Chapter 3
Structures and Analysis of Carotenoid Molecules

Delia B. Rodriguez-Amaya

Abstract Modifications of the usual C_{40} linear and symmetrical carotenoid skeleton give rise to a wide array of structures of carotenes and xanthophylls in plant tissues. These include acyclic, monocyclic and dicyclic carotenoids, along with hydroxy and epoxy xanthophylls and apocarotenoids. Carotenols can be unesterified or esterified (monoester) in one or two (diester) hydroxyl groups with fatty acids. *E-Z* isomerization increases the array of possible plant carotenoids even further. Screening and especially quantitative analysis are being carried out worldwide. Visible absorption spectrometry and near infrared reflectance spectroscopy have been used for the initial estimation of the total carotenoid content or the principal carotenoid content when large numbers of samples needed to be analyzed within a short time, as would be the case in breeding programs. Although inherently difficult, quantitative analysis of the individual carotenoids is essential. Knowledge of the sources of errors and means to avoid them has led to a large body of reliable quantitative compositional data on carotenoids. Reverse-phase HPLC with a photodiode array detector has been the preferred analytical technique, but UHPLC is increasingly employed. HPLC-MS has been used mainly for identification and NMR has been useful in unequivocally identifying geometric isomers.

Keywords Carotenoids • Structures • Carotenes • Xanthophylls • E-Z isomers • Screening • Quantitative analysis • HPLC methods • UHPLC methods • HPLC-DAD-MS • NMR

3.1 Introduction

Carotenoids are widely distributed, lipophilic, naturally occurring yellow, orange, or red pigments. They are noted for their structural diversity and multiple functions and actions. It is estimated that about 100 million tons of these compounds are produced annually in nature (Isler et al. 1967). About 750 carotenoids, exclusive

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of *E* (*trans*) and *Z* (*cis*) isomers, have been isolated and characterized from natural sources (Britton et al. 2004). Most of these carotenoids come from higher plants, algae, bacteria and fungi.

3.2 Nomenclature

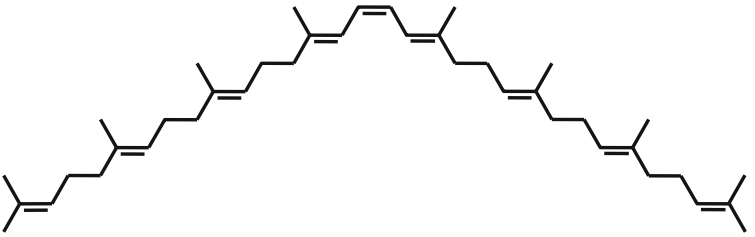
A semisystematic nomenclature for carotenoids that conveys structural information, including stereochemistry/three-dimensional structure, was devised by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (IUPAC/IUB 1975; Weedon and Moss 1995). The names are based on the stem name "carotene," preceded by Greek-letter prefixes (β, ε, ψ, κ), denoting the two end groups. The numbering for the carotenoid skeleton is shown for lycopene and β-carotene in Figs. 3.1 and 3.2. Changes in hydrogenation and the presence of oxygen-containing substituents are indicated by standard prefixes and suffixes used in organic chemistry. The absolute stereochemistry of chiral, optically active carotenoids is indicated by the R/S designation.

Carotenoids have trivial names, usually derived from the biological sources from which they were first isolated. For the sake of simplicity, these short and familiar trivial names will be used throughout this chapter. The trivial names are accompanied by the semi-systematic names in Figs. 3.1, 3.2, 3.3, and 3.4. The *E/Z* designation is now preferred to indicate the configuration of the double bonds and will be used in this chapter instead of the still widely used *trans*/*cis* terminology.

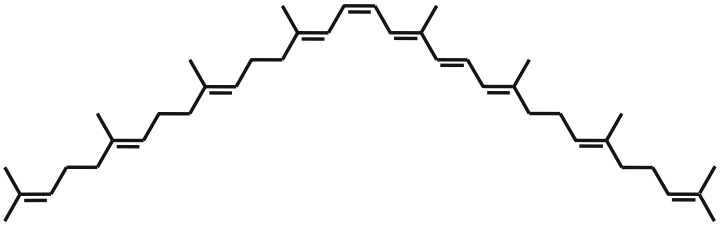
3.3 Structures

Carotenoids in plants are mostly C_{40} tetraterpenes/tetraterpenoids formed from eight C_5 isoprenoid units joined head-to-tail, except at the center where a tail-to-tail linkage reverses the order (Fig. 3.1). The basic linear and symmetrical skeleton has lateral methyl groups separated by six C-atoms at the center and the others by five C-atoms. The most distinctive structural feature is a centrally located, long system of alternating double and single bonds, in which the π-electrons are effectively delocalized throughout the polyene chain, although electron density appears to be greater at or towards the end of the chain. This conjugated double-bond system constitutes the light-absorbing chromophore that gives carotenoids their attractive color and is mainly responsible for their special properties and many functions. On the other hand, it renders the molecule susceptible to geometric isomerization and oxidative degradation.

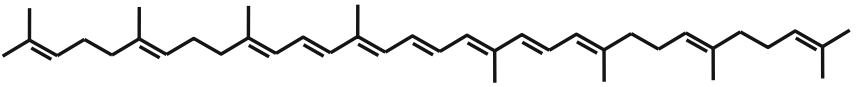
The basic structure is modified in many ways, including cyclization, hydrogenation, dehydrogenation, introduction of oxygen-containing groups, migration of double bonds, rearrangement, chain shortening or elongation, or combinations thereof, resulting in an immense array of structures.



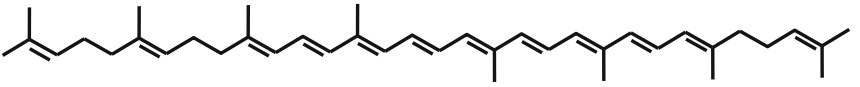
Phytoene 7,8,11,12,7′,8′,11′12′-octahydro-ψ,ψ-carotene



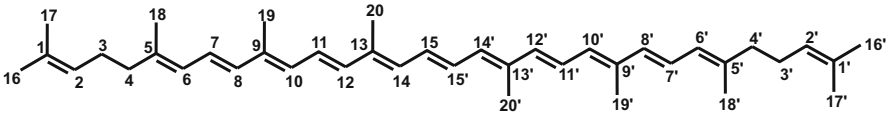
Phytofluene 7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene



ζ-Carotene 7,8,7′,8′-tetrahydro-ψ,ψ-carotene

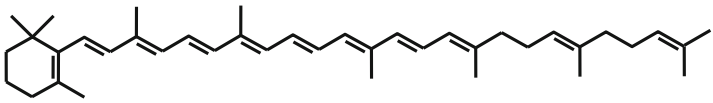


Neurosporene 7,8-dihydro-ψ,ψ-carotene

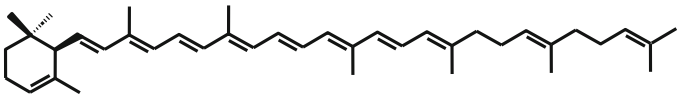


Lycopene ψ,ψ-carotene

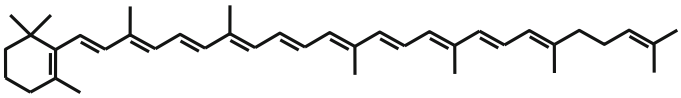
Fig. 3.1 Structures of acyclic plant carotenes



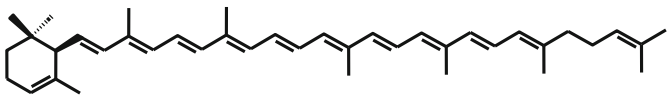
β-Zeacarotene 7′,8′-dihydro-β,ψ-carotene



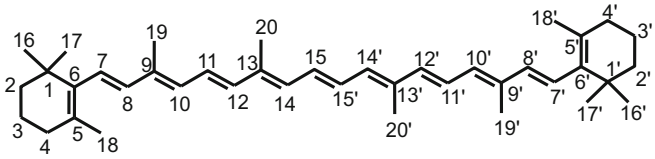
α-Zeacarotene (6R)-7′,8′-dihydro-ε,ψ-carotene



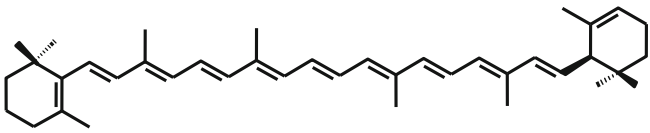
γ-Carotene β,ψ-carotene



δ-Carotene (6R)-ε,ψ-carotene



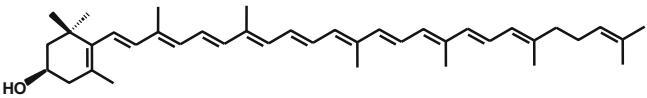
β-Carotene β,β-carotene



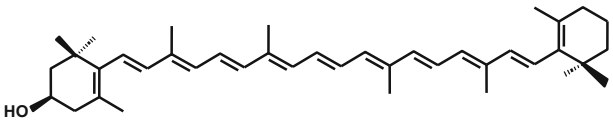
α-Carotene (6′R)-β,ε-carotene

Fig. 3.2 Structures of monocyclic and dicyclic plant carotenes

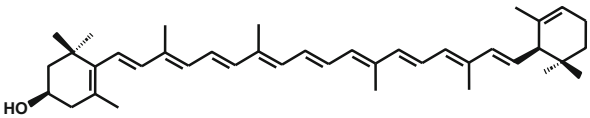
Hydrocarbon carotenoids (e.g. β-carotene, lycopene) are collectively called carotenes. Derivatives containing oxygen functions are termed xanthophylls. Common oxygen-containing substituents are hydroxyl- (as in β-cryptoxanthin), keto- (as in canthaxanthin), epoxy- (as in violaxanthin), and aldehyde (as in β-citraurin, (3R)-3-hydroxy-8′-apo-β-caroten-8′-al).



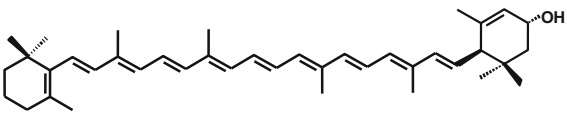
Rubixanthin (3R)-β,ψ-caroten-3-ol



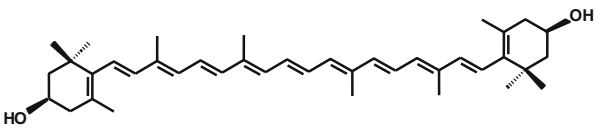
β-Cryptoxanthin (3R)-β,β-caroten-3-ol



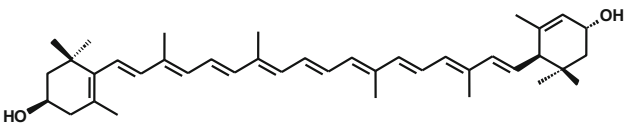
Zeinoxanthin (3R,6′R)-β,ε-carotene-3-ol



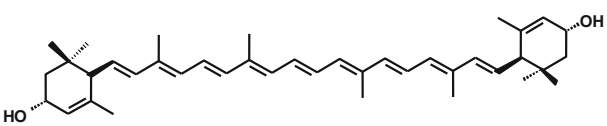
α-Cryptoxanthin (3′R,6′R)-β,ε-caroten-3′-ol



Zeaxanthin (3R,3′R)-β,β-carotene-3,3′-diol



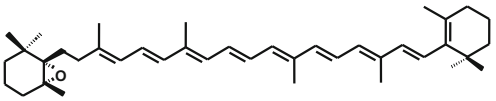
Lutein (3R,3′R,6′R)-β,ε-carotene-3,3′-diol



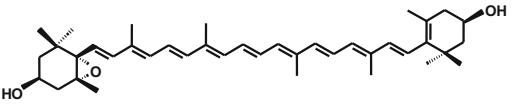
Lactucaxanthin (3R,6R,3′R,6′R)-ε,ε-carotene-3,3′-diol

Fig. 3.3 Structures of plant carotenols (hydroxy carotenoids)

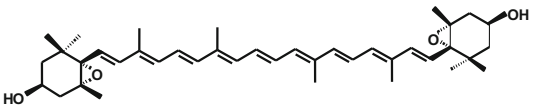
Fig. 3.4 Structures of plant epoxy carotenoids



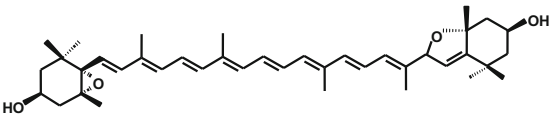
β-Carotene-5,6-epoxide (5R,6S)-5,6-epoxy-5,6-dihydro-β,β-carotene



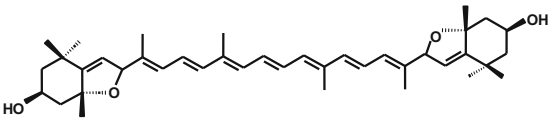
Antheraxanthin (3S,5R,6S,3′R)-5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol



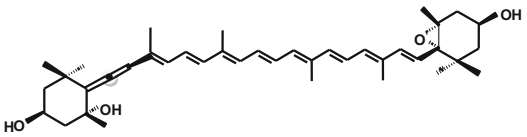
Violaxanthin (3S,5R,6S,3′S,5′R,6′S)-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol



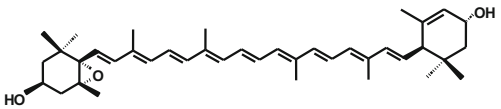
Luteoxanthin 5,6,5′,8′-diepoxy-5,6,5′,8′-tetrahydro-β,β-carotene-3,3′-diol



Auroxanthin (3S,5R,8RS,3′S,5′R,8′RS)-5,8,5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene-3,3′-diol



Neoxanthin (3S,5R,6R,3′S,5′R,6′S)-5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol



Lutein-5,6-epoxide (3S,5R,6S,3′R,6′R)-5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3′-diol

Carotenoids may be acyclic (e.g. lycopene, ζ-carotene) or may have a six-membered ring at one end (e.g. γ-carotene, δ-carotene) or at both ends (e.g. β-carotene, α-carotene) of the molecule. Exceptionally, capsanthin and capsorubin have five-membered rings.

Because plants are able to synthesize carotenoids *de novo*, along with the principal carotenoids, low levels of their biosynthetic precursors and derivatives are also found. The carotenoid composition is variable and often complex.

3.3.1 Carotenes

Of the uncyclized carotenes (Fig. 3.1), lycopene and ζ-carotene are the most common. Lycopene is a $C_{40}H_{56}$ carotene with 11 conjugated and 2 non-conjugated double bonds. ζ-carotene, with a molecular formula of $C_{40}H_{60}$, has seven conjugated double bonds and four isolated double bonds. The colorless phytoene ($C_{40}H_{64}$) and phytofluene ($C_{40}H_{62}$) contain, respectively, three and five conjugated double bonds and six and five isolated double bonds. Neurosporene ($C_{40}H_{58}$) has nine conjugated and three unconjugated double bonds.

The monocyclic β-zeacarotene ($C_{40}H_{58}$) and γ-carotene ($C_{40}H_{56}$) contain 9 and 11 conjugated double bonds, respectively. Both have one end cyclized into a β-ring in which one of the conjugated double bonds is situated; the other end has an isolated double bond. α-Zeacarotene ($C_{40}H_{58}$) and δ-carotene ($C_{40}H_{56}$), with 8 and 10 double bonds, respectively, possess an *ε*-ring in which the double bond is out of conjugation.

β-carotene is a ($C_{40}H_{56}$) carotene with both ends of the molecule cyclized into β-rings (Fig. 2.2). It has 11 conjugated double bonds, two of which are located in the β-rings. The ring double bonds are not coplanar with those of the polyene chain. Also bicyclic, α-carotene ($C_{40}H_{56}$) has a β-ring, an *ε*-ring and 10 conjugated double bonds.

3.3.2 Xanthophylls

A diversity of xanthophylls is found in plants. Figure 3.3 shows the structures of common carotenols, with the hydroxyl groups located at the C-3 and C-3′ positions. Rubixanthin and β-cryptoxanthin (both $C_{40}H_{56}O$, 11 conjugated double bonds) are monohydroxy derivatives of γ-carotene and β-carotene, respectively, with the hydroxyl group at C-3 of the β-ring (Fig. 3.3).

The hydroxylation of α-carotene produces two monohydroxylated derivatives (both $C_{40}H_{56}O$, 10 conjugated double bonds): zeinoxanthin with the hydroxy group in the β-ring and α-cryptoxanthin with the allylic hydroxyl in the *ε*-ring (Fig. 3.3).

Lutein and zeaxanthin are dihydroxy, dicyclic carotenoids derived from α- and β-carotene, respectively, both having a molecular formula of $C_{40}H_{56}O_2$ (Fig. 3.3).

Both have hydroxyl groups at the 3 and 3′ positions. They differ only in the location of a single double bond, resulting in lutein having a β-ring and an ε-ring and zeaxanthin having two β-rings. Lutein has 10 conjugated double bonds, one of which is in the β-ring; an isolated double bond is in the ε-ring. Zeaxanthin contains 11 conjugated double bonds, two of which are located in β-rings.

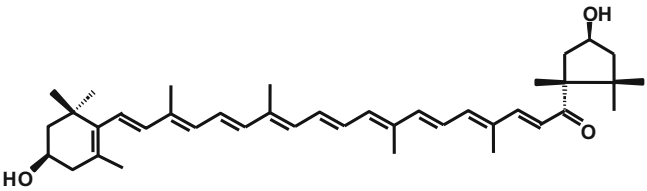
Carotenoids with both ends cyclized into ε-rings are rare. The dihydroxy derivative of ε-carotene ($C_{40}H_{56}$, (6R, 6′R)-ε,ε-carotene), lactucaxanthin ($C_{40}H_{56}O_2$, 9 conjugated double bonds, 2 non-conjugated double bonds in ε-rings), is a major carotenoid of lettuce, but has not been encountered in other leaves.

Carotenols in green leaves (Kobori and Rodriguez-Amaya 2008) are not esterified and those of corn (Rodriguez-Amaya and Kimura 2004; de Oliveira and Rodriguez-Amaya 2007) are also mostly unesterified. Carotenols in ripe fruits are generally esterified with fatty acids. Exceptions are the carotenols of a few fruits, particularly those that remain green when ripe such as kiwi (Gross 1987), which undergo limited or no esterification. Lutein, the main carotenoid, occurs free or esterified in one (monoester) or both (diester) hydroxyl groups in the edible nasturtium (Niizu and Rodriguez-Amaya 2005) and marigold (Breithaupt et al. 2002) flowers, with the esters predominating. Esterification, which occurs progressively during maturation, appears to be of physiological importance. Acylation increases the lipophilic character of the xanthophylls, facilitating their accumulation in the chromoplasts (Gross 1987).

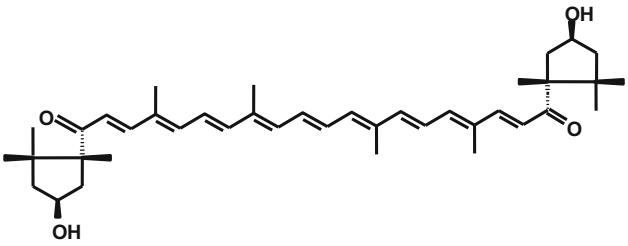
Epoxy carotenoids comprise a large group of plant xanthophylls (Fig. 3.4). The zeaxanthin-derived dihydroxy epoxides, antheraxanthin ($C_{40}H_{56}O_3$, 10 conjugated double bonds), violaxanthin ($C_{40}H_{56}O_4$, 9 conjugated double bonds), luteoxanthin ($C_{40}H_{56}O_4$, 8 double bonds), auroxanthin ($C_{40}H_{56}O_4$, 7 conjugated double bonds) and neoxanthin ($C_{40}H_{56}O_4$), are widely distributed. While antheraxanthin has one 5,6-epoxy substituent, violaxanthin has both 5,6- and 5′,6′-epoxides. Luteoxanthin contains epoxy groups in the 5,6 and 5′,8′ (often referred to as furanoid) positions; auroxanthin has two furanoid groups. Neoxanthin has a more complex structure, having an allene and a 5′,6′-epoxy groups, with hydroxyls at positions 3, 5 and 3′. The 5,8-epoxy derivative of neoxanthin, neochrome ($C_{40}H_{56}O_4$), is occasionally detected, this is also the case with the 5,6-epoxide of lutein ($C_{40}H_{56}O_3$).

The epoxy carotenoids derived from β-carotene, β-carotene-5,6-epoxide, β-carotene-5,8-epoxide (mutatochrome), β-carotene-5,6, 5′,6′-diepoxide, β-carotene-5,6, 5′,8′-diepoxide (luteochrome) and β-carotene-5,8, 5′,8′-diepoxide (aurochrome), and those of β-cryptoxanthin, especially β-cryptoxanthin-5,6-epoxide and β-cryptoxanthin-5,8-epoxide (cryptoflavin), are also encountered in plant tissues.

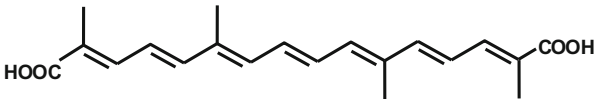
Except for violaxanthin and neoxanthin, epoxy carotenoids are usually detected in trace levels. In spite of their wide distribution, their natural occurrence is often questioned because they can be generated during analysis.



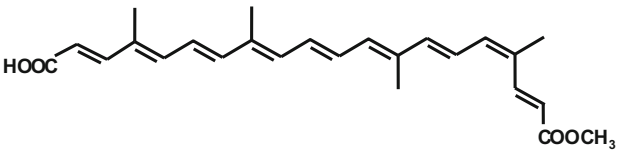
Capsanthin (3R,3′S,5′R)-3,3′-dihydroxy-β,κ-caroten-6′-one



Capsorubin (3R,5R,3′S,5′R)-3,3′-dihydroxy-κ,κ-carotene-6,6′-dione



Crocetin 8,8′-diapocarotene-8,8′-dioic acid



Bixin methyl hydrogen (9′*Z*)-6,6′-diapocarotene-6,6′-dioate

Fig. 3.5 Structures of major carotenoids in food colorants

Species-specific carotenoids (Fig. 3.5) also occur. The most prominent examples are capsanthin ($C_{40}H_{56}O_3$) and capsorubin ($C_{40}H_{56}O_4$), the predominant pigments of red pepper. Capsanthin has one end cyclized into a β-ring and the other end cyclized into a five-membered trimethylcyclopentyl κ-ring (Fig. 3.5). It has two hydroxyls attached at the 3 and 3′ positions and a carbonyl substituent at the 6′ position. With the carbonyl double bond, the total number of conjugated double bonds is 11. Capsorubin has κ-rings at both ends, 2 carbonyl groups, 11 conjugated double bonds.

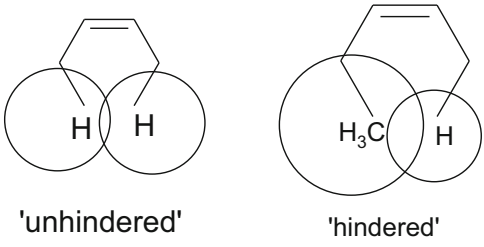
3.3.3 Apocarotenoids

Some carotenoids have their carbon skeletons shortened by the removal of fragments from one (apocarotenoids) or both (diapocarotenoid) ends of the usual C_{40} molecules (Weedon and Moss 1995). Natural examples are bixin, the major pigment of the food colorant annatto, and crocetin, the main yellow coloring component of saffron. Bixin ($C_{25}H_{30}O_4$) is the monomethyl ester of a dicarboxylic *Z*-diapocarotenoid, having a total of 11 conjugated double bonds, 9 carbon-carbon and two carbon-oxygen double bonds (Fig. 3.5). It blends with turmeric or paprika oleoresin, giving rise to yellow to reddish-orange color range in the food. Crocetin, which has a molecular formula of $C_{20}H_{24}O_4$, is a symmetrical diapocarotenoid with seven carbon-carbon double bonds and carboxylic groups at both ends.

3.3.4 Z-Isomers

In nature, carotenoids occur primarily in the generally more thermodynamically stable all-*E* form. Notable exceptions are the first two carotenoids formed biosynthetically, phytoene and phytoflene, which have the 15-*Z* configuration in most natural sources. Another exception is bixin, the principal pigment of the colorant annatto, which occurs naturally in the *Z* form. With the greatly improved efficiency of chromatographic separations, small amounts of *Z* isomers have been detected in plant foods, which increase appreciably during thermal processing and light exposure.

In principle, each carbon-carbon double bond in the polyene chain of carotenoids can isomerize from the *E* to the *Z* form. Some double bonds, however, are prevented from undergoing such isomerization because the *Z* configuration is sterically hindered. This is the case with the C-7,8, C-11,12, C-7′,8′ and C-11′,12′ double bonds, in which steric hindrance can be observed between a hydrogen atom and a methyl group. As illustrated below, this prevents the *Z* configuration for this double bond (Zechmeister et al. 1941; Weedon and Moss 1995; Liaan-Jensen 2004). The *Z* isomers of symmetrical β-carotene (Fig. 3.5) (Lessin et al. 1997; Marx et al. 2000; Dachtler et al. 2001) and zeaxanthin (Dachtler 2001; Humpries and Khachik 2003; Updike and Schwartz 2003; Aman et al. 2005) that are commonly found in foods are the 9-*Z*-, 13-*Z*- and the 15-*Z*-isomers (Fig. 3.6), the formation of which possess relatively little hindrance as it comes from two hydrogen atoms.



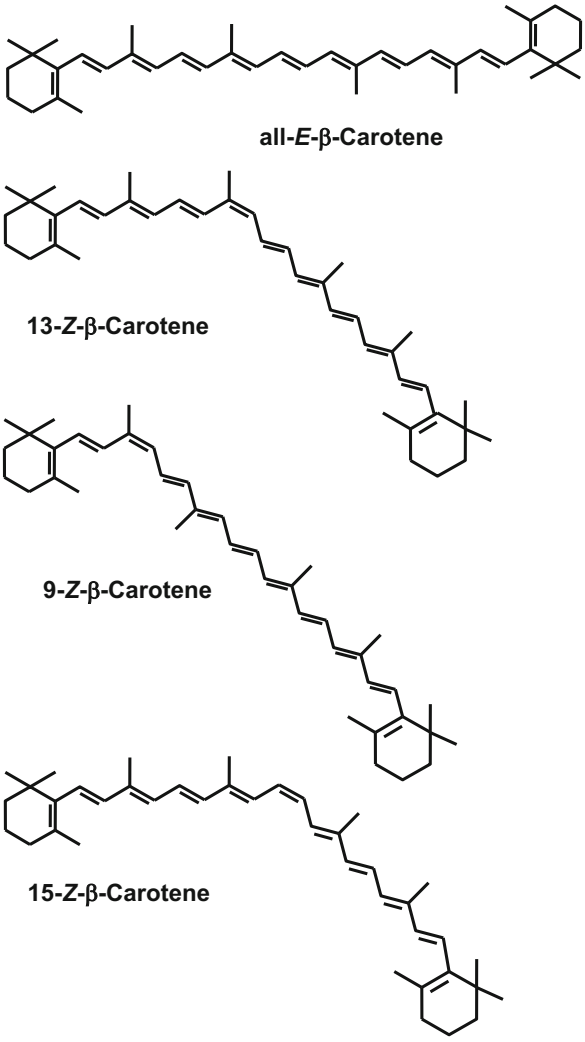


Fig. 3.6 Common geometric isomers of β-carotene

Carbon-carbon double bonds located in the cyclic part of the carotenoid structure, as the C-5,6 double bond in β-carotene, are also sterically hindered and are not isomerized. However, this double bond in the acyclic lycopene is unhindered and 5-*cis*-lycopene is increasingly detected in tomato and tomato products, along with the 9-*Z*-, 13-*Z*-, and 15-*Z*-isomers (Fig. 3.7) (Tiziani et al. 2006; Li et al. 2012; Stinco et al. 2013).

The unsymmetrical all-*E*-α-carotene (Lessin et al. 1997), all-*E*-β-cryptoxanthin (Lessin et al. 1997) and all-*E*-lutein (Fig. 3.8) (Dachtler et al. 2001; Humphries and Khachick 2003; Updike and Schwartz 2003; Aman et al. 2005; Achir et al. 2010) give rise to 13′-*Z*- and 9′-*Z*-isomers in addition to 13-*Z*-, 9-*Z*-, 15-Z-isomers (Fig. 3.8).

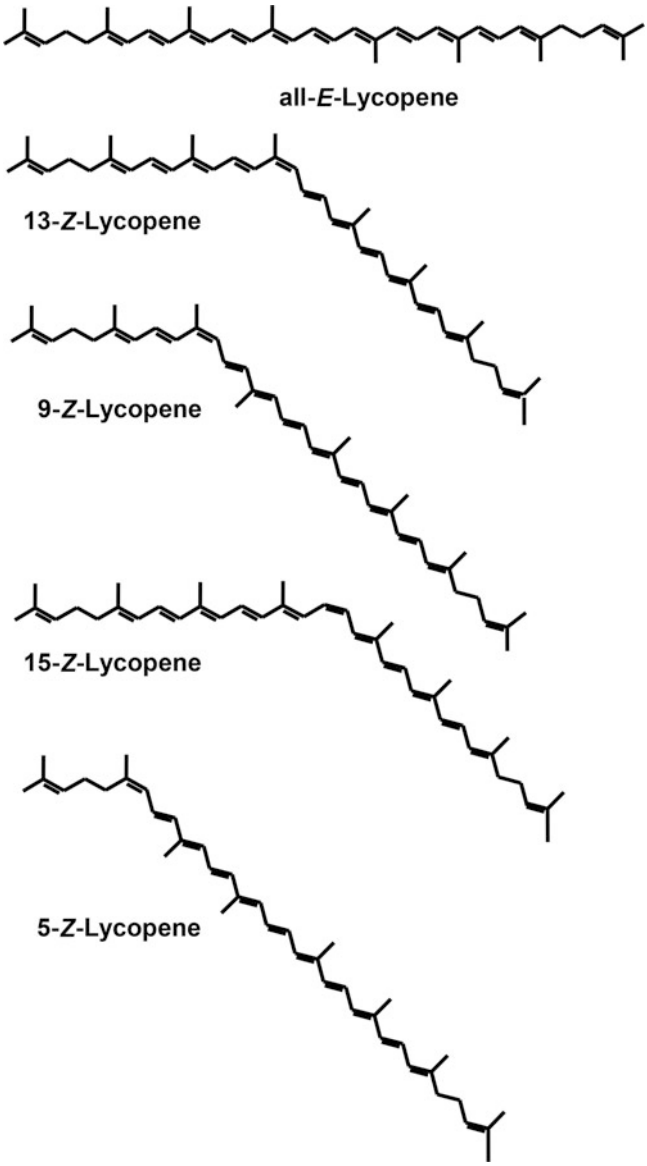


Fig. 3.7 Common geometric isomers of lycopene

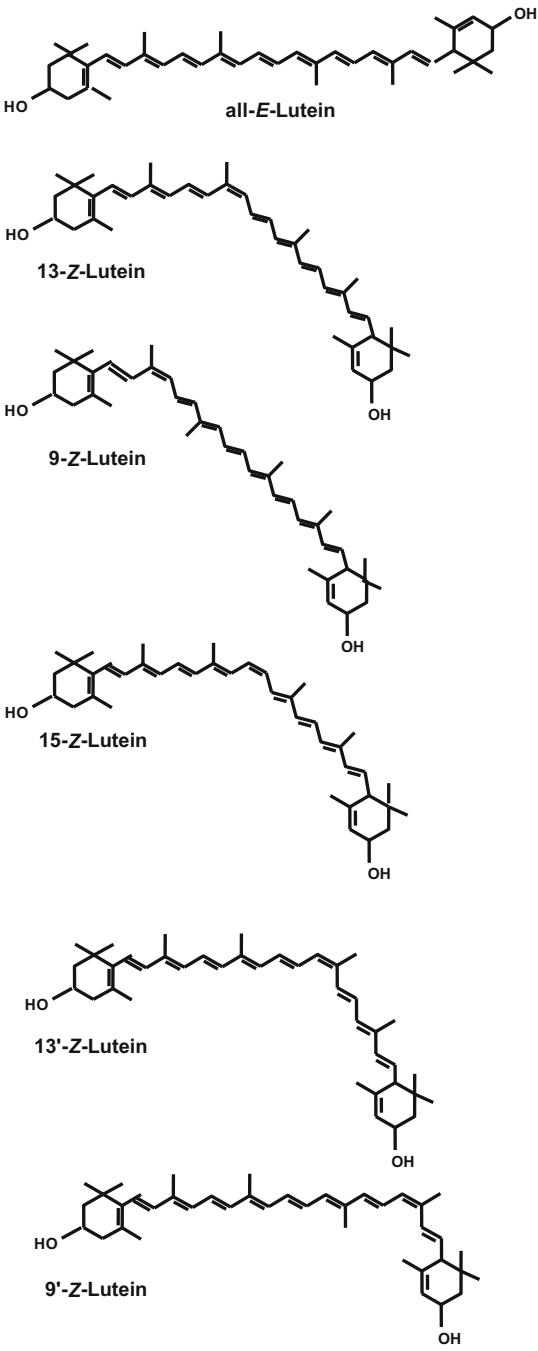


Fig. 3.8 Common geometric isomers of lutein

3.4 Carotenoid Analysis

The development or adoption of methods is generally guided not only by the methods' performance but also by the intended use of the analytical data. Trends in carotenoid analysis have reflected advances and refinements in analytical methodology and instrumentation, commensurate with the evolving knowledge on the actions and functions of these important compounds.

3.4.1 Raman Mapping

Raman mapping provides detailed information on the relative contents and spatial distribution of carotenoids in carrot roots of different colors (Baranska et al. 2006a, b). Near-infrared Fourier transform (NIR-FT) Raman spectroscopy measures β-carotene, lycopene and α-carotene/lutein (with strong bands at 1520, 1510 and 1527 cm^{-1}, respectively) *in situ* without preliminary sample preparation. The Raman mapping technique revealed that β-carotene in the secondary phloem increased gradually from the periderm towards the core, but declined in cells close to the vascular cambium. α-Carotene was deposited in younger cells at a higher rate than β-carotene, while lycopene in red carrots accumulated throughout the whole secondary phloem at the same level. The same research group utilized NIR-FT-Raman spectroscopy in the *in situ* analysis of a variety of raw plant tissues of various species: vegetables (yellow and orange carrots, red tomato, green French bean pods, red pepper), fruits (nectarine, apricot, watermelon), flowers (marigold, chamomile), leaves (ivory, begonia, *Euonymus fortune*) and saffron stigmas (Schulz et al. 2005). The results showed the usefulness of Raman spectroscopy in evaluating the distribution of individual carotenoids in intact plant tissues and in investigating the *E-Z* isomerization of carotenoids during processing.

Raman contour mappings of carrot root cross-sections were also obtained by Gonzalvez et al. (2014) over the spectral range of 500 to 2500 cm^{-1} as a function of the root radius. The Raman intensity signal was very low throughout the xylem tissue, but was high, indicating high carotene concentration, in the secondary phloem and periderm.

3.4.2 Screening

In certain cases (e.g. breeding experiments), a large number of samples has to be analyzed within a short time. Estimating the total carotenoid content is no longer considered adequate for most carotenoid research, but can be employed for this initial screening (Rodriguez-Amaya and Kimura 2004; Kimura et al. 2007). Briefly, the carotenoids are extracted with acetone, transferred to petroleum ether and the

visible absorption spectrum is taken. The total carotenoid content is calculated using the absorbance at the wavelength of maximum absorption and the absorption coefficient of the major carotenoid in the solvent used.

In recent years, near infrared reflectance spectroscopy (NIRS) has been used as a fast, simple, safe and nondestructive technique for the estimation of total carotenoid and/or principal carotenoid contents, principally to assist breeding programs. Sample treatment is minimal, involving only drying and grinding of the sample. NIRS has been employed in carotenoid content estimation in maize (Brenna and Berardo 2004), tritordeum (Atienza et al. 2005), banana (Davey et al. 2009b), potato (Bonierbale et al. 2009), cassava (Sánchez et al. 2014), and summer squash (Martínez-Valdivieso et al. 2014), mostly for germplasm characterization and breeding projects.

Visible spectrometric determination of the total carotenoid content has the disadvantages of being comparatively more lengthy and laborious than NIRS measurement, and it uses organic solvents for extraction. However, the equipment required is usually available in analytical laboratories.

Several analytical techniques and approaches relying on photothermal phenomena have been shown to be applicable for pretreatment-free and rapid screening of carotenoids in foods (Bicanic et al. 2010; Bicanic 2011). Laser photoacoustic spectroscopy (PAS) was used for estimating the total carotenoid content of corn and sweetpotato flours, using UV-visible spectrometry as reference method (Luterotti et al. 2011).

3.4.3 Quantitative Analysis

Since carotenoids differ in their properties (e.g. polarity, stability, bioavailability, antioxidant activity) and health-promoting actions and efficacy, accurate identification and quantification of individual carotenoids are essential. Because of the varied nature of plant matrices and the qualitative and quantitative variation in the carotenoid composition, a single analytical method/procedure is not likely to be appropriate for different plant samples (Rodriguez-Amaya and Kimura 2004; Rodriguez-Amaya 1999; Kimura et al. 2007). Method optimization (e.g de Sá and Rodriguez-Amaya 2004), development/validation (e.g. Hart and Scott 1995; Kimura and Rodriguez-Amaya 2002; Kimura et al. 2007; Akhtar and Bryan 2008) and the evaluation of performance in the analyst's laboratory, even when using a validated method (e.g. Dias et al. 2008), should precede data generation.

Quantitative carotenoid analysis generally consists of (1) sampling, (2) preparation of the analytical sample, (3) extraction, (4) partition to a solvent compatible with the subsequent chromatographic step, (5) saponification and washing (optional), (6) concentration or evaporation of solvent, (7) chromatographic separation, (7) identification, (8) quantification, and (9) data processing and interpretation.

A detailed description of the procedures for quantitative carotenoid analysis is beyond the scope of this chapter. Instead, a brief discussion of the different steps,

sources of errors and precautions to avoid errors will be presented. The readers are referred to Rodriguez-Amaya (1999) and Rodriguez-Amaya and Kimura (2004) for detailed procedures (including weights, volumes and calculations). Quality assurance for carotenoid analysis is discussed in Rodriguez-Amaya (2010).

3.4.3.1 Inherent Difficulties

Several factors make quantitative carotenoid analysis of plant samples challenging (Rodriguez-Amaya 1999; Rodriguez-Amaya and Kimura 2004):

- There is a large number of naturally occurring carotenoids. Conclusive identification is a pre-requisite to accurate quantification.
- The carotenoid composition varies substantially, qualitatively and quantitatively. The analytical procedure should be adapted to the carotenoid composition of the plant sample under investigation.
- The carotenoid concentrations in a given sample vary over a wide range. Preparation of standard solutions and construction of standard curves are done at widely differing levels.
- The plant matrices are diverse and complex. The analytical procedure, especially the preparation of the analytical sample and extraction, should be optimized for each type of matrix.
- The distribution of carotenoids between samples and in a fruit, leaf, seed or root is not uniform. Thus, statistically sound sampling and preparation of the analytical sample should be established.
- The highly unsaturated carotenoid molecules are prone to isomerization and oxidation during analysis and during storage of samples and standards.
- Carotenoids occur at low levels (μg/g), together with much higher concentrations of other compounds that can interfere in the analysis or at least make complete extraction difficult.

The inherent difficulty in carrying out quantitative carotenoid analysis was evident in an interlaboratory study among 26 US and European laboratories, using a baby food composite (Sharpless et al. 1999). The relative expanded uncertainties were higher than those generally expected for certified values. Certified concentrations were provided for some carotenoids, but only reference values could be given for other carotenoids. For lycopene, for example, only reference values were provided because of greater variation in the values obtained, which was attributed to degradation of this analyte in some participating laboratories.

That the difficulties vary with the sample matrix is shown in the results of a European study, involving 14 laboratories. β-carotene and its *cis*-isomers were determined in commercially processed foods (margarine, vitamin drink, pudding powder, natural mixed vegetable), which were chosen according to their type of matrix, their range of concentration and their availability in food stores (Schüep and Schierle 1997). The best repeatability and reproducibility were obtained with the supplemented drink and the worst results with the pudding powder.

Isomerization and oxidation of carotenoids are major causes of analytical errors. Irrespective of the analytical method chosen, precautionary measures to avoid formation of artifacts and quantitative losses should be taken. These include (Schiedt and Liaaen-Jensen 1995; Rodriguez-Amaya 1999): completion of the analysis within the shortest possible time, exclusion of oxygen, protection from light, avoiding high temperature and contact with acid, use of solvents free from damaging impurities (e.g. peroxides), addition of antioxidants (e.g. butylated hydroxytoluene, pyrogallol and ascorbyl palmitate) and neutralizing agents (e.g. MgCO3) (Schiedt and Liaaen-Jensen 1995; Rodriguez-Amaya 1999) and adequate storage conditions or execution of analysis immediately after sample collection (Rodriguez-Amaya 1999).

Loss or change of color at any time during the analysis is an indication of degradation or structural modification.

Oxygen, especially in combination with light and heat, is highly destructive. The presence of even traces of oxygen in stored samples (even at deep freeze temperatures) and of peroxides in solvents (e.g. diethyl ether and tetrahydrofuran) or of any oxidizing agent in extracts of carotenoids can rapidly lead to degradation and the formation of artifacts, such as epoxy carotenoids and apocarotenals (Britton 1991). Oxygen can be excluded at several steps during analysis and during storage with the use of vacuum and a nitrogen or argon atmosphere. Antioxidants may also be used, especially when the analysis is prolonged. They can be added during sample disintegration and saponification or added to solvents and isolates.

Carotenoid analysis from the extraction step must be done under subdued light. The speed of manipulation and the protection from light, especially direct sunlight and ultraviolet light, are particularly important in extracts containing chlorophylls (e.g. extracts of green leafy and non-leafy vegetables) or other potential sensitizers. In the presence of these sensitizers, photodegradation and isomerization can occur very rapidly, even with brief exposure to light. Gold lighting, used by some laboratories to filter out visible light, was shown by O'Neil and Schwartz (1995) to only slow down, not to prevent, sensitized photoisomerization.

3.4.3.2 Storage of Samples and Standards

Ideally, samples should be analyzed as soon as they are collected because it is difficult to avoid changes in the carotenoid composition during storage, even at very low temperature. Moreover, because carotenoid concentration is expressed per unit weight of sample, changes in the sample's weight (e.g. due to loss or gain of water during storage) also affect the final result.

When storage is unavoidable, raw plant samples are better stored intact. Tissue disintegration should be postponed until after storage and then carried out immediately before or simultaneously with extraction to avoid enzymatic oxidation of the carotenoids (Rodriguez-Amaya 1999). Loss of moisture and volatile compounds is also greater with disintegrated tissues, concentrating the carotenoids and leading to overestimation of the concentrations, if corrections are not made. Storage of extracts

should also be avoided or limited to a very short period. Once extracted, carotenoids lose the natural protection of the plant's cellular structure and degrade rapidly, even at low temperature. In carotenoid extracts from banana, for example, a breakdown rate of around 5 % per day was observed even in the dark at −20 °C and in the presence of antioxidants (Davey et al. 2009a).

Lyophilization has been considered the appropriate means of preserving samples that need to be stored before analysis. Davey et al. (2006, 2009a) reported no significant difference in the recoveries of total provitamin A carotenoids from lyophilized and fresh banana, and no further significant loss during storage of lyophilized tissue in the dark at −20 °C, whereas variable losses occurred in frozen pulp tissues. On the other hand, degradation of carotenoids, especially of lycopene, does occur during lyophilization (Craft et al. 1993), and it increases sample porosity and consequently the exposure to oxygen during storage. Moreover, to transform the carotenoid concentrations obtained with lyophilized samples to those of the foods as purchased or as consumed, the preferred form in databases, the moisture content is used. The determination of moisture is simple but it is a proximate analysis; the error from this macrocomponent analysis can be carried over and appreciably affect the microcomponent concentration.

Fractions or isolates should be kept dry under nitrogen or argon or dissolved in a hydrocarbon solvent such as petroleum ether or hexane, and kept at −20 °C or lower when not in use. Leaving carotenoids in solvents such as cyclohexane, dichloromethane, diethyl ether (Craft and Soares 1992) and acetone can lead to substantial degradation. Carotenoids extracted with acetone should be immediately transferred to petroleum ether.

Standard carotenoid crystals should be sealed in ampoules under nitrogen or argon and stored at −20 °C or preferably at −70 °C until use. Stock and working solutions, even when kept at low temperature, have limited validity. The analyst should know when degradation commences under his laboratory's conditions.

3.4.3.3 Sampling

The analytical data can only be reliable and truly useful if the sample taken for analysis is representative of the lot under investigation and is adequately prepared for analysis. Errors introduced in these initial steps cannot be corrected or compensated for in the subsequent steps. Natural variation should be clearly distinguished from analytical errors and compositional data should be accompanied by pertinent information such as the variety, stage of maturity, geographical origin, season, and part of the plant analyzed.

Carotenoids are highly affected by genetic and environmental factors (Rodriguez-Amaya et al. 2008). Aside from the considerable variation between plant materials, in a given fruit, vegetable or other material, compositional variation also occurs due to factors such as cultivar/variety, maturity at harvest, climate/season/geographic site of production, part of the plant utilized, farming practices, harvesting and post-harvest handling, processing and storage conditions.

Sampling is therefore not an easy task (Rodriguez-Amaya 1999; Rodriguez-Amaya and Kimura 2004). All factors that can potentially influence the composition should be accounted for in the sampling plan. Several sample lots are individually submitted to analysis and mean values are reported, accompanied by a statement of uncertainty, usually expressed in terms of the standard deviation.

The more heterogeneous the plant material is, the greater the difficulty and effort needed to obtain the representative composition. Representativity can be enhanced by increasing the sample size, the weight of the subsample submitted to analysis (analytical sample), the degree of comminuting and the number of analytical runs. Because of the heterogeneity of plant samples, a large number of samples should ideally be analyzed. In practice, however, the sampling and sample preparation procedures adopted are usually a compromise between heterogeneity considerations and operational costs. Moreover, the current initiative to apply green, environmentally friendly analytical chemistry recommends minimal sample size and a minimal number of samples (Gałuszka et al. 2013)

The number of analytical runs needed per plant material depends on the compositional variability. In tomato fruit, Darrigues et al. (2008) observed that between-plot field variation accounted for 50 and 52 % of the total variation for lycopene and β-carotene, respectively. The corresponding contributions of within-plot variation were 7 % and 3 %, and of uncontrolled error 43 % and 45 %. There was no significant variance due to replicated extraction or replicated HPLC injection for either carotenoid. Also working with tomato, Dias et al. (2008) concluded from the results of 12 samples harvested in the same region that analyzing five primary samples would be sufficient to estimate the population mean value with a level of confidence of 95 %.

In African bananas and plantains (Davey et al. 2007), analysis of the between-plant, within-plant, within-hand and within-fruit variations of *Musa* varieties cultivated under standardized field conditions demonstrated that the provitamin A carotenoid content varied significantly across all sample groups. It was necessary to collect fruits from hands at the proximal end, the middle and the distal end of the bunch to obtain representative values.

3.4.3.4 Preparation of the Analytical Sample

The sample that is collected and brought to the laboratory is usually too large for analysis, both in bulk and particle size. A small homogeneous, representative subsample should be obtained for analysis. The procedure should be adapted to the nature of the sample, the analyte, the analytical method, and the distribution of the analyte in the sample. Sample preparation and extraction are the most time-consuming and error-prone steps in the analytical process.

Variation in the carotenoid concentration along the longitudinal and across the transversal axes of the cassava root was shown by Chávez et al. (2008). The

carotenoid content was higher in the part of the root closest to its attachment to the stem (proximal section), gradually decreasing towards the opposite end (distal section). Across the root, carotenoid content was higher in the core, lower towards the periphery. Quartering roots and fruits longitudinally is therefore the appropriate manner of reducing the sample's volume to obtain the analytical sample.

Subsampling and homogenization may be done simultaneously. Physical operations, such as chopping, cutting into pieces, mixing, milling, blending and sieving, are carried out, along with bulk reduction, for example, by quartering and riffling. The process can be done manually or through commercially available mills, blenders, grinders, riffle cutters, etc.

Once homogenized, the analytical sample should be weighed and extraction should immediately follow, because tissue disruption releases enzymes (e.g. lipoxygenase) that catalyze carotenoid oxidation and acids that promote geometric isomerization. Usually, sample maceration, homogenization and extraction with an organic solvent are carried out simultaneously.

Currently, the major errors in carotenoid analysis appear to be due to the sampling/sample preparation schemes. In many papers, the sampling plan and reduction of the gross sample to the analytical sample are not described at all or if they are, only superficially. Errors from these initial steps are not observed in intralaboratory and interlaboratory evaluations in which the same homogenized samples are analyzed by the participating analysts/laboratories.

3.4.3.5 Extraction

For efficient extraction, the solvent should be able to penetrate the food matrix and efficiently dissolve the range of carotenoids in the sample, without altering or degrading them. The solvent should not pose toxic effects to the analyst. Because carotenoids are found in a variety of plant materials, the extraction procedure should be adapted to suit the sample being analyzed. Plant samples generally contain large amounts of water, thus water-miscible organic solvents, such as acetone, methanol, ethanol or mixtures thereof are used as extracting solvents to allow good solvent penetration. Dried materials can be extracted with water-immiscible solvents, but extraction is usually more efficient if the samples are rehydrated prior to extraction with water-miscible solvents.

Extraction should be carried out under a fume hood to protect the analyst from inhaling solvent vapor. The sample is generally homogenized with celite (or Hyflosupercel) and the solvent in a suitable mechanical blender for 1–2 min or with a mortar and pestle. Celite facilitates both tissue disintegration and the subsequent filtration. A Waring blender is fast and efficient for mechanical disruption and homogenization of soft fruits and juice. The Polytron homogenizer is widely used because it provides rapid and uniform homogenization. Vortexing has also been employed, but it should only be used for finely ground and easy to extract samples. For samples such as fresh leaves, the simple mortar and pestle is better because small pieces of leaves, which can escape the homogenizer's blades, can be

ground well. Leaves and other difficult-to-extract matrices may also need previous soaking in the extracting solvent (about 15 min for leaves) to soften the cell wall. Prolonged soaking should be avoided to prevent isomerization and degradation of the carotenoids.

Acetone has been the traditional solvent for carotenoid extraction. It has three advantages:

– It dissolves both carotenes and xanthophylls efficiently
– It penetrates the plant matrix well
– Subsequent partitioning to an apolar solvent occurs more easily.

Our HPLC method (Fig. 3.9), which makes use of acetone as extracting solvent, has been validated in our laboratory using a certified reference material (Kimura et al. 2007) and in an international interlaboratory study (Rodriguez-Amaya et al. 2012).

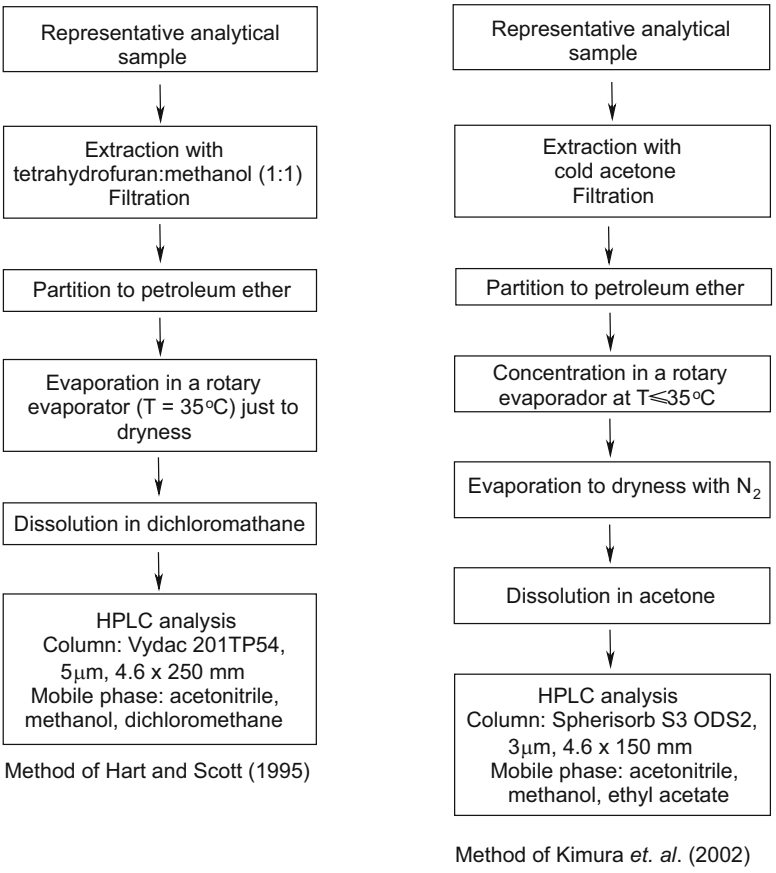


Fig. 3.9 Schematic diagrams of two widely used, validated HPLC methods for the quantitative analysis of carotenoids

Tetrahydrofuran (THF) became a popular extracting solvent when HPLC was introduced. Solubility of both β-carotene and lutein in this solvent was shown to be excellent (Craft and Soares 1992). The widely used method of Hart and Scott (1995) employs THF:methanol (Fig. 3.9). This method was validated in the European interlaboratory study of Scott et al. (1996).

Other solvents, such as hexane, petroleum ether, methanol and ethanol have also been used. The first two solvents readily dissolve carotenes but not the xanthophylls; on the other hand, methanol and ethanol dissolve the xanthophylls efficiently but not the carotenes. Thus, mixtures of solvents such as hexane:ethanol (Taungbodhitham et al. 1998; Lin and Chen 2003; Cortés et al. 2004), hexane:acetone:ethanol (Periago et al. 2004; Barba et al. 2006) and hexane:ethanol:acetone:toluene (Chen et al. 2004) have been employed.

Carotenoids may decompose, dehydrate, or isomerize in the presence of acids. 5,6-Epoxy carotenoids, such as violaxanthin and neoxanthin, readily undergo rearrangement to the 5,8-epoxides. A neutralizing agent (e.g., calcium carbonate, magnesium carbonate, or sodium bicarbonate) may be added during extraction to neutralize acids liberated from the sample itself. Extracting solvents such as chloroform, which has traces of hydrochloric acid, should be avoided. Strong acids and acidic reagents should not be used in rooms where carotenoids are handled. Most carotenoids are stable towards alkali.

In our experience, using cold acetone (left in the refrigerator for a short time before use) and doing the extraction immediately or simultaneously with sample maceration, not only prevents enzymatic oxidation but also makes the addition of neutralizing agents unnecessary.

Filtration can be done with a sintered glass funnel or with a Buchner funnel. The solid residue is returned to the homogenizer and re-extracted with fresh solvent; extraction and filtration are repeated until the residue is colorless (three extractions are usually sufficient).

Using the Placket-Burman experimental design, Periago et al. (2007) examined 15 factors that affect the extraction and quantification of lycopene from tomato and tomato products: sample weight; volume of extraction solution; antioxidant (BHT) concentration; neutralizing agent ($MgCO_3$) concentration; light presence during extraction; homogenization velocity and time; agitation time; temperature during the extraction process; water volume for separation of polar/nonpolar phases; presence of inert atmosphere throughout the process; time, temperature and light presence during separation of phases and time delay for reading. The sample weight, neutralizing agent concentration and water volume for separation of polar/nonpolar phases could be considered the key factors for raw and processed tomato. For tomato sauce, sample weight and volume of extraction solution had the greatest impact on the results.

A European study, with 17 participating laboratories, investigated various possible problem areas, including the chromatographic systems, standardization of carotenoid stock solutions, extraction procedures and data handling, using a lyophilized vegetable mix (Scott et al. 1996). The effect of the chromatographic

system did not appear to be the major variable. In the more experienced laboratory, variation in the standardization of the carotenoid solution was also not thought to be a significant problem. The results suggested that the preparation of the extract might account for about 13 % of the overall variance of around 23 %. Similarly, in an international interlaboratory study involving 19 Asian, African, European, Latin American and US laboratories with widely differing laboratory conditions and experience in carotenoid analysis, using sweetpotato, cassava and corn flours as test materials, incomplete extraction was found to be a major problem (Rodriguez-Amaya et al 2012).

3.4.3.6 Partition

The extract usually contains a substantial amount of water, which can be removed by the partition to hexane, petroleum ether, diethyl ether, or dichloromethane or mixtures of these solvents. This partition also serves as a clean-up step. Diethyl ether or a mixture of ether with hexane or petroleum ether is preferred for extracts with large amounts of xanthophylls, part of which is lost with the washing water during the partition to pure hexane or petroleum ether (Kobori and Rodriguez-Amaya 2013).

A procedure, which has been used for years, still seems to be the best way of carrying out the partition. Small portions of the extract are added to petroleum ether or another appropriate solvent in a separatory funnel (Rodriguez-Amaya 1999). After each addition, water is added gently to avoid formation of an emulsion, preferably by allowing the water to flow along the walls of the funnel. The two layers are allowed to separate, without agitation, and the lower aqueous phase (with the water miscible extracting solvent) is discarded. When the entire extract has been added, the petroleum ether phase is washed four or five times with water to remove residual extracting solvent. In our experience, this procedure is efficient and emulsions are less likely to form.

Alternatively, the acetone extract can be added to petroleum ether in the separatory funnel all at once, followed by addition of water. Some workers then agitate the mixture, but this practice leads to the formation of emulsion, which is difficult to break and results in loss of carotenoids to the aqueous phase. After separation of phases, the lower layer is drawn off and re-extracted with fresh petroleum ether. The combined petroleum ether solution is then washed 4 or 5 times with water.

The solvents used in the extraction or partition is subsequently removed or at least reduced by evaporation, thus, solvents with low boiling points are chosen to avoid prolonged heating. The lower-boiling fractions of petroleum ether (b.p. 30-60 °C or 40-60 °C) is used instead of the higher-boiling fractions. Dichloromethane (b.p. 42 °C) is preferred instead of chloroform (b.p. 61 °C).

3.4.3.7 Saponification

Saponification removes chlorophyll and unwanted lipids; it also hydrolyzes the carotenol esters, simplifying the chromatographic separation. However, it extends the analysis time and is error prone. Considerable losses during this step and the subsequent washing have been reported through the years, especially of lutein, zeaxanthin, violaxanthin and other dihydroxy, trihydroxy and epoxycarotenoids (Khachik et al. 1986; Rodriguez-Amaya et al. 1988; Kimura et al. 1990; Marsili and Callahan 1993; Riso and Porrini 1997; Yue et al. 2006), although provitamin A carotenoids (α-carotene, β-carotene, γ-carotene, β-cryptoxanthin) appeared stable (Rodriguez-Amaya et al. 1988; Kimura et al. 1990). The extent of carotenoid degradation depends on the conditions used, being greater with higher concentration of alkali and hot saponification (Kimura et al. 1990).

Saponification should therefore be included in the analytical procedure only when indispensable. It is unnecessary, for example, in the analysis of leafy vegetables, tomato and carrot, all of which are low-lipid materials and essentially free of carotenol esters. The chlorophylls co-extracted with carotenoids from leaves can be separated during chromatography. For the high-lipid dry corn, saponification is dispensable when gradient elution is used (Rodriguez-Amaya and Kimura 2004).

If required, this step should be thoroughly evaluated and optimized, and the subsequent washing carefully done to avoid losing carotenoids with the discarded aqueous phase (de Sá and Rodriguez-Amaya 2004). Complete hydrolysis of carotenol esters to the free carotenoids should also be verified.

Losses of lutein during saponification and the subsequent washing are frequently underestimated, as in leafy vegetables. Except in lettuce, if the lutein content is almost equal to or lower than that of β-carotene in a leafy vegetable, as is sometimes seen in the literature, lutein loss during analysis is strongly indicated.

Concern about the negative effects of saponification has led analyst to shorten the duration of ambient temperature saponification (e.g., to 1 or 2 h). In our experience, however, longer saponification times are required for complete hydrolysis of carotenol esters.

Saponification is best done after partition of the carotenoids to petroleum ether or hexane, by adding an equal volume of 10 % methanolic potassium hydroxide. The mixture is left overnight at room temperature in the dark, after which the carotenoid solution is washed about 5 times with water to remove the alkali. To avoid losing carotenoids into the washing water, especially the more polar xanthophylls, this step should be done in the same manner as in the partition, described above. When apocarotenals (e.g., β-citraurin in citrus) are present in the sample, all traces of acetone must be removed before saponification to avoid aldol condensation between the apocarotenals and acetone.

3.4.3.8 Concentration or Evaporation of the Solvent

The carotenoid solution, after partitioning for unsaponified sample or after washing for saponified samples, is dried with anhydrous sodium sulfate and concentrated in a rotary evaporator at reduced pressure and a temperature $\leq$35 °C. If complete removal of the solvent is desired, concentration in the rotary evaporator is done first, and evaporation to dryness is accomplished with a stream of nitrogen or argon. Bringing the carotenoid solution to complete dryness in the rotary evaporator increases the possibility of degradation, especially of lycopene (Tonucci et al. 1995), and may leave the carotenoids tightly adhered to the glass walls, making quantitative removal from the flask difficult.

3.4.3.9 Chromatographic Separation

Reversed-phase high performance liquid chromatography (HPLC) has been the chromatographic technique most widely used for quantitative carotenoid analysis for over two decades. Compilations of HPLC methods for carotenoids can be found in review articles (Su et al. 2002; Rivera and Canela-Garayoa 2012; Amorim-Carrilho et al. 2014). Two examples of widely used, validated HPLC methods are shown in Fig. 3.9.

Two developments in HPLC advanced quantitative carotenoid analysis considerably:

- Columns that provide baseline separation of the range of carotenoids in a given food, up to the separation of geometric isomers
- The photodiode array detector that provides the visible absorption spectra of the separated carotenoids on-line.

The most widely used column for carotenoid separation for some time was the polymeric C_{18} Vydac 201TP54 (250 x 4.6 mm, 5 μm) column. It has been surpassed by the polymeric C_{30} YMC (250 x 4.6 mm, 5 μm) column. Especially designed for the separation of carotenoid isomers (Sander et al. 1994), the C_{30} stationary phase has gained wide application in food analysis in general (Sander et al. 2000).

Carotenoid separation has been carried out with 5 μm spherical particles packed in a 250 × 4.6 mm column. The C18 columns with 5 μm particles have often been found not to provide adequate separation of food carotenoids, especially the geometric isomers. Shorter columns with smaller particles have demonstrated greater separation efficiency and requires much less mobile phase. The C18 Spherisorb ODS 2 (150 x 4.6 mm, 3 μm) column used in our laboratory, for example, has provided excellent chromatograms for the carotenoids of different foods, including those with complex carotenoid composition, such as leafy vegetables (Kimura and Rodriguez-Amaya 2002; Kobori and Rodriguez-Amaya 2008), squashes and pumpkins (Azevedo-Meleiro and Rodriguez-Amaya 2007), pepper (Azevedo-Meleiro and Rodriguez-Amaya 2009) and the tropical fruit *Eugenia uniflora* L (Azevedo-Meleiro and Rodriguez-Amaya 2004).

As mobile phases for carotenoids, the primary solvents have been acetonitrile and methanol. In all our work using the 3 μm C_{18} column and in most of those employing the Vydac column, the mobile phase has these two solvents. Ammonium acetate improves the recovery of carotenoids from the column when added to acetonitrile-based solvents. The addition of triethylamine to the mobile phase, which contains ammonium acetate, further increases recovery, from around 60 % to over 90 % (Hart and Scott 1995).

For the C_{30} column, a combination of methanol and methyl-*tert*-butyl ether has been used in majority of the studies.

Since geometric isomers of carotenoids have different bioavailability and antioxidant activity, when measurable amounts of the *Z*-isomers are present, their separation and quantification should be carried out, especially in health-oriented studies. Examples of foods in which quantification of the *Z*-isomers is important are thermally prepared or processed foods (Lessin et al. 1997), such as tomato and tomato products (Tavares and Rodriguez-Amaya 1994), prepared/processed corn (Aman et al. 2005; de Oliveira and Rodriguez-Amaya 2007), carrot juice (Marx et al. 2000) and cooked spinach (Glaser et al. 2003). Fresh foods generally have negligible amounts of *Z*-isomers (Godoy and Rodriguez-Amaya 1994, 1998). An exception is cassava root in which appreciable levels of (9-*Z*)-β-carotene and (13-*Z*)-β-carotene accompany the principal carotenoid, (all-*E*)-β-carotene (Kimura et al. 2007). The two columns cited above can be used for this separation: the 3 μm C18 column and the C30 column. Identification is easier in the former because the *Z*-isomers elute close to the corresponding all-*E*-carotenoid, but separation of the isomers is better in the latter. Equivalent results were obtained for (all-*E*)-, (9-*Z*-)- and 13-*Z*-β-carotene of cassava when these two columns were used (Kimura et al. 2007).

Gradient elution should be used only when the analysis cannot be done isocratically. Isocratic separation is rapid and results in stable baseline and more reproducible retention times. Gradient elution, on the other hand, has the advantages of greater resolving power, improved sensitivity and elution of strongly retained compounds. It is more likely to resolve the whole range of carotenoids found in a given food. However, it has several disadvantages:

- Increased complexity
- Need for column re-equilibration between runs
- Greater differential detector's response (i.e., different detector's signals for the same concentration of different compounds)
- Often poor reproducibility

Good solvent miscibility is required to prevent baseline disturbance resulting from outgassing and refractive index effects (Craft et al. 1992).

In the 1990s, lycopene had drawn analytical concern because of reported low recoveries from the HPLC column (Konings and Roomans 1997), high intralaboratory (Hart and Scott 1995) and interlaboratory (Scott et al. 1996) coefficients of variation and low range of linearity (Riso and Porrini 1997). Each laboratory should verify if their analytical procedure and/or chromatographic system lead to loss of this carotenoid.

3.4.3.10 Identification

Since inconclusive or even erroneous identifications could be found in the literature, Schiedt and Liaaen-Jensen (1995) recommended the following minimum criteria for the identification of carotenoids:

1. The visible (or ultraviolet for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least two different solvents must be in agreement with the chromophore suggested
2. Chromatographic properties must be identical in two systems, preferably TLC (R_F) and HPLC (t_R), and co-chromatography with an authentic sample should be demonstrated
3. A mass spectrum should be obtained, which allows at least the confirmation of the molecular mass.

The chromatographic behavior and the ultraviolet and visible absorption spectrum provide the first clues for the identification of carotenoids. Both the position of the absorption maxima (λmax) and the shape (fine structure) of the spectrum reflect the chromophore. Tables giving the λmax values for carotenoids are available in many reviews and books published through the years (e.g. Britton 1995; Rodriguez-Amaya 1999). Identification of carotenoids based solely on the retention times/co-chromatography with standards or the absorption spectra may lead to erroneous conclusions. Different carotenoids can have the same retention time in a given chromatographic system, and different carotenoids may have the same chromophore, thus presenting the same absorption spectrum. However, carotenoids with well-known structure can be conclusively identified by the combined and judicious use of chromatographic behavior, UV-visible absorption spectra and, for xanthophylls, specific group chemical reactions (Azevedo-Meleiro and Rodriguez-Amaya 2004). In the absence of mass spectra, the chemical tests can confirm the type, location and number of functional groups (Eugster 1995; Rodriguez-Amaya 1999).

The absorption spectra of carotenoids are markedly solvent dependent. This has to be remembered in HPLC when spectra are taken by the photodiode array detector in mixed solvents in isocratic elution and in varying mixed solvents in gradient elution. The λmax values recorded in hexane, petroleum ether, diethyl ether, methanol, ethanol and acetonitrile are practically the same, but higher by 2–6 nm in acetone, 10–20 nm in chloroform, 10–20 nm in dichloromethane and 18–24 nm in toluene (Britton 1995).

Many laboratories around the world now have access to HPLC-MS. Aside from the molecular formula, important characteristic fragment ions facilitate the identification of carotenoids. Enzell and Back (1995) tabulated data for 170 different carotenoid end groups.

Several ionization methods have been used for the MS analysis of carotenoids: electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI) atmospheric pressure photoionization (APPI), and atmospheric pressure solids analysis probe (ASAP) (Enzell and Back 1995; van Breemen 1995, 1997; Dachtler et al. 2001; Aman et al. 2005).

EI was the most common ionization method. Carotenoids generally give good molecular ions and many fragmentations diagnostic of particular structural features had been identified (Enzell and Back 1995).

APCI has rapidly become the most widely used ionization technique. APCI produces molecular ions and/or protonated or deprotonated molecules, depending upon the mobile phase conditions and abundant fragment ions, especially for xanthophylls (van Breemen 1997). The main advantage of APCI is its high linearity of detector response over a carotenoid concentration range of at least three orders of magnitude, suggesting that APCI LC/MS might be the preferred mass spectrometric technique for carotenoid quantification. Disadvantages of APCI include the multiplicity of molecular ion species, which might lead to ambiguous molecular weight determinations because it tends to reduce the abundance of the molecular ions.

Fruits and flowers have a complicated mixture of esterified carotenoids, which are difficult to identify in the ester form. HPLC-APCI/MS was used for the identification of carotenoid esters in mango (Pott et al. 2003), red orange essential oils (Dugo et al. 2008), marigold flowers, several fruits (Breithaupt et al. 2002), tritordeum grains (Mellado-Ortega and Hornero-Méndez 2012), wolfberries, Chinese lanterns, orange pepper and sea buckthorn (Weller and Breithaupt 2003).

Tandem mass spectrometry (MS/MS) offers many advantages for the analysis of carotenoids. Analyzing zeaxanthin and its oxidation products in biological samples, Prasain et al. (2005) affirmed that tandem mass spectrometric analysis offers added selectivity and specificity and requires minimal sample clean-up, leading to high sample throughput. It reduces interference by impurities in the extract and allows the following (Rivera and Canela-Garayoa 2012):

- Minimal sample clean-up
- Distinguishing between carotenoids that coelute
- Information about structural isomers
- A decrease in overall analysis time.

A limitation of MS for carotenoids is its inability to distinguish between all-*E*- and *Z*-isomers and between 5,6- and 5,8-epoxy carotenoids. HPLC-NMR (Dachtler et al. 2001; Glaser et al. 2003; Aman et al. 2005) and off-line NMR (Tiziani et al. 2006) have been utilized particularly for the unambiguous identification of geometric isomers. One- and two-dimensional NMR successfully determined the carotenoid profile of typical tomato juice, including the Z-isomers, with minimal purification procedures (Tiziani et al. 2006).

Xanthophylls undergo functional group chemical reactions that can serve as simple and rapid tests for the identification of carotenoids (Eugster 1995; Rodriguez-Amaya 1999). Primary and secondary hydroxyl groups are acetylated by acetic anhydride in pyridine. Allylic hydroxyls, isolated or allylic to the chromophore, are methylated with acidic methanol. Both the acetylated and methylated products have unchanged chromophores, thus unaltered UV-visible spectra, but are less polar than the original carotenoids.

Epoxy groups in the 5,6 or 5,6,5′,6′ positions are easily detected by their facile conversion to the furanoid derivatives in the presence of an acid catalyst, reflected by a hypsochromic shift of 20–25 nm or 40–50 nm, respectively.

Apocarotenals undergo reduction with LiAlH4 or NaBH4, manifested by the appearance of the three-maxima spectra of the resulting hydroxyl carotenoid, in lieu of the single broad band of the original apocarotenal.

Iodine-catalyzed *cis-trans* isomerization, which can be done directly in the spectrometer cuvette, results in a 3–5 nm shift of the λmaxs of all-*E*-carotenoids to lower wavelengths, whereas those of *cis* carotenoids (such as 15-*cis* and 13-*cis*-β-carotene) will shift to longer wavelengths after 1–5 min exposure to light.

3.4.3.11 Quantification

HPLC quantification is usually carried out by external calibration with the respective standards, although internal standardization has also been used. Because carotenoids absorb maximally at different wavelengths and have different coefficients of absorption, a carotenoid cannot be quantified accurately using another carotenoid as standard, as is sometimes seen in the literature.

In the calibration process, for each carotenoid being quantified, the analyst has to prepare standard solutions of varying concentrations (usually 5 concentrations), inject each solution usually in triplicate, and construct the standard curve (Rodriguez-Amaya and Kimura 2004). The standard curve should be linear, pass through the origin, and should bracket the expected concentrations of the food samples.

The concentration of the standard solution is determined by visible absorption spectrometry, using the absorption coefficient of the carotenoid being quantified (Rodriguez-Amaya and Kimura 2004). Absorption coefficients of the major all-*E*-carotenoids in foods in specific solvents are available (Britton 1995; Rodriguez-Amaya 1999). For *Z*-carotenoids, the absorption coefficients of the corresponding all-*E*-carotenoid is used. Since the coefficients for *Z*- isomers are generally lower, this practice underestimates the *Z*-isomer contents (Liaaen-Jensen 2004).

Since quantification is based on the detector's response for the analyte compared to the response for standard solutions of known concentrations, errors introduced in the preparation of the standard solutions, determination of the concentrations and construction of the calibration curves will be reflected in the analytical results. The purity of the standards should be verified and the concentrations of the standard solutions corrected accordingly. If necessary (i.e., low purity), the standards should be repurified.

Carotenoid standards are costly, unstable and often unavailable. Open column chromatography can be used to separate and isolate standards with purity comparable or even better than that of commercial standards (Kimura and Rodriguez-Amaya 2002; Rodriguez-Amaya and Kimura 2004; Kimura et al. 2007). This procedure has been used in other laboratories (e.g. Morris et al. 2004; Gama and de Sylos 2007; Giuffrida et al. 2007; Griffiths et al. 2007).

Even when HPLC-MS is used for identification, quantification has been carried out mostly with the DAD detector. As exceptions, Frenich et al. (2005), Matsumoto et al. (2007), and Slavin et al. (2009) quantified with HPLC-MS by selective ion monitoring (SIM). Weller and Breithaupt also quantified zeaxanthin esters by HPLC-MS.

3.4.3.12 UHPLC-DAD Methods

HPLC-DAD has provided a large body of reliable and detailed data on carotenoid composition of foods. However, because of environmental and economic concerns, the search for faster methods that use less organic solvents has continued.

Developments in column technology and instrumentation have led to ultra-high-performance liquid chromatography (UHPLC). This technique, in comparison with HPLC, uses narrow bore, shorter columns (2.1×50 mm vs. 4.6×200 mm); less run time (10 min vs. 30 min); lower flow rate (0.3 mL/min vs. 1 mL/min); lower injection volume (1.4 μL vs. 20 μL) and solvent volume/sample (4.0 mL vs. 27 mL); smaller particle size (2 μm vs. 2–5 μm); much higher back pressure (up to 103.5 MPa vs. 35–40 MPa) (Boboyo-Gil et al. 2012; Rivera and Canela-Garayoa 2012). High strength silica (HSS) C18 and T3 and ethylene bridged hybrid (BEH) C18 stationary phases have been successfully used to separate carotenoids.

Several advantages have been cited for UHPLC over conventional HPLC, including: (a) faster analyses due to shorter retention times; (b) narrower peaks, giving increased signal-to-noise ratio, and (c) higher resolution and sensitivity (Rivera and Canela-Garayoa 2012). Moreover, it is estimated that UHPLC typically saves at least 80 % of mobile phase compared to HPLC (Chen and Kord 2009); much lower solvent consumption is in accordance with green analytical chemistry.

Nevertheless, as with the HPLC methods, UHPLC methods have to undergo rigorous method standardization, intralaboratory and interlaboratory validation to guarantee data reliability. Special attention should be given to sampling and sample preparation because the smaller the analytical sample, the more difficult it is to guarantee representativity. There is also a tendency to transform the sample into dried or lyophilized, ground fine powder (Li et al 2012, 2013; Rivera and Canela 2012; Kaulmann et al. 2014), considered to be more amenable to small-scale analysis. Both drying and grinding can lead to substantial losses of carotenoids (Rodriguez-Amaya 1999, 2010).

UHPLC has been used for the quantification of carotenoids in corn (Rivera and Canela 2012; Rivera et al. 2013), Brassica vegetables (Guzman et al. 2012), durum wheat (Hung and Hatcher 2011), milk (Chauveau-Duriot et al. 2010), tomato (Li et al. 2012, 2013; van Meulebroek et al. 2012), honeybee pollen, pumpkin, and nectarine (Boboyo-Gil 2012), buckthorn berries and leaves (Pop et al. 2014), *Brassica oleraceae* and plum varieties (Kaulmann et al. 2014). In milk, the UHPLC method gave similar concentration of (all-*E*)-β-carotene to that obtained by an HPLC method (Chauveau-Duriot et al. 2010). UHPLC was compared to HPLC, using seven carotenoid standards, and the quantified concentrations were statistically indistinguishable (Boboyo-Gil et al. 2012)

3.4.3.13 Other Techniques and Methods

Carotenoid extraction with organic solvents generates large amounts of waste, the disposal of which is an environmental problem. An environmentally friendly alternative is supercritical fluid extraction with CO_2, which also has the advantages of inertness, low toxicity and reactivity. This technique has been applied to the extraction of carotenoids from food samples without a modifier (Gómez-Prieto et al. 2003) or with ethanol or methanol as modifier (Barth et al. 1995; Mathiasson et al. 2002). Several vegetable samples obtained with a supercritical CO_2 procedure with ethanol as modifier, performed in approximately 30 min, averaged 23 % higher than those of ethanol-pentane extraction, performed in 90 min (Marsili and Callahan 1993).

Other environmentally friendly extraction methods have been introduced, such as microwave-assisted extraction (Xiao et al. 2012; Hiranvarachat et al. 2013), accelerated solvent extraction (Breithaupt 2004; Sun et al. 2012), ultrasound-assisted extraction (Sun et al. 2006; Yue et al. 2006; Lianfu and Liu Zelong 2008; Li et al. 2013). Microwave-assisted extraction, which shortens the extraction time and improves the yield of the extraction, is simple and is not limited by solvent selectivity (Kiss et al. 2000). Ultrasound assisted extraction was used in quantifying lutein from chicken liver by HPLC (Sun et al. 2006). With three samples, the concentrations of lutein in the ultrasound assisted solvent extracted samples had higher levels (6.0, 10.4 and 5.5 μg/g) than those of saponified solvent extracted samples (2.9, 4.5, 2.5 μg/g).

Matrix solid-phase dispersion has been proposed for the rapid, mild (without artifact formation), complete and reproducible extraction of carotenoid *Z/E* isomers as shown with raw and cooked spinach samples (Glaser et al. 2003). It was also used for carotenoids in corn flour and kiwi (Gentili and Caretti 2011). In comparison to liquid-liquid extraction (LLE) and solid-phase extraction (SPE), matrix solid-phase dispersion (MSPD) is easy to handle because the solid or viscous biological tissue can be directly mixed with the sorbent material; it combines the advantages of being time-saving and requiring less solvent (Dachtler et al. 2001). As the carotenoids elute in a small fraction, the evaporation of large amounts of solvent, as is usual in liquid-liquid extraction, is not necessary. With MSPD, the carotenoids are protected against oxidation and isomerization during the quick and gentle extraction process.

Isocratic separation and determination of carotenoids in vegetables by capillary electrochromatography was found to effectively resolve β-carotene, lycopene and lutein (Herrero-Martínez et al. 2006). The concentrations of β-carotene and lutein in carrot and spinach, and of lycopene in tomato obtained with this technique were within the ranges reported in the literature. Recently, ultra-high performance supercritical fluid chromatogaphy (UHPSFC) was applied to carotenoids in paprika oleoresin (Berger and Berger 2013).

Attenuated total reflectance infrared spectroscopy (ATR-IR) (Baranska et al. 2006b; de Nardo et al. 2009), Fourier transform infrared spectroscopy (FTIR) (Rubio-Diaz et al. 2011), Raman spectroscopy (Bhosale et al. 2004; Darwin et al. 2007) and NIRS (Pedro and Ferreira 2005; Clément et al. 2008) have been proposed for the determination of carotenoids. Values obtained by NIRS and

HPLC were found to be in good agreement (Berardo et al. 2004; Brenna and Berardo 2004), indicating the potential of NIRS as a quantitative method. However, in a comparison of Fourier transform-Raman (FT-Raman), ATR-IR, and NIRS for measuring lycopene and β-carotene, ATR-IR showed the best statistics. The prediction quality of Raman was poorer, and NIRS had the worst prediction potential (Baranska et al. 2006b).

It was also shown that X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) could be used for direct measurement of the major carotenoid in the annatto seed (Felicissimo et al. 2004).

As with the HPLC methods, these alternative procedures or methodologies should undergo standardization and validation, including interlaboratory studies, before they can be adopted for wider use.

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