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Review

Chromatographic determination of carotenoids in foods

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Abstract

In recent years, there has been particular emphasis on obtaining more accurate data on the types and concentrations of carotenoids in foods for various health and nutrition activities. The analysis of carotenoids is complicated because of the diversity and the presence of *cis–trans* isomeric forms of this group of compounds. In addition, a wide variety of food products of vegetal and animal origin, vegetables and animal samples contain carotenoids, and a great range of carotenoids can be found in these samples. The characteristic conjugated double bond system of carotenoids produces the main problem associated with work and manipulation on carotenoids, that is their particular instability, especially towards light, heat, oxygen and acids. For this reason, several precautions are necessary when handling carotenoids. Another problem associated with analysis of carotenoids is the difficulty in obtaining standard compounds. High-performance liquid chromatographic methods for the determination of carotenoids in foods are reviewed. The sample extraction and treatment, carotenoid purification and standard manipulation are briefly commented on. We present a critical assessment of chromatographic methods developed for the determination of carotenoids in foods. Finally, some methods for carotenoid ester separation are reviewed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Food analysis; Reviews; Carotenoids

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1. Introduction

The carotenoids are one of the most important group of natural pigments, because of their wide distribution, structural diversity and numerous functions. Although the classical source of carotenoids is plants, they are also found in animals and microorganisms [1,2]. The structure of the carotenoids confers very special and remarkable properties which are the basis of their varied functions and actions in all kinds of living organisms. The carotenoids are essential for photosynthesis and for life in an oxygen atmosphere [3]. In addition to the provitamin A activity of some carotenoids, these pigments have recently been implicated in the prevention of or protection against serious human health disorders such as cancer, heart disease, macular degeneration and cataracts [2,4–9]. Carotenoids have also been successfully used for many years in the treatment of individuals suffering from photosensitivity disease, for example, erithropoietic protoporphyria [10,11].

Numerous observational studies has found that increased intake of carotenoid-rich foods, fruits and vegetables, have a protective effect against several human chronic diseases [6,7,12,13]. However, more recently, intervention trials have not been conclusive in the evaluation of the β-carotene supplementation effect on cardiovascular disease and cancers [13,14]. In well-nourished populations, β-carotene supplementation is of little or no value in preventing these human health disorders [15,16]; on the contrary, in smokers, β-carotene supplementation increases rather than decreases lung cancer incidence [17,18]. In a recent study, a strong proliferative response in lung tissue was observed in ferrets (animals that metabolize β-carotene in much the same way as humans) supplemented with high-dose of β-carotene, and this response was enhanced by exposure to tobacco smoke [19]. It was concluded that diminished retinoid signaling could be a mechanism to enhance lung tumorigenesis after high-dose β-carotene supplementation and exposure to tobacco smoke [19].

In epidemiological studies we can find associations between dietary components and the incidence of various chronic diseases but do not define specific cause–effect relationships. Unmeasured variables are always present in this kind of study. Also in many studies, there was exclusive focusing on provitamin A carotenes or β-carotene. In general, food composition databases reported the vitamin A, provitamin A or β-carotene content of foods; at most, total carotenoid content was reported [20,21]. A list of individual non-provitamin A carotenoid content of some foods, both raw and cooked, has been recently published [22–24].

Other biological functions have been ascribed to carotenoids [4,14]: stimulants of the immune response at different levels [25–27], enhancers of gap-junction communication [28,29], and quenchers of free radicals [30]. A role in the control of the cell growth and differentiation has been proposed through the modulation of the expression of transcriptor factors and nuclear binding proteins [31–33]. There is strong in vitro evidence that carotenoids are powerful growth inhibitory agents in several human and murine tumor cell lines but not in normal cells [14]. Fucoxanthin retarded the growth of human neuroblastoma GOTO cell lines [34], lycopene was capable of inhibiting the growth of mammary and lung cancer cells [35], and crocin and crocetin inhibited the growth of HeLa cells [36].

Nowadays the physiological and metabolic functions of the carotenoids are still not well understood [37]. The greatest problem is the structural diversity of the carotenoids, more than 700 have been characterized. Moreover, individual carotenoids are present naturally in different *cis–trans* isomeric forms, which may affect their biochemistry [38]. It is necessary to have good, reliable data on carotenoid composition of foods, thus, in this way, nutritional studies may obtain more realistic and valuable conclusions. In this aspect, the analytical methodologies of carotenoids are the pathways to achieve these objectives. Chromatographic methods have been the traditional methods to separate and quantify the carotenoids, but the physical, chemical and biological features of this group of compounds have not facilitated the development of simple, easy-to-apply methods. For example, the AOAC official method [39] for the determination of carotenes and xanthophylls in plants is still based on an open-column separation and does not attempt to separate the individual carotenoids.

The continuing development of chromatographic methods is parallel to the evolution of the technique

and the hardware. The use of photodiode array detection (DAD), which is capable of measuring simultaneously at a wide range of wavelengths, is widespread. The software associated with the DAD systems enables the manipulation of the wavelength spectra of any chromatographic peak.

In the present paper, the chromatographic methods for the determination of carotenoids in foods are reviewed. The methods for the separation of carotenoid esters are discussed in a separate section. Prior to these discussions, however, sample extraction and treatment, carotenoid purification and standard manipulation are briefly commented on.

2. Carotenoids

2.1. Structure

Structurally, the carotenoids are polyisoprenoid compounds, which are synthesized by tail-to-tail linkage of two C_{20} geranylgeranyl molecules. All the carotenoids are produced by variations of the parent C_{40} skeleton. We can distinguish between the hydrocarbon carotenoids, named *carotenes* made only of C and H, and the oxidized carotenoids, named *xanthophylls* (or oxycarotenoids) that present some O-substituent groups such as hydroxy, keto and epoxy groups. The spectrophotometric features of the carotenoids are produced by the conjugated double bond system. At the extremes of the molecule, the carotenoids present lineal or cyclic groups, cyclohexane and cyclopentane. The combination of these end-groups with the addition of oxygen-containing functional groups and changes in the hydrogenation level permit the majority of the structures of the carotenoids. Fig. 1 shows the chemical structure of several common carotenoids.

2.2. Extraction and sample treatment

The characteristic conjugated double bond system of carotenoids produces the main problems associated with work and manipulation on carotenoids, that is their particular instability, especially towards light, oxygen and heat. Acid and alkaline conditions can also be detrimental to certain carotenoids. Any of these factors may produce the degradation and/or the transformation of the carotenoids that are present in the sample, and then the change in the carotenoid composition of the sample. For this reason, several precautions are necessary when handling carotenoids, and have been extensively revised in some other reviews [40–43]. For example, the use of antioxidants, laboratory experiments should be carried out in dim lighting, in order to avoid as far as possible the contact with direct sunlight, evaporation should be performed by rotary evaporation and/or under a stream of nitrogen, and samples should be stored in the dark, under nitrogen or argon, at about −20°C.

The use of antioxidants is one of the most common strategies to prevent oxidation during the extraction and sample treatment, especially when the samples are saponified to obtain free carotenoids. Ethoxyquin, pyrogallol, ascorbic acid and sodium ascorbate are examples of antioxidants used [44], but butylated hydroxytoluene (BHT) is the most extensively used antioxidant. Normally BHT is used at 0.01% or 0.1% in the extraction solution.

2.2.1. Extraction

There is no standard extraction procedure for carotenoids because of the wide variety of food products and animal samples containing these compounds, and the great range of carotenoids that can be found in these samples.

The extraction procedures applied to food products begin with an extraction with methanol (MeOH) or a mixture of MeOH and other more apolar dissolvents. Hart and Scott [23], for example, used a MeOH–tetrahydrofuran (THF) (1:1, v/v) solution in the carotenoid analysis of a wide variety of vegetables and fruits, both raw and cooked. MeOH and diethyl ether [45], MeOH and chloroform [46], MeOH and hexane [47], and MeOH and acetone–hexane [48,49] have also been reported. Other groups prefer the use of acetone alone [50] or in combination with light petroleum [51]. In a water convolvulus (*Ipomoea aquatica*) and carrot carotenoid analysis, Chen and co-workers [52,53] used a more complex mixture extracting solution, hexane–acetone–MeOH–toluene (10:7:6:7, v/v). After the initial extraction of the samples, the extract still contains some polar lipid contaminants, which can be removed by partitioning

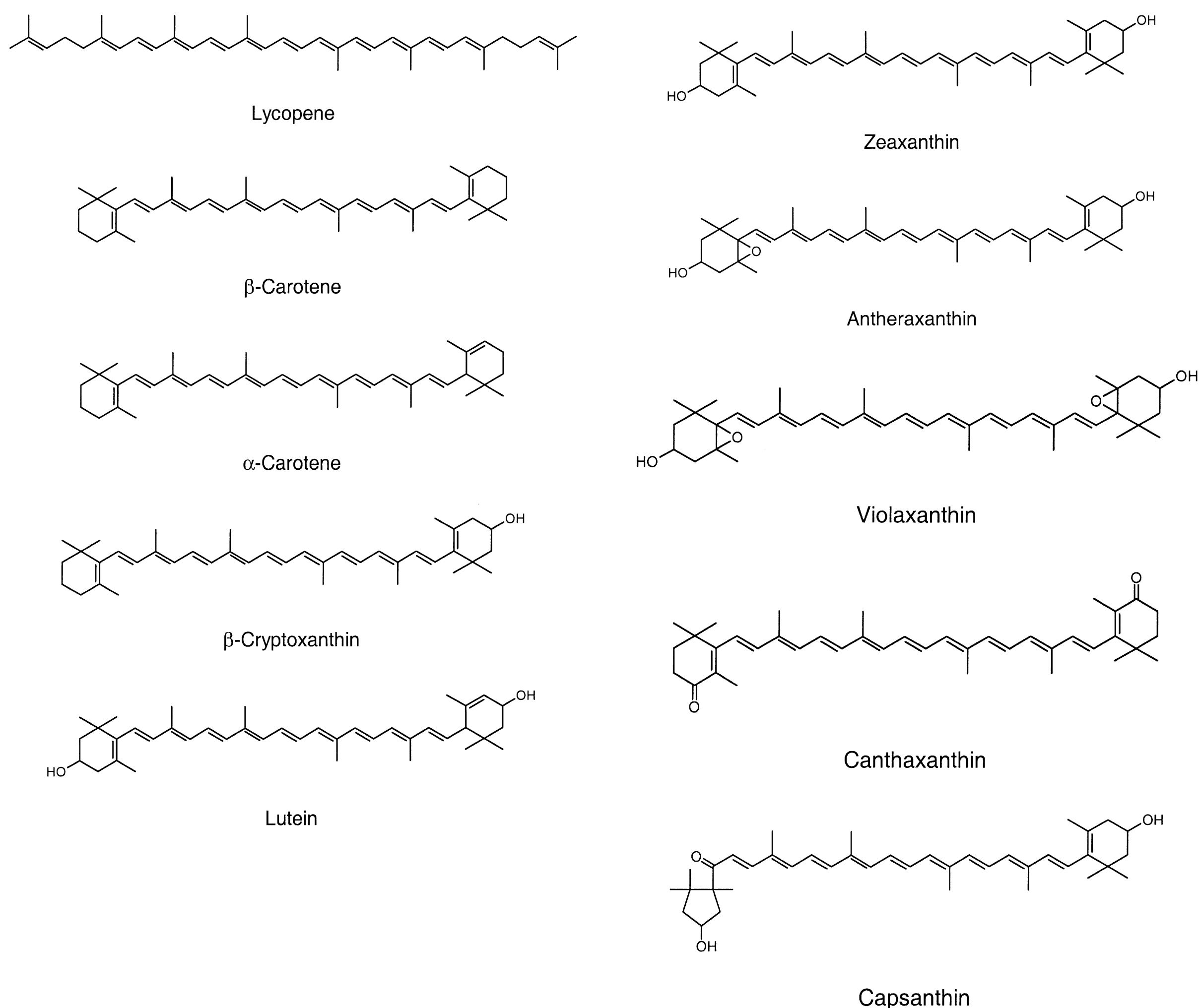

Fig. 1. Chemical structures of several all-*trans* carotenoids.

the extract against water or aqueous salt solutions [41].

The extraction procedure in other samples, serum or plasma, is more constant. A first step with ethanol (EtOH) to precipitate proteins is usually used, with a second step of hexane extraction [49,54] or butanol–ethyl acetate (1:1, v/v) extraction [55].

Supercritical fluid extraction (SFE) technique is a rapid, selective and easier to automate method for sample preparation prior to their characterization by other analytical methods (for review see Ref. [56]). Carotenoids have become a focus of many studies in analytical-scale SFE [56]. Tonucci and Beecher [57] investigated static and dynamic extractions using CO_2 with different modifiers at 1% (MeOH, EtOH and 2-propanol) at different pressures and temperatures. Extraction yields vary depending on the sample composition with higher yields (96%) in high fat food [57]. In sweet potatoes, extraction yield varies depending on the moisture content of the sample, dehydration method and particle size has been described [58], with better recoveries in a freeze–dried sample.

SFE methods for β-carotene from leaves and vegetables [59] and from vitamin supplements [60] have been described. Both methods use SFE-grade

CO_2 without a modifier at 31 mPa with a 1-min static extraction followed by a 40-min dynamic extraction at a 2 ml/min flow-rate at 40°C. Compared with a liquid–liquid extraction method (MeOH–chloroform, 4:1, v/v) the recoveries of β-carotene were similar or somewhat higher for the SFE protocol [60].

2.2.2. Saponification

After the sample extraction, traditionally, the second step in the protocol of carotenoid determination is the alkaline saponification. Carotenoids in vegetables and fruits are predominantly esterified by fatty acids [42]. In addition, the degree of esterification can be different in function of the number of hydroxyls present in the xanthophylls. 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. Thus, the identification of peaks in high-performance liquid chromatography (HPLC) of these carotenoid esters can be difficult [61]. In order to simplify the separation, most methods for quantitative carotenoid analysis have a previous phase of extract saponification [53].

Normally, the saponification is carried out in a KOH solution: aqueous, ethanolic or methanolic solution, and the concentration may vary from 10 to 60% (w/v) [62]. The conditions under which the saponification is carried out may be at ambient temperature overnight or under heating which reduces the time of the reaction [44]. After the saponification, the sample is extracted with diethyl ether or hexane then the extract is washed several times with water until all the KOH is removed.

The degradation and loss of total carotenoid content [63] or individual carotenoids [24,46,50,64] during the saponification have been described. However, this loss depends on the conditions of the saponification [50,64] and on the sample composition [46]. In recent years, many chromatographic methods for the simultaneous determination of free and esterified carotenoids in fruit samples have been described (see above), thus avoiding the saponification step.

The carotenoid extraction of milk and breast milk is more problematic because of the composition of these samples and the fact that, in general, the simultaneous determination of retinoids and carotenoids is required. Saponification is required because of the high fat content (~45%) and carotenoids are contained in the lipid matrix [65]. An enzymatic and bile salts treatment is necessary in milk samples, also with EtOH protein precipitation and finally hexane extraction [54,66]. Another milk saponification method with classical alkaline solution treatment has been described [67,68], but it must be noted that retinoids are even more unstable compounds than carotenoids.

2.3. Purification

One of the main problems associated with analysis of carotenoids is the unavailability of appropriate standard compounds. The diversity, the inherent instability and the presence of isomers of this group of compounds means that most of them are not available commercially. The more common and widespread carotenoids (β-carotene, α-carotene, lutein, zeaxanthin, lycopene, β-cryptoxanthin, etc.), even some more rare carotenoids (capsanthin, capsorubin, etc.), may be purchased commercially. However, the purity of some of these commercial carotenoids is not sufficient for a chromatographic standard, and thus require some additional purification steps.

Traditionally the carotenoid standards were a self-made preparation. From fruits and vegetables and with a purification process, each research obtained standard preparations of the most important carotenoids of their samples. Phase separation, thin-layer chromatography (TLC) and liquid chromatography (LC) methods are used to obtain pure carotenoid standards, also more often nowadays a preparative HPLC method has been described for this purpose.

Normally, to obtain a pure enough carotenoid solution almost two or three steps of purification are required. The best sources of each individual carotenoid are used, for example, neoxanthin from green cabbage, violaxanthin from spinach, antheraxanthin from potatoes, α-cryptoxanthin from carrot leaves, capsanthin and capsorubin from paprika, etc. The spectrophotometric features of carotenoids are used to identify and quantify the individual carotenoid that has been purified. There are many bibliographic references with spectral data of many carotenoids in

different solvents [40,43,69]. The purity degree of standard solution must be ensured by spectrophotometry and HPLC, for both commercial and self-purified standards.

Until the development of HPLC methods, open column separations were used to quantify carotenoids. The AOAC official method for the determination of carotenes and xanthophylls still uses an open column method [39]. In an interlaboratory study of procedures for the analysis of vitamins in foods [70] three laboratories out of 10 use an open column method.

Alumina, silica gel, MgO, $MgCO_3$, $Ca(OH)_2$, $CaCO_3$, Celite, etc., have been used to purify carotenoids [41,43] with different solvent systems. Different proportions of light petroleum, diethyl ether, toluene and EtOH, alone or in combination, have been described to purify most groups of carotenoids by TLC on silica gel [43]. After the separation on TLC the bands are scraped off the layer and eluted from the silica gel with a suitable solvent; for the pigment identification and quantification the wavelength spectra is recorded.

The last development in the TLC method applied to the separation of carotenoids is reversed-phase TLC which consists of a separation on C_{18} plates. This method has been applied to the separation of various carotenoid standards using different proportions of light petroleum–acetonitrile (MeCN)–MeOH [71] achieving better separations than normal-phase TLC [44]. Chen and Chen [72] used a solvent system of hexane–acetone–chloroform–MeOH (70:25:10:5) to separate xanthophylls and β-carotene on silica gel to confirm the identification of a HPLC method of water convolvulus. A preparative TLC using 5–10% acetone in light petroleum on silica gel plates for separation of xanthophyll esters of fruits has been described [73].

More recently, a high-performance TLC (HPTLC) method has been described to detect lycopene added to alcoholic and non-alcoholic beverages [74]. TLC was carried out on high-performance silica gel plates (Whatman LHPKDF) and developed with dichloromethane (DCM)–MeOH (1:1, v/v). A direct quantification procedure was applied successfully. The in situ spectra of each band were recorded with a densitometer (370–700 nm) and the maximum absorption wavelength was used to quantify the pigment with a calibration curve of authentic standards at different concentrations.

Like HPLC for carotenoid determination, preparative HPLC is more often cited for carotenoid purification. In contrast with analytical HPLC, the preparative HPLC methods are characterized by the use of columns with a bigger particle size (10 or 7 μm) of stationary phase, bigger diameter column and/or bigger flow-rate. For example, Wingerath et al. [75] used a 7 μm C_{18} reversed-phase column and MeOH–MeCN–DCM–*n*-hexane (20:40:20:20, v/v) as solvent system at a flow-rate of 4 ml/min to separate carotenol fatty acid esters of fruit juice. Yuang and Chen [76] used an Ultrasphere C_{18} (5 μm) semi-preparative column with MeOH–water–DCM (90:8:2, v/v) as mobile phase at a flow-rate of 3 ml/min to separate and purify *trans*-astaxanthin from algae.

During the preparation and handling of standard carotenoids, special precautions are necessary to avoid the degradation or isomerization of purified pigments. A stock solution must be prepared and stored in the dark, at −20°C, under nitrogen atmosphere and using solvents with antioxidants. To prepare a standard solution, an appropriate aliquot of the stock solution is diluted with suitable solvent and the concentration of the solution must be calculated by spectrophotometry. The purity of the standard solution should be confirmed by HPLC.

3. Chromatographic determination of carotenoids

The classical open column chromatographic methods have been replaced by the new HPLC method, because of the rapidity, non-destructiveness and ease in automating these techniques. The development of the HPLC apparatus is parallel with the development of the laboratory hardware. The new HPLC chromatographs are more compact, more precise, more automated and their use is simpler. Most HPLC chromatographs have special software to control all the apparatus parameters, to obtain chromatograms and, at the same time, data integration and result calculation automatically.

The spectrophotometric HPLC detectors, which are used to detect carotenoids, have shown develop-

ments similar to chromatographs. The new spectrophotometric detectors have a high sensibility and accuracy, are capable of selecting any wavelength and in some cases are programmable, during the chromatographic separation the wavelength can be changed. The more sophisticated detectors applied to the HPLC separation of compounds with characteristic absorption spectra, such as carotenoids, are the DAD systems. These detection systems are capable of recording the entire spectral range (from 190 to 800 nm) during analysis. Thus any chromatogram at selected wavelengths can be monitored, and the absorption spectra of any peak chromatogram and at any moment during the chromatographic separation can be recorded.

All the properties of DAD make these detectors suitable for carotenoid chromatographic separation. Nowadays, the published analytical carotenoid methods use DAD more often. The wavelength range of DAD in a carotenoid separation depends on the purpose of the method, for example, our group in a recent chromatographic method for carotenoid determination uses a detection range of 350–550 nm [46]. However, in HPLC analysis of serum samples the DAD system was set at 250–550 nm to allow the simultaneous determination of retinol, tocopherols and carotenoids [77].

The majority of carotenoid separations reported in the literature involve the use of reversed-phase HPLC, very few normal-phase HPLC have been reported in the last years. Recently, Khachik et al. [54] used a normal-phase separation in an exhaustive qualitative and quantitative analysis of carotenoids in human milk and serum. However, normal-phase and reversed-phase HPLC separations are described in this reference. In an interlaboratory study of methods for the determination of fat-soluble vitamins, only two normal-phase HPLC separations have been reported as opposed to five reversed-phase separations and three open-column chromatographic methods [70].

It is well known that traditional gas chromatography (GC) is not suitable for the analysis of carotenoids, because of their inherent instability and their low volatility [44], extensible to GC–mass spectrometry (MS) [78]. A recent review on the latest innovations in LC–MS applied to carotenoid analysis has been published [78,79], and two different kinds of MS detectors have been proposed as suitable for carotenoid LC–MS, namely, LC–electrospray ionization (ESI) MS [80,81] and LC–atmospheric pressure chemical ionization (APCI) MS [82].

Information on normal-phase HPLC and any other chromatographic separations (TLC, LC, etc.) of carotenoids has been reported in numerous previous reviews [38,41,44,83]. In the present report, we summarize the latest published HPLC methods for the determination and quantification of carotenoids in foods, despite the previous commented reference, and all of them are reversed-phase separations. In a separate section, the simultaneous chromatographic determinations of free carotenoids and carotenoid esters published during the last years are reported.

3.1. Determination of carotenoids by high-performance liquid chromatography

Granado et al. [22] analyzed the carotenoid composition in raw and cooked Spanish vegetables using Spheri-5-RP-18 or Spheri-5-ODS columns, MeCN–DCM–MeOH (70:20:10, v/v) as mobile phase and a flow-rate of 1.8 ml/min. For the separation of lutein from zeaxanthin, the mobile phase was substituted with MeCN–MeOH (85:15, v/v). In this work, both non-provitamin A (lutein, zeaxanthin and lycopene) and provitamin A (β-cryptoxanthin, γ-carotene, α-carotene and β-carotene) carotenoids were reported, whereas β-apo-8′-carotenal and canthaxanthin were not detected in any of the samples. Echinenone or retinyl palmitate were used as internal standard. Carotenoids were detected at 450 nm and retinyl palmitate at 325 nm with a programmable multiwavelength detector.

A carotenoid analysis of carrot, spinach, tomatoes, corn (canned) and tangerines using a stainless steel (250×4.6 mm I.D.) column packed with Vydac 201 TP, 5 μm particle size, and a mobile phase consisting of MeOH–THF (95:5, v/v) have been described [84]. The flow-rate was at 1 ml/min, the chromatogram was recorded at 450 nm and ethyl-β-apo-8′-carotenoate was used as internal standard. Lutein, zeaxanthin, α-carotene, β-carotene and lycopene were separated and quantified with this method.

Muller [24] used three different mobile phases for the carotenoid separation and determination of fruit and vegetable samples on Vydac 5 μm C_{18} analytical

column with a flow-rate of 1 ml/min and column temperature of 20°C. An isocratic mobile phase system was a mixture of MeOH–acetone (95:5, v/v) (previously described in Ref. [65]), another suitable isocratic system consisted of MeOH–MeCN–ammonium acetate (85:15:0.01, v/v/w); and, a gradient separation using two solvents, MeCN–2-propanol (40:60, v/v) and water (previously described [50]). The samples were saponified and losses were occasionally observed especially for xanthophylls. Lycopene was the most unstable xanthophyll with saponification treatment.

Ben-Amotz and Fishler [85] reported extensive data on common fruit and vegetable carotenoid composition. In this study, a Vydac 201 TP54 C_{18} 5 μm column maintained at 30°C and MeOH–MeCN (9:1, v/v) mobile phase at a flow-rate of 1 ml/min is described. β-Apo-8′-carotenal or echinenone was used as internal standard. Some carotenoid isomer content has been reported with special emphasis on 9-*cis*-β-carotene.

A reversed-phase ion-pair HPLC applied to the separation of carotenoids and chlorophylls in olive has been described [86]. Separation was carried out on a Spherisorb ODS-2 5 μm particle size column and with a non-linear gradient of eluent A (water–solution P–MeOH, 1:1:8, v/v/v) and eluent B (acetone–MeOH, 1:1, v/v) at a flow-rate of 2 ml/min. The ion-pair reagent (solution P) is 0.05 *M* tetrabutylammonium acetate and 1 *M* ammonium acetate in water. Eighteen pigments, including chlorophylls and carotenoids, were separated in 30 min. Spectral data of carotenoids identified in the eluent was reported.

A non-aqueous reversed-phase HPLC using an isocratic mobile phase consisting of MeCN–DCM–MeOH (65:25:10, v/v/v) has been described [87]. An Analytichem C_{18} column was used, flow-rate was 1 ml/min and the chromatographic separation was monitored using DAD. The peak spectrum was used to identify some carotenoids of red grapefruit. DAD was used for spectral characterization of the separated pigments, some spectral information was reported.

Another simultaneous determination of chlorophylls and carotenoids has been proposed by Chen and Chen [72]. The column was packed with Ultremex C_{18}, 5 μm particle size and flow-rate was 1 ml/min of MeCN–MeOH–chloroform–hexane (75:12.5:7.5:7.5, v/v). β-Apo-8′-carotenal was used as internal standard. For a similar purpose, the use of a 3 μm ODS-Hypersil column and a linear gradient of two eluents (eluent A: MeOH–MeCN–water–0.2 *M* Tris–HCl buffer, 15:65:19:1, v/v; eluent B: MeOH–hexane, 7:1, v/v) has been described.

Mínguez-Mosquera and Hornero-Méndez [88] developed a reversed-phase method to quantify red peppers, paprika and oleoresin carotenoids. The separation was carried out on a Spherisorb ODS C_{18}, 5 μm particle size column using a non-linear gradient of acetone and water at a flow-rate of 1.5 ml/min. Spectral data of carotenoids identified were reported in the mobile phase.

Weissenberg et al. [45] optimized the separation of red pepper pigments with a non-aqueous ternary mobile phase. Different proportions of MeCN, 2-propanol and ethyl acetate at 0.8 ml/min as flow-rate were assessed in two C_{18} columns with different length (25 and 12.5 cm, LiChrospher 100 RP-18, 5 μm and Superspher RP-18, 4 μm, respectively). The best separation of capsorubin and capsanthin was achieved in a 12.5 cm column length and MeCN–2-propanol–ethyl acetate (80:10:10, v/v) as a mobile phase; however, canthaxanthin and lutein ran together. Peak distortion was observed with increased MeCN proportion of the solvent.

Previously, Craft and Wise [65] optimized an isocratic HPLC separation of carotenoids in a polymeric C_{18} column. Nine solvent modifiers were investigated using a MeOH-based mobile phase. Column temperature was also investigated. 3 to 5% THF in MeOH and a column temperature of 20°C result in good separation of a mixture of seven carotenoids (lutein, zeaxanthin, β-cryptoxanthin, echinenone, α-carotene, β-carotene and lycopene).

Multiple peak formation and peak distortion have been described associated with the analysis of carotenoids by HPLC [89,90]. These problems have been associated with the solubility of the carotenoids in the HPLC eluent, the nature of the injection solvent [89,90] and also with the reaction between carotenoids and metal surfaces [90]. Particularly, lycopene seems a very unstable compound in stainless steel columns with metal frits, so recoveries lower than 60% have been described [84,90]. The addition of antioxidants (BHT) in the stock solvent

solution or to store the stock solution dried has been shown to improve lycopene recovery [90]. Scott [90] also reported a between-column variation using columns containing different batches of packing material (Vydac TP20154): changes in peak responses, elution times and differences in the peak profile separation were shown. A progressively worse result has been reported on C_{18} NovaPak columns that previously were useful for carotenoid separation [90].

Epler et al. [91] observed bad carotenoid separations (no peaks were observed) on columns that previous carotenoid separations were acceptable after a minute exposition to trifluoroacetic acid. The same column after treatment with MeOH–aqueous 0.01 *M* ammonium acetate (50:50, v/v) for 45 min was restored to its initial good carotenoid-separating state. What is more, five columns which in a previously published evaluating paper of the same research group [92] had poor recovery (<48%), after treatment with MeOH–ammonium acetate and using a mobile phase with 0.05% TEA the columns become suitable for carotenoid separation (recovery >87%) [91].

A study of the column temperature effect on the carotenoid chromatographic determination has been published [93]. The column used in this study was a 5 μm Spherisorb ODS 2 column and the mobile phase consisted of MeCN–MeOH–DCM (75:20:5, v/v) containing 0.1% BHT at a 1.5 ml/min flow-rate. The sample was a reference standard mixture of lutein, zeaxanthin, β-cryptoxanthin, echinenone, lycopene, α-carotene and β-carotene. A reduction of about 1 min in elution time for every 1°C rise in temperature was shown, and the optimum temperature was at 20–22.5°C.

Differences between monomeric and polymeric octadecylsilica (ODS) reversed-phase columns in chromatographic separation of carotenoids using MeOH-based mobile phase have been described [92,94]. In general, the polymeric ODS stationary phase has a better selectivity for carotenoids than monomeric ODS stationary phase. MeOH-based solvents provided higher recoveries than MeCN-based solvents [92]; the most appropriate solvent system was MeOH–THF (90:10, v/v) [94]. Lutein and zeaxanthin were difficult to separate in the monomeric ODS phases [92].

Interest in the carotenoid isomer analysis has grown in the last years, numerous chromatographic methods specially focused on carotenoid isomers have been described (see Ref. [38] for review). The traditional reversed-phase chromatographic columns seldom have an adequate ability to separate the *cis–trans* isomers of a particular carotenoid. A better performance for this purpose has been attributed to polymeric ODS columns [92].

Stahl et al. [95] achieved the separation of five isomers of β-carotene and seven isomers of lycopene in human serum and tissues. The separation was carried out on 5 μm particle size Suplex PKB 100 column with either MeOH–MeCN–2-propanol (54:44:2, v/v) or MeOH–MeCN–2-propanol–water (10:40:40:10, v/v). An eluent system of MeCN–MeOH–DCM (75:15:10, v/v) or MeCN–MeOH (90:10 or 5:95) has been described as a better mobile phase for the separation of β-carotene isomers and MeOH–DCM (99:1, v/v) for the separation of α-carotene isomers and for the simultaneous separation of α- and β-carotene isomers [53].

The configurational isomers of the all-*trans*-, and most of the configurational isomers of the 9-*cis*-, 13-*cis*- and 15-*cis*-astaxanthin have been separated on a chiral HPLC separation [96]. Recently, a polymeric C_{30} stationary phase was developed to optimize carotenoid reversed-phase chromatography [97]. This novel stationary phase has demonstrated superior resolution for carotenoid isomer separation (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene) in comparison to traditional C_{18} columns [97–99].

Sander et al. [97] achieved a good resolution and identification of four isomers of β-carotene (all-*trans*, 9-*cis*, 13-*cis* and 15-*cis*) in a not fully optimized separation of a carotene and xanthophyll standard solution using C_{30} stationary phase column and a gradient of 81:15:4 to 6:90:4 of MeOH–methyl-*tert*.-butyl ether (MTBE)–water during 90 min. Both monomeric and polymeric C_{18} columns were unable to separate β-carotene isomers in this standard solution [97]. Similar separation has been described in a canned sweet potatoes extract with a linear gradient from 85:15 to 10:90 of MeOH–MTBE containing 1 m*M* ammonium acetate over 60 min [80].

A superior resolution of C_{30} stationary phase

respect to traditional C_{18} columns have been described to separate several standard solutions of individual carotenoids and xanthophylls (β-carotene, α-carotene, zeaxanthin, lutein, β-cryptoxanthin and lycopene) using different proportions of MeOH–MTBE as mobile phase [98,99].

The electrochemical detection of carotenoids in chromatographic separations has been described, but with utilization of conventional one or two channel electrochemical cells [100–103]. Recently, a HPLC carotenoid determination with a coulimetric electrochemical array detector equipped with eight channels in series has been described [49]. The potential settings were from 100 to 520 mV in 60 mV increments. The chromatographic conditions were 5 μm polymeric C_{30} as stationary phase and a gradient with different concentrations of MeOH–MTBE–ammonium acetate (eluent A, 95:3:2, eluent B, 25:73:2). The electrochemical detectors have shown to be 100- to 1000-fold more sensitive than conventional spectrophotometric detectors [49,102]. In addition to the high sensitivity, the electrochemical array detector signal was characteristic for each individual carotenoid (lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene), even for *cis–trans* isomers of β-carotene (all-*trans*, 9-*cis*, 13-*cis* and 15-*cis*) [49].

3.2. Determination of carotenoid esters by high-performance liquid chromatography

Hydroxycarotenoids in vegetables are predominantly esterified by fatty acids [42]. The carotenoid composition of fruits is not constant, depends on variety, fruit ripening, environmental conditions, etc. Similarly, in some fruits, during ripening the esterification degree of carotenoids increases [104,105]. In addition, a high stability against possible thermo-, photo- and enzymatic oxidation reactions has been related to the esterification degree [106,107]. In recent years, the methods for the carotenoid determination without saponification have increased. The use of DAD has facilitated the identification of the esterified forms of carotenoids.

In Table 1, recent chromatographic methods to separate carotenoid esters in fruits and foods are summarized. Because of the wide range of apolarity of the carotenoids and carotenoid esters, the chromatographic methods are characterized by a reversed-phase HPLC on C_{18} columns. Most of the methods listed use DAD to facilitate the identification of the carotenoid esters comparing peak spectra along the chromatogram. Seven isocratic [107–109] and eight gradient [46,48,50,51,75,76,110,111] mobile phase systems have been described. In general, both isocratic and gradient mobile phases are based on MeOH, acetone, 2-propanol and MeCN with some modifiers (water, hexane, ethyl acetate or DCM).

Independently of the mobile phase composition, there are similar patterns of chromatographic separation. For example, in paprika samples (fruits, powder or food with high content of paprika) [46,48,107,108,110,111] the chromatogram could be divided into four zones: the first zone corresponds to free carotenoids, the second zone to monoesterified pigments, the third zone to α-carotene, β-carotene and their isomers, and finally the fourth zone to diesterified carotenoids. The fatty acid moiety of carotenoid esters has been identified in some references [48,73,75,109,110,112,113], and there are a great variety of saturated fatty acids, from decanoic acid to oleic acid which have been reported. The degree of esterification varied between carotenoids, for example in red pepper fruits, zeaxanthin is predominantly found in the monoesterified form and capsorubin in the diesterified form [114]. Goda et al. [113] have shown in paprika a preferential position of esterification group of the capsanthin hydroxyl group pertaining to the cyclopentane versus the cyclohexane group.

4. Nomenclature

BHT	Butylated hydroxytoluene
DCM	Dichloromethane
EtOH	Ethanol
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC	Liquid chromatography
MeCN	Acetonitrile
MeOH	Methanol

Table 1
HPLC methods for carotenoid ester separations

Column	Eluent	Detection	Samples	Ref.
ODS Resolve C_{18}	Linear gradient of MeOH and ethyl acetate Flow-rate: 1.8 ml/min	470 nm	Red bell peppers	[48]
5 μm Zorbax C_{18}	Gradient of acetone–water (75:25, v/v) and acetone–MeOH (75:25, v/v). Flow-rate: 1 ml/min	460 nm	Paprika	[110]
10 μm Chromsil C_{18}	Acetone–water (90:10, v/v), acetone–water–hexane (85:10:5, v/v) or MeCN–2-propanol–water (39:57:5:4, v/v). Flow-rate: 1 ml/min	290 nm, 350 nm and 438 nm	Paprika fruits and powder	[106]
5 μm ODS RP-18e and 5 μm ODS RP-8 serially connected	Non-linear gradient of MeCN–2-propanol (40:60, v/v) and water. Flow-rate: 0.8 ml/min.	DAD (462 nm)	Pepper fruits and powder paprika	[50]
6 μm LiChrosorb C_{18}	MeCN–2-propanol–MEOH–water (39:52:5:4, v/v). Flow-rate: 0.9–1.5 ml/min	DAD	Spice red pepper, tomato, carrot, orange and mandarin	[107]
5 μm Suplex pKB 100 or 5 μm LiChrospher C_{18}	Linear gradient of MeOH–MeCN–DCM–hexane at 10:85:2.5:2.5 (v/v) and 10:45:22.5:22.5 (v/v). Flow-rate: 0.7 ml/min	DAD	Tangerine and orange concentrates	[75]
YMC-Pack ODS-A. Column temperature: 30°C	MeOH–ethyl acetate (62:38 or 88:12, v/v) or MeOH–water–ethyl acetate (80:10:10). Flow-rate: 1 ml/min	470 nm	Banana peel	[108]
5 μm Spherisorb ODS1	Non-linear gradient of MeOH–MeCN (50:50, v/v), water and ethyl acetate. Flow-rate: 1.5 ml/min	DAD (450 nm)	Paprika extract	[109]
5 μm Ultrasphere C_{18} Column temperature: 25°C	Linear gradient of DCM–MeOH–MeCN–water at 5:85:5.5:4.5 (v/v) and 22:28:45.5:4.5 (v/v). Flow-rate: 1 ml/min	DAD	Alga (*Haematococcus pluvialis*)	[76]
5 μm Spherisorb C_{18} ODS2	Non-linear gradient of acetone–water at 100:50 to 100:5. Flow-rate: 1 ml/min	DAD ("max plot")	Powder paprika and fat cured crude sausage (Sobrassada)	[46]
5 μm Spherisorb C_{18} ODS-2 Column temperature: 40°C	Non-linear gradient of different proportions of acetone, MeOH and water. Flow-rate: 1 ml/min	DAD	Aqueous paprika extract and orange juice	[51]

MS Mass spectrometry
MTBE Methyl-*tert*.-butyl ether
ODS Octadecylsilica
SFE Supercritical fluid extraction
THF Tetrahydrofuran
TLC Thin-layer chromatography

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