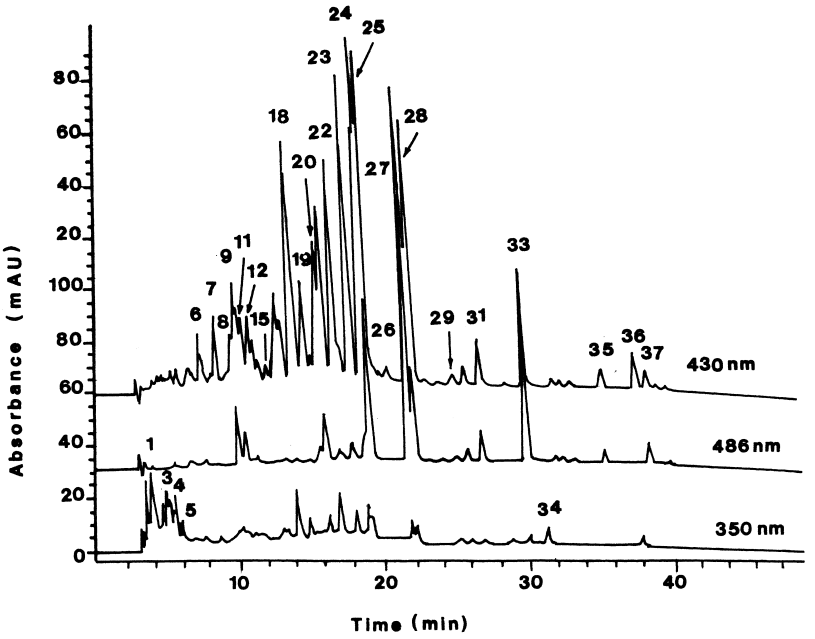

Fig. 7. Saponified carotenoids in orange juice. Peaks are labeled and identified in Table 7. Chromatographic conditions are given in the text. Chromatograms from absorbance monitoring at 430, 486, and 350 nm, are shown, all at identical attenuations. Peak identification: 1–3, 5, 8, 10, 13–17, 21, 26, 29, 30, 32, 38–39=unidentified; 4=valenciaxanthin; 6=neochrom; 7=tollichrom; 9=antherxanthin; 11=*cis*-antherxanthin; 12=neoxanthin; 18=auroxanthin A; 19=auroxanthin B; 20=*cis*-violaxanthin; 22=leutoxanthin; 23=mutatoxanthin A; 24=mutatoxanthin B; 25=lutein; 27=zeaxanthin; 28=isolutein; 31=α-cryptoxanthin; 33=β-cryptoxanthin; 34=phytofluene; 35=α-carotene; 36=ζ-carotene; 37=β-carotene. Reprinted with permission from Ref. [53].

temperatures (from −7 to +30°C) and different flow-rates 0.8–2.0 ml/min). It was found that column temperature exerts a considerable effect on the retention time and elution order as demonstrated in Table 5. Adequate separation of enantiomers can be achieved only at low column temperatures. The method has been proposed for the routine analysis of β-carotene and lycopene isomers in tomato-based products.

The carotenoid content of tomato paste, tomato sauce and tomato soup has been measured using a similar method [56]. Carotenoids were separated on a C_{18} column (250×4.6 mm I.D., particle size 5 μm). Mobile phase was acetonitrile–methanol–di-

Table 5
Retention times of lycopene and four β-carotene isomers, using different column temperatures between 0 and 30°C, flow-rate 2.0 ml/min (further chromatographic conditions see text)

Temperature (°C)	Retention time (min)				
	Lycopene	(*E*)-β-Carotene	9(*Z*)-β-Carotene	13(*Z*)-β-Carotene	15(*Z*)-β-Carotene
+30	12.77	16.00	17.43	18.43	18.43
+25	16.21	19.19	21.28	22.13	22.13
+20	26.64	26.64	30.71	30.71	30.71
+15	41.47	36.03	41.47	41.47	41.47
+10	80.14	55.45	65.72	60.74	60.74
+5	159.04	86.99	105.44	92.13	86.99
0	293.73	133.25	164.16	133.25	124.27

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chloromethane–hexane (85:10:2.5:2.5, v/v) for 0–10 min, then changed to acetonitrile–methanol–dichloromethane–hexane (45:10:22.5:22.5, v/v) between 10 and 40 min. A typical chromatogram is shown in Fig. 8. indicating that pigments are well separated under the HPLC conditions, therefore, the method can be employed for study of antioxidants and their mode of action in diet and health. The carotenoid compositions of some food products are compiled in Table 6. The same technique has been previously employed for the determination of the pigment composition tomatoes and green vegetables [57].

The β-carotene content of some green leafy vegetables has also been measured by HPLC [58]. Fresh samples were extracted with acetone followed by hexane. Overnight saponification was carried out with 5% KOH then the suspensions were extracted with light petroleum (b.p. 40–60°C). Total carotenoids were determined with visible spectrophotometry, β-carotene was separated on a C_{18} column (250×4.6 mm I.D., particle size 5 μm), isocratic mobile phase consisting of acetonitrile–methanol–dichloromethane (90:8:2, v/v). It has been stated that the results can be successfully used for dietary planning.

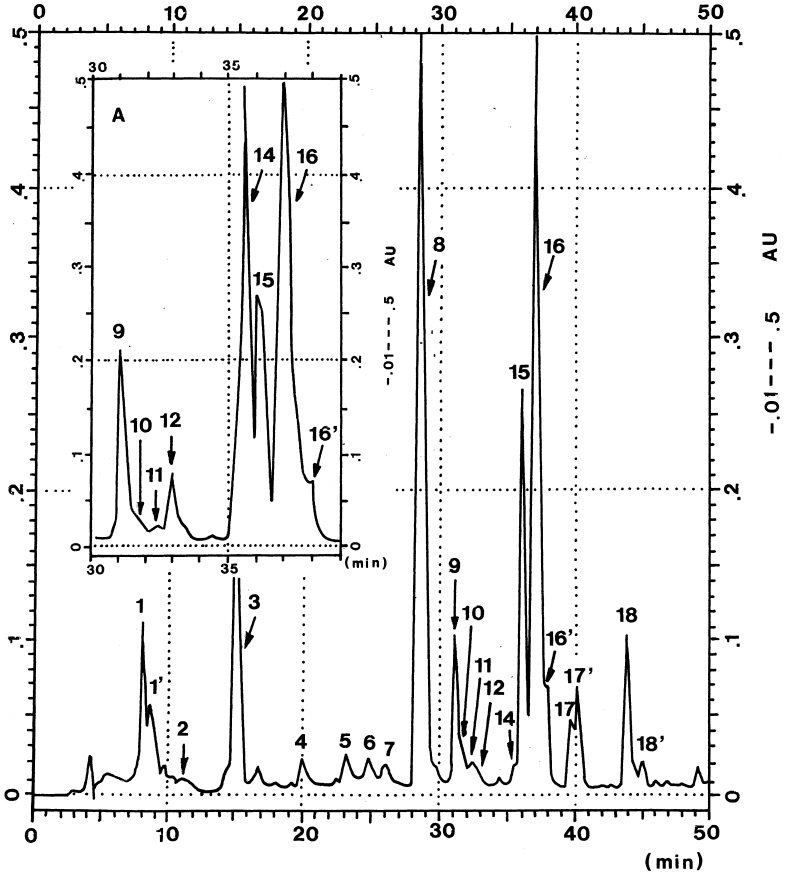

Fig. 8. HPLC profile of carotenoids in an extract of vegetarian vegetable soup. HPLC conditions are described in the text. An expansion of the profile from 30 to 39 is shown in the inset. Peak identification: 1+1′=all-*trans*-lutein and *cis*-lutein; 2=5,6-dihydroxy-5,5-dihydrolycopene (lycopene-5,6-diol); 3=β-apo-8′-carotenal (I.S.); 4=lycopene 1,2-epoxide; 5=lycopene 5,6-epoxide; 6=1,2-dimethoxy-prolycopene (tentative identification); 7=5,6-dimethoxy-5,6-dihydrolycopene; 8=lycopene; 9=pheophytin *b*; 10=neurosporene; 11=τ-carotene; 12=pheophytin *a*; 14=pheophytin *a* isomer and ζ-carotene; 15=α-carotene; 16+16′=all-*trans*-β-carotene and *cis*-β-carotene; 17+17′=all-*trans*- or *cis*-phytofluene; 18+18′=all-*trans*- or *cis*-phytoene. Reprinted with permission from Ref. [56].

Table 6
Carotenoids (mg per 100 g) in tomato-based soups, tomato juice, vegetable juice, and the quality control sample

Sample	α-Carotene	β-Carotene	τ-Carotene	ζ-Carotene	Lutein	Lycopene	Neurosporene	Phytoene	Phytofluene	Lycopene 5,6-diol
Tomato soup										
Mean±SD	nd	0.23±0.047	1.95±0.41	0.17±0.016	0.09±0.02	10.92±2.92	1.53±0.20	1.72±0.172	0.72±0.176	0.11±0.03
RSD (%)		20	21	9.5	21	27	13	10	25	29
N	6	6	4	6	6	6	4	6	6	6
Vegetable beef soup										
Mean±SD	0.46±0.05	1.51±0.29	C	C	0.11±0.03	0.31±0.034	C	0.35±0.06	0.79±0.02	tr
RSD (%)	11	19			28	11		16	10	
n	4	4	4	4	4	4	4	4	4	4
Minestrone soup										
Mean±SD	0.21±0.12	0.92±0.39	C	C	0.15±0.08	1.48±0.83	C	0.28±0.11	0.17±0.09	nd
RSD (%)	56	43			53	56		38	52	
n	5	5	5	5	5	5	5	4	4	5
Vegetarian veg soup										
Mean±SD	0.41±0.18	1.50±0.37	C	C	0.16±0.04	1.93±0.51	C	0.60±0.07	0.31±0.04	tr
RSD (%)	44	25			26	26		12	13	
n	5	5	5	5	5	5	5	4	5	5
Tomato juice										
Mean±SD	nd	0.27±0.04	1.74±0.20	0.18±0.003	0.06±0.02	10.77±1.07	1.23±0.27	1.90±0.19	0.83±0.19	0.11±0.03
RSD (%)		13	11	19	24	10	22	10	17	29
n	6	6	6	6	6	6	6	6	6	6
Vegetable juice										
Mean±SD	0.21±0.03	0.83±0.14	C	C	0.08±0.02	9.66±0.12	C	1.71±0.16	0.69±0.07	0.05±0.01
RSD (%)	14	17			21	1		9	10	10
n	3	3	3	3	3	3	3	3	3	3
Quality control (vegetable juice)										
Mean±SD	0.25±0.01	0.91±0.11	C	C	0.09±0.01	6±0.53	c	1.69±0.07	0.73±0.037	0.08±0.01
RSD (%)	5	12			9	6		4	5	12
n	9	9	9	9	9	9	9	9	9	9

SD=standard deviation; nd=not detected; RSD=relative standard deviation; *n*=number of values used to calculate the mean and SD; C=coeluted with pheophytins; tr=trace, below 0.005 mg/100 g. Reprinted with permission from Ref. [56].

The separation and identification of carotenoids of vegetable juices was carried out on a C_{30} stationary phase using DAD and atmospheric pressure chemical ionization (APCI) mass spectrometry [54]. Pigments were extracted by mixing 2 ml of juice with 1 ml of methyl *tert*.-butyl ether (MTBE) and the organic phase directly injected into the column (250×4.6 mm I.D., particle size 3 μm). The mobile phase was a mixture of methanol–MTBE (70:30, v/v), the flow-rate was 1 ml/min. Pigments were detected in the range of 320–700 nm. Mass spectra were taken between *m/z* 200–700 for the determination of detection limit, for the analysis of carotenoid mixture and vegetable juice, and between *m/z* 80–1000 for the detection of β-carotene isomers. It was established that the detection limit of the method is 1 pmol β-carotene. It was concluded from the results that the HPLC–APCI-MS method is rapid, separates well carotenoids even in complicated matrices without time-consuming sample preparation procedure.

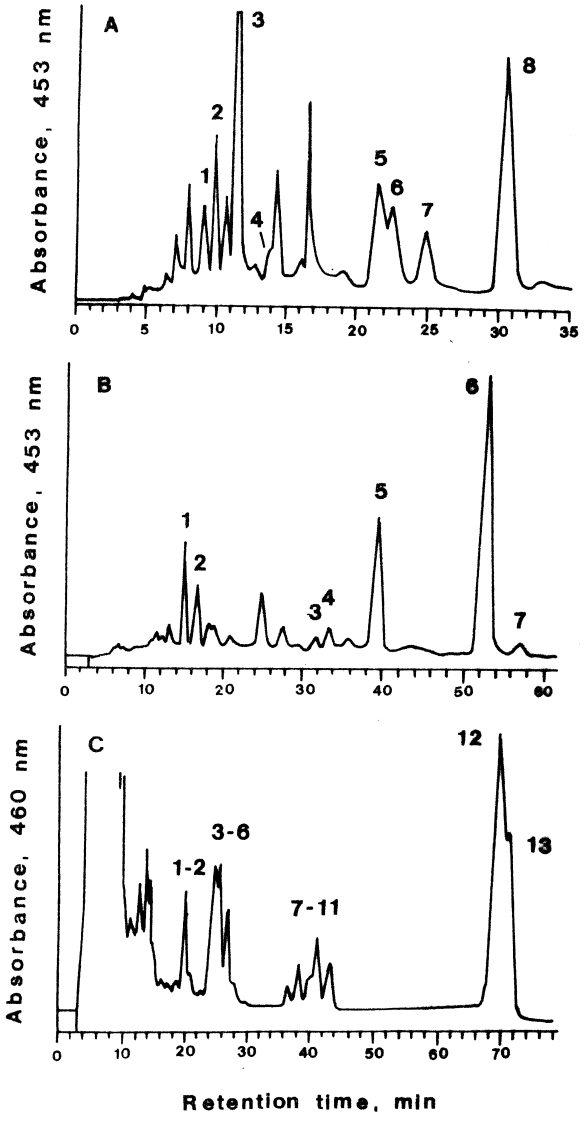

Fig. 9. Comparison of the separation of carotenoid stereoisomers on a C_{30} column. (A) (Spinach): 0–21 min acetone–water (86:14, v/v); 21–25 min linear gradient; 25–40 min, acetone–water (97:3, v/v); (B) (ox-retina); 0–30 min acetone–water (86:14, v/v); (C) (isomerized standard mixture of zeaxanthin and lutein): 0–30 min acetone–water (86:14, v/v). For other chromatographic conditions see text. Reprinted with permission from Ref. [55].

Not only MS but also nuclear magnetic resonance (NMR) coupling has been employed for the analysis of carotenoid isomers in spinach and in retina [55]. Separations were performed on a C_{30} column (250× 4.6 mm I.D., particle size 3 μm) using both isocratic and gradient elution of acetone–water. Column was thermostated at 25°C. Flow-rate was 1 ml/min, carotenoids were detected at 450 nm, and with MS and NMR.

Carotenoids from the samples of spinach and ox-retina were extracted and preconcentrated by solid-phase extraction (SPE). Chromatograms illustrating the separation of carotenoids from spinach, and ox-retina are shown in Fig. 9. The chromatograms indicate that the method is suitable for the separation of the stereoisomers of carotin, lutein and zeaxanthin. The concentration of various carotenoid stereoisomers found in spinach samples are compiled in Table 7. It was found that the method can be employed for the separation of carotenoid stereoisomers in spinach and retina.

The geometrical isomers of carotenoids have been separated using a similar method [59]. Pigments have been extracted from human serum, raw and thermally processed carrots (116°C, 75 min), an algae preparation and poultry feed supplement. Extracts were dried over anhydrous sodium sulfate and carotenoids were preconcentrated on a neutral alumina SPE cartridge. Carotenoids have been separated on a C_{30} column (250×4.6 mm I.D., particle size μm). Isocratic mobile phases were MTBE–methanol mixtures (from 3:97 to 38:62, v/v). The chromatograms of pigments extracted from carrots

Table 7
Quantitation of carotenoid stereoisers of various spinach samples (μg/g spinach)

	Spinach after 5 weeks raw	Spinach after 5 weeks cooked	Spinach after 8 weeks raw	Spinach after 8 weeks cooked	Spinach commercial raw	Spinach commercial cooked
13-*Z*-Lutein	1.7	2.5	2.1	2.8	1.2	1.3
13′-*Z*-Lutein	1.7	3.1	2.4	2.5	1.3	1.4
all-*E*-Lutein	76.5	75.9	93.3	98.1	57.1	48.6
9-*Z*-Lutein	3.6	3.8	7.1	7.4	2.8	2.2
9′-*Z*-Lutein	5.2	6.3	10.2	10.4	2.0	2.4
Total lutein	88.7	91.6	115.1	121.2	64.4	57.1
all-*E*-Zeaxanthin	3.9	36.6	6.3	6.5	1.1	1.3
13,15-*ZZ*β-Carotene	1.4	1.6	1.7	1.6	1.2	1.0
13-*Z*β-Carotene	3.1	3.3	3.3	3.7	1.5	1.2
9,13-*Z*β-Carotene	3.6	3.1	5.5	3.9	4.8	4.3
all-*E*β-Carotene	61.5	63.5	70.5	74.0	37.5	36.0
9-*Z*β-Carotene	9.2	10.2	10.2	7.2	5.1	4.7
Total β-Carotene	78.8	81.7	91.7	90.4	50.1	47.2
Total carotenoids	171.4	176.9	213.1	218.1	115.6	105.6

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and human serum are shown in Figs 10 and 11, respectively. Recovery values were 64 and 52% for α- and β-carotenes. These values are markedly lower than those obtained on a C_{18} column [60]. However, it was observed that the addition of 0.1% (v/v) triethylamine to the mobile phase [61] enhanced the recoveries to 89 and 85%. The method has been proposed for the separation of geometrical isomers of carotenoids from food and biological samples.

HPLC methods have also been developed for the determination of natural dyes added to food products [62]. Separation of carotenoids was performed on a C_{18} column (150×4.6 mm I.D.) using gradient elution. It was found that the efficacy of extraction procedure considerably depends on the type of pigments to be extracted and on the composition of the food product [i.e., tetrahydrofuran (THF) for carotenoids and xanthophylls]. It was emphasized that both liquid–liquid extraction and SPE have to be employed for a successful extraction procedure.

Carotenoids have been measured in the powder of Spirulina Pacifica (*Spirulina platensis* strain pacifica microalgae) also [60]. Pigments were separated on two C_{18} columns (200×2.1 mm I.D., 5 μm; 100×2.1 mm I.D., 5 μm) connected in series. The isocratic mobile phase consisted of acetonitrile–methanol (0.1 *M* ammonium acetate)–dichloromethane (72:22:7, v/v). Carotenoids were detected at 449 nm, and with electrospray mass spectrometry in the positive ion (PI) mode. Mass spectra were acquired between *m/z* 500–650 in steps of 0.1 u (dwell time, 2 ms). Quantitative analysis was performed using SIM (single ion monitoring: *m/z* 568 for lutein and zeaxanthin; *m/z* 564 for canthaxanthin; *m/z* 552 for β-cryptxanthin; *m/z* 536 for β-carotene; and *m/z* 619 for the sodium adduct of astaxanthin). The total ion current chromatogram of the extract of *S. platensis* algae allowed the identification of zeaxanthin, β-cryptoxanthin and β-carotene. It was found that the concentration values found by DAD and MS detection are commensurable: 119.0±1.0 μg/ml and 98.4±1.3 μg/ml for zeaxanthin; 10.5±0.3 μg/ml and 12±2 μg/ml for β-cryptoxanthin and 228±7 μg/ml and 225±4 μg/ml for β-carotene (first concentration values were measured by DAD). It was stated that the method is sensitive enough to be employed for the separation and quantitative determination of carotenoids in complicated matrices.

High-speed counter-current chromatography (HSCCC) has also found application in the analysis of pigments. The preparative separation and purification of lutein from the microalga *Chlorella vulgaris* by HSCCC has been reported [61]. Preparative separation by HSCCC was performed using a polytetrafluoroethylene (PTFE) tube (total capacity, 230 ml). The mobile phase consisted of *n*-hexane–ethanol–water (4:3:1, v/v). The flow-rate was 1 ml/min, fractions were detected at 254 nm. Sample size was

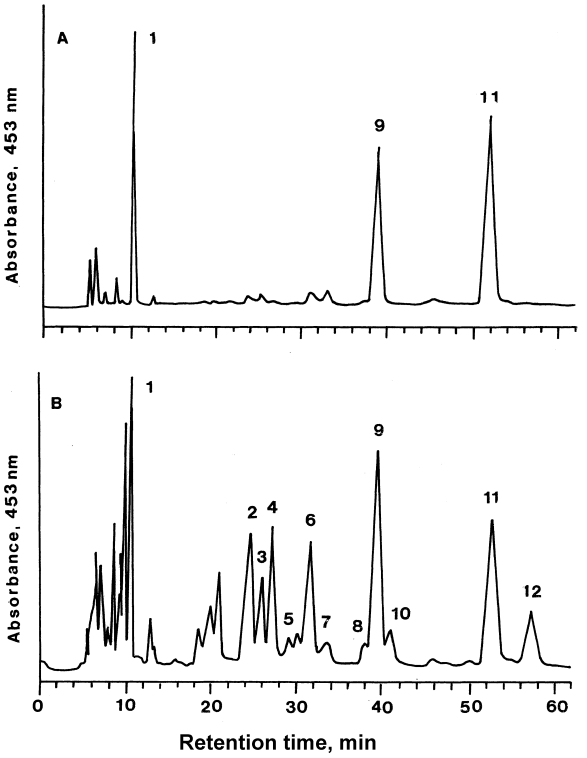

Fig. 10. C_{30} chromatograms of carotenoids extracted from (A) raw and (B) thermally processed carrots, MTBE–methanol (11:89, v/v) mobile phase. Tentative peak identifications: 1, all-*trans*-lutein; 2, 13-*cis*-α-carotene; 3, a *cis*-α-carotene isomer; 4, 13′-*cis*-α-carotene; 5, 15-*cis*-β-carotene; 6, 13-*cis*-β-carotene; 7–8, *cis*-β-carotene isomers; 9, all-*trans*-α-carotene; 10, 9-*cis*-α-carotene; 11, all-*trans*-β-carotene; and 12, 9-*cis*-β-carotene. Reprinted with permission from Ref. [59].

200 mg. The purity of fractions was checked by RP-HPLC on a C_{18} column (250×4.6 mm I.D., particle size, 5 μm). The mobile phase was methanol–dichloromethane–acetonitrile–water (67.5:22.5:9.5:0.5, v/v). It was stated that HSCCC can be successfully employed for the separation of bioactive compounds from alga and may have potential applications in other biotechnological processes.

3. Conclusions

Although it is well recognized that the sensitivity of pigments towards oxidation, elevated temperature, pH and moisture content requires special sample preparation methods the majority of papers fail to indicate what kind of precautions they have taken to assure the decomposition-free treatment of pigments.

Because of their considerable commercial importance much effort has been devoted to the separation and quantitative determination of carotenoids and carotenoid derivatives with various liquid chromatographic methods, such as adsorption and reversed-phase TLC and HPLC. Although the separation capacity, reproducibility and sensitivity of TLC techniques is lower than that of the corresponding HPLC techniques they can be successfully applied for preliminary investigations and as a pilot method for HPLC [63,64]. The versatility, low equilibration time and low environmental impact made RP-HPLC technique a method of choice for the analysis of pigments. A wide variety of RP-HPLC separation procedures have been developed and employed for the determination of carotenoids in various foods and food products the selection of the method depending on the product to be analyzed and on the carotenoids to be determined. Unfortunately, studies comparing the capacity of various reversed-phase stationary phases (endcapped and non endcapped, high or low carbon loading, polymeric or monomeric coating) for the separation of the same mixture of pigments have never been carried out.

4. Future trends

Methods for sample preparation are very important in the liquid chromatography of pigments. The classical liquid–liquid extraction techniques are time consuming and require a considerable amount of organic solvents increasing in this way environmental pollution. The more general application of up-to-date extraction processes (SPE, microwave-assisted extraction, pressurized liquid extraction [65] may result in increased extraction efficiency, reduced extraction time and solvent consumption.

The increase of selectivity and/or the enhancement of sensitivity are the most important purposes of the development of new chromatographic techniques. The advantageous characteristics of microbore HPLC columns (higher theoretical plate number, lower detection limit and solvent consumption, etc.) have not been entirely exploited in the analysis

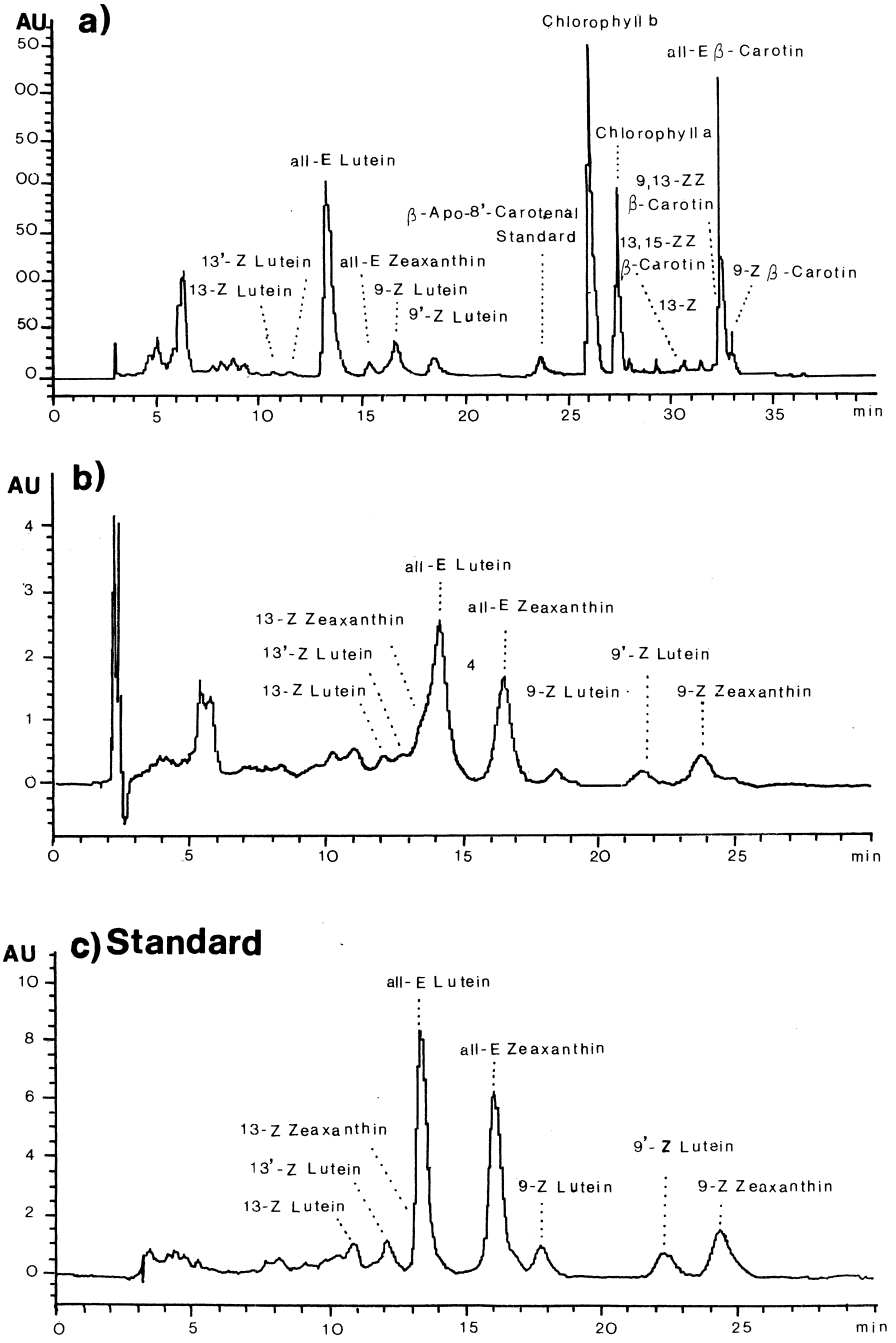

Fig. 11. C_{30} chromatograms of carotenoids extracted from human serum: (a) xantophylls fraction, MTBE–methanol (7:93, v/v) mobile phase; (b) α- and β-carotene fractions, MTBE–methanol (11:89, v/v) mobile phase; (c) lycopene fraction, MTBE–methanol (38:62, v/v) mobile phase. Tentative peak identifications: (a) 1, 13-*cis*-lutein; 2, 13′-*cis*-lutein; 3, all-*trans*-lutein; 4, zeaxanthin; 5–7 unidentified β,ε-carotenoids; and 8, β-cryptoxanthin; (b) 1–2, unidentified ζ-carotene isomers; 3, 15-*cis*-β-carotene; 4, 13-*cis*-β-carotene; 5, all-*trans*-α-carotene, 6, all-*trans*-β-carotene; 7, 9-*cis*-β-carotene; and (c) 1–11 and 13, *cis*-lycopene isomers; and 12, all-*trans*-lycopene. Reprinted with permission from Ref. [59].

of color pigments in foods. Their widespread application can be expected in the future.

In spite of the high separation power electrically driven separation methods such as capillary zone electrophoresis and miceller electrokinetic chromatography have not been frequently used in pigment analysis. The efficacy of pigment separation can be markedly increased by the use of such techniques.

The safe identification of individual pigments cannot be performed with any of the chromatographic techniques using traditional detection methods. The coelution of the standard with the unknown compound does not prove their identity, however, it is generally accepted as a proof of identity. Hyphenated techniques employing MS or MS–MS detection can play a considerable role in the identification of pigments. Other detection methods using Fourier transform infrared (FTIR), NMR or Raman spectroscopy can also contribute to the elucidation of the molecular structure. The future application of these hyphenated techniques in the chromatographic analysis of pigments is to be expected.

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