# A GUIDE TO CAROTENOID ANALYSIS IN FOODS

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# PREFACE

There is a worldwide consensus — in different fields of studies and in programs to control micronutrient deficiency and promote better human health — that more extensive and accurate data on the carotenoid composition of foods are urgently needed. Carotenoid analysis, however, is inherently complicated. Nevertheless, the difficulty can be eased if the analyst is provided with sufficient background information about these fascinating compounds and is well informed of the problems associated with their identification and quantification.

For many years we have worked on various aspects of food carotenoids. This monograph is an attempt to pass on our accumulated experience in the hope that others can study these compounds without much frustration, in less time, at lower cost, and with greater reliability. Although written with the would-be carotenoid analysts in mind, some informations herein presented and discussed may also be useful to workers in this area.

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I greatly appreciate the efforts of several people who contributed to the publication of this work: Drs. Frances Davidson and Penelope Nestel who saw it through to completion; Drs. Gary Beecher and Steven J. Schwartz for carefully evaluating the scientific content; the OMNI Research staff, Suzanne Harris, Paula Trumbo, and Dorothy Foote; Judith Dickson for editing; Kenn Holmberg for the layout; and Marcos Antonio de Castro for preparing the first manuscript.

Delia B. Rodriguez-Amaya

# NATURE OF CAROTENOIDS IN FOODS

Food carotenoids are usually $C_{40}$ tetraterpenoids built from eight $C_5$ isoprenoid units, joined so that the sequence is reversed at the center. The basic linear and symmetrical skeleton, which can be cyclized at one or both ends, has lateral methyl groups separated by six C atoms at the center and five C atoms elsewhere. Cyclization and other modifications, such as hydrogenation, dehydrogenation, double-bond migration, chain shortening or extension, rearrangement, isomerization, introduction of oxygen functions, or combinations of these processes, result in a myriad of structures. A distinctive characteristic is an extensive conjugated double-bond system, which serves as the light-absorbing chromophore responsible for the yellow, orange, or red color that these compounds impart to many foods. Hydrocarbon carotenoids (i.e., carotenoids made up of only carbon and hydrogen) are collectively called carotenes; those containing oxygen are termed xanthophylls. In nature, they exist primarily in the more stable all-*trans* isomeric form, but *cis* isomers do occur. The first two $C_{40}$ carotenoids formed in the biosynthetic pathway have the 15-*cis* configuration in plants. The presence of small amounts of *cis* isomers of other carotenoids in natural sources has been increasingly reported.

Because plants are able to synthesize carotenoids de novo, the carotenoid composition of plant foods is enriched by the presence of small or trace amounts of biosynthetic precursors, along with derivatives of the main components. Although commonly thought of as plant pigments, carotenoids are also encountered in some animal foods. Animals are incapable of carotenoid biosynthesis, thus their carotenoids are diet derived, selectively or unselectively absorbed, and accumulated unchanged or modified slightly into typical animal carotenoids.

In the early stages of carotenoid biosynthesis, the $C_5$ primer for chain elongation undergoes successive additions of $C_5$ units, yielding in sequence $C_{10}$, $C_{15}$, and $C_{20}$ compounds. Dimerization of the latter produces phytoene, the first $C_{40}$ carotenoid. The succeeding transformations are schematically shown in Figure 1, a perusal of which, though complicated at first glance, makes carotenoid composition of foods comprehensible.

The sequential introduction of double bonds at alternate sides of phytoene (3 conjugated double bonds) gives rise to phytofluene (5 conjugated double

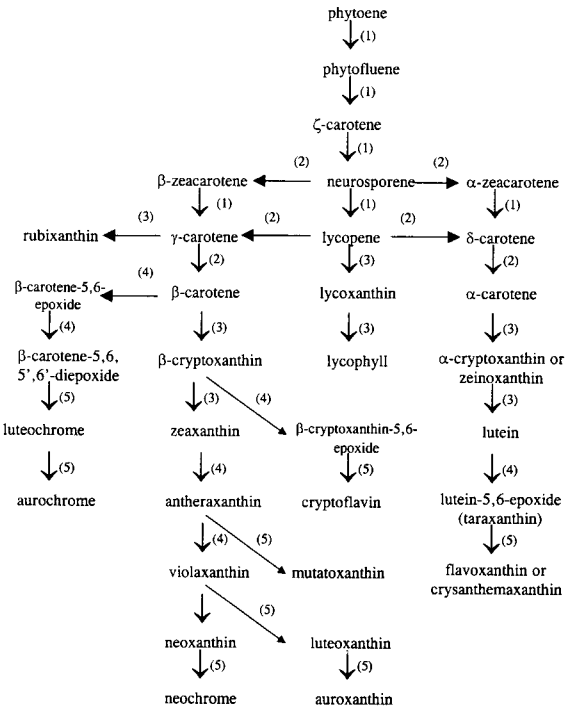


**Figure 1.** Later stages of carotenoid biosynthesis and possible transformations of carotenoids. Reactions: 1) desaturation, 2) cyclization, 3) hydroxylation, 4) epoxidation, and 5) epoxide-furanoxide rearrangement.

bonds), ζ-carotene (7 conjugated double bonds), neurosporene (9 conjugated double bonds), and lycopene (11 conjugated double bonds). With the cyclization of one or both ends of the molecule, the biosynthetic pathway branches out, forming the monocyclic β-zeacarotene and γ-carotene and the bicyclic β-carotene on one side and the monocyclic α-zeacarotene and δ-carotene and the bicyclic α-carotene on the other side. α-Carotene may also be produced through γ- carotene, the β ring being formed before the ε ring. Hydroxylation leads to the formation of rubixanthin (monohydroxy) from γ-carotene and to lycoxanthin (monohydroxy) and lycophyll (dihydroxy) from lycopene. Introduction of a hydroxyl group in β-carotene results in β-cryptoxanthin and of a second hydroxyl group, in zeaxanthin. Similar modifications of α-carotene produces the monohydroxy α-cryptoxanthin or zeinoxanthin and the dihydroxy lutein. Epoxidation of β-carotene, β-cryptoxanthin, zeaxanthin, and lutein yields a large number of epoxy carotenoids.

A semisystematic nomenclature, that conveys structural information, has been devised for carotenoids (Table 1), but for the sake of simplicity, the better known trivial names will be used throughout this monograph. Also, although the E/Z designation is now favored to indicate the configuration of the double bonds, the still widely used *cis*/*trans* terminology will be retained because it is more readily recognized by workers in the food field. Absolute configuration will not be dealt with.

## Common Food Carotenoids

Of the acyclic carotenes (Figure 2), lycopene and ζ-carotene are the most common. Lycopene is the

**Table 1.** Trivial and semisystematic names of common food carotenoids

| Trivial name | Semisystematic name |
|---|---|
| Antheraxanthin | 5,6-epoxy-5,6-dihydro-β,β-carotene-3,3′-diol |
| Astaxanthin | 3,3′-dihydroxy-β,β-carotene-4,4′-dione |
| Auroxanthin | 5,8,5′,8′-diepoxy-5,8,5′,8′-tetrahydro-β,β-carotene-3,3′-diol |
| Bixin | methyl hydrogen 9′-*cis*-6,6′-diapocarotene-6,6′-dioate |
| Canthaxanthin | β,β-carotene-4,4′-dione |
| Capsanthin | 3,3′-dihydroxy-β,κ-caroten-6′-one |
| Capsorubin | 3,3′-dihydroxy-κ,κ-carotene-6,6′-dione |
| α-Carotene | β,ε-carotene |
| β-Carotene | β,β-carotene |
| β-Carotene-5,6-epoxide | 5,6-epoxy-5,6-dihydro-β,β-carotene |
| β-Carotene-5,8-epoxide (mutatochrome) | 5,8-epoxy-5,8-dihydro-β,β-carotene |
| β-Carotene-5,6,5′,6′-diepoxide | 5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene |
| δ-Carotene | ε,ψ-carotene |
| γ-Carotene | β,ψ-carotene |
| ζ-Carotene | 7,8,7′,8′-tetrahydro-ψ,ψ-carotene |
| Crocetin | 8,8′-diapocarotene-8,8′-dioic acid |
| α-Cryptoxanthin | β,ε-caroten-3′-ol |
| β-Cryptoxanthin | β,β-caroten-3-ol |
| Echinenone | β,β-caroten-4-one |
| Lutein | β,ε-carotene-3,3′-diol |
| Lutein-5,6-epoxide (taraxanthin) | 5,6-epoxy-5,6-dihydro-β,ε-carotene-3,3′-diol |
| Lycopene | ψ,ψ-carotene |
| Neoxanthin | 5′,6′-epoxy-6,7-didehydro-5,6,5′,6′-tetrahydro-β,β-carotene-3,5,3′-triol |
| Neurosporene | 7,8-dihydro-ψ,ψ-carotene |
| Phytoene | 7,8,11,12,7′,8′,11′12′-octahydro-ψ,ψ-carotene |
| Phytofluene | 7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene |
| Rubixanthin | β,ψ-caroten-3-ol |
| Violaxanthin | 5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-β,β-carotene-3,3′-diol |
| α-Zeacarotene | 7′,8′-dihydro-ε,ψ-carotene |
| β-Zeacarotene | 7′,8′-dihydro-β,ψ-carotene |
| Zeaxanthin | β,β-carotene-3,3′-diol |
| Zeinoxanthin | β,ε-carotene-3-ol |

principal pigment of many red-fleshed fruits and fruit vegetables, such as tomato, watermelon, red-fleshed papaya and guava, and red or pink grapefruit. ζ-Carotene is more ubiquitous but it is usually present at low levels except in Brazilian passion fruit (Mercadante et al. 1998) and in carambola (Gross et al. 1983), in which it occurs as a major pigment. Phytoene and phytofluene are probably more widely distributed than reported; because they are both colorless and vitamin A–inactive, their presence may often be overlooked. Neurosporene has limited occurrence and is normally found in small amounts.

The bicyclic β-carotene (Figure 3) is the most widespread of all carotenoids in foods, either as a minor or as the major constituent (e.g., apricot, carrot, mango, loquat, West Indian Cherry, and palm fruits). The bicyclic α-carotene and the monocyclic γ-carotene sometimes accompany β-carotene, generally at much lower concentrations. Substantial amounts of α-carotene are found in carrot and some varieties of squash and pumpkin (Arima and Rodriguez-Amaya 1988, 1990) and substantial amounts of γ-carotene are found in rose hips and *Eugenia uniflora* (Cavalcante and Rodriguez-Amaya 1992). Less frequently encountered is δ-carotene, although it is the principal carotenoid of the high delta strain of tomato and the peach palm fruit (Rodriguez-Amaya et al., unpublished).

The hydroxy derivatives of lycopene, lycoxanthin and lycophyll (Figure 4), are rarely encountered; they are found in trace amounts in tomato. Rubixanthin, derived from γ-carotene, is the main pigment of rose hips and also occurs in an appreciable level in *E. uniflora* (Cavalcante and Rodriguez-Amaya 1992).

The xanthophylls α-cryptoxanthin and zeinoxanthin (Figure 4) are widely distributed, although generally at low levels. β-Cryptoxanthin is the main pigment of many orange-fleshed fruits, such as peach, nectarine, orange-fleshed papaya, persimmon, fruit of the tree tomato, and *Spondias lutea*, but occurs rarely as a secondary pigment.

In contrast to the relative abundance of the parent carotenes, α- and β-carotene, respectively, lutein is normally present in plant tissues at considerably higher levels than is zeaxanthin. Lutein is the predominant carotenoid in leaves, green vegetables, and yellow flowers. Except for yellow corn and the Brazilian fruit *Cariocar villosium*, in which it is the major pigment (Rodriguez-Amaya et al., unpublished), zeaxanthin is a minor food carotenoid. This is not surprising considering that the precursor β-carotene is the

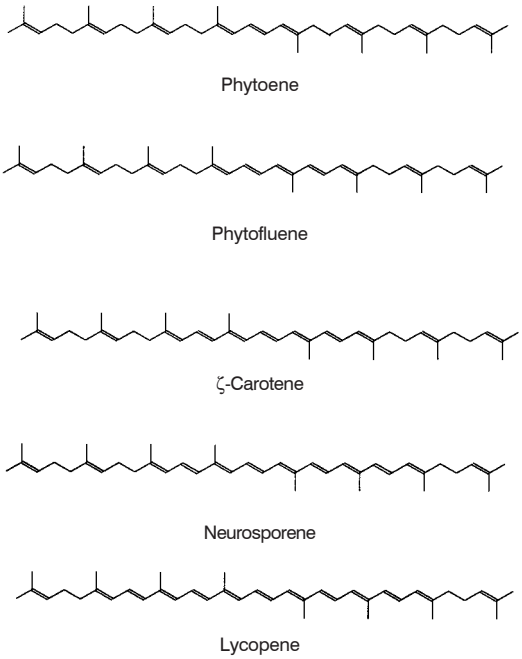


**Figure 2.** Acyclic carotenes.

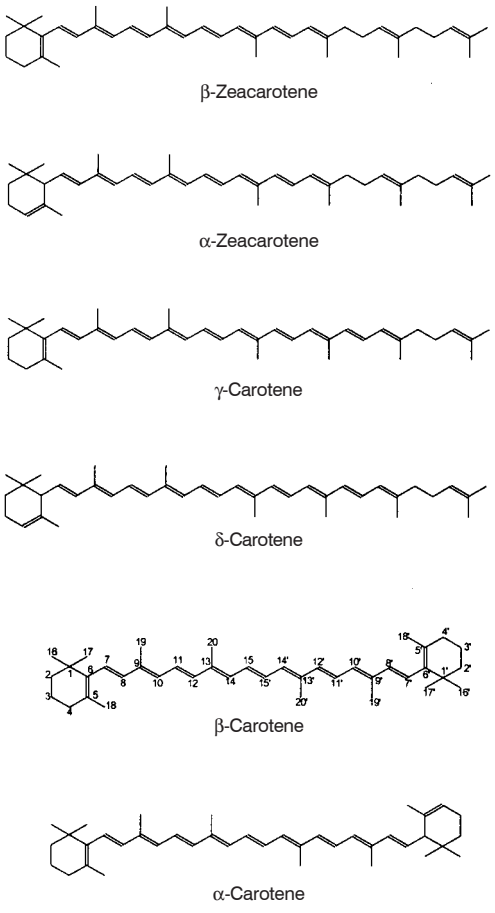


**Figure 3.** Cyclic carotenes.

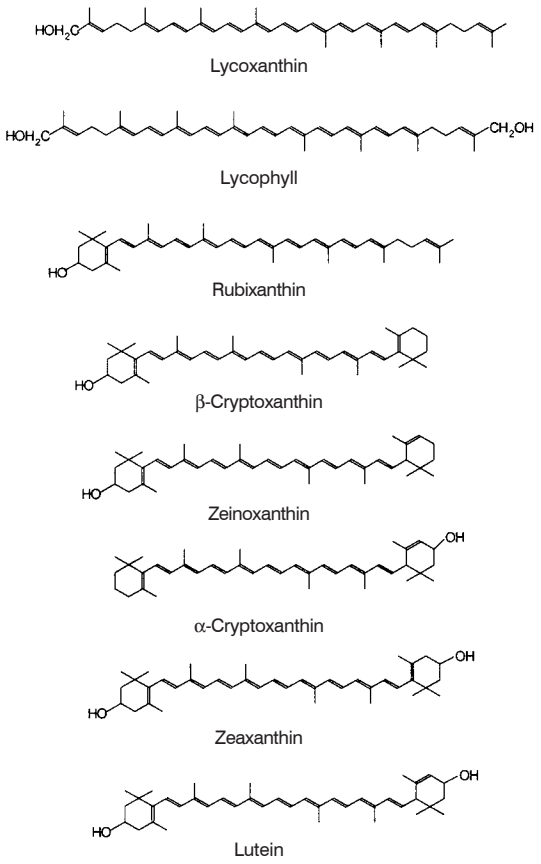


**Figure 4.** Carotenols (hydroxycarotenoids).

preponderant pigment of many foods and whatever zeaxanthin is formed is easily transformed to antheraxanthin and, especially, violaxanthin (Figure 5). Lutein appears to undergo limited epoxidation.

Because of its facile degradation, the epoxycarotenoid violaxanthin may be underestimated in foods, as was shown for mango (Mercadante and Rodriguez-Amaya 1998). Other epoxides (Figure 5) are also frequently encountered, but because they can be formed during analysis, their natural occurrence is often questioned.

The existence of uncommon or species-specific carotenoids (Figure 6) has also been demonstrated. The most prominent examples are capsanthin and capsorubin, the predominant pigments of red pepper. Other classical examples of unique carotenoids are bixin, the major pigment of the food colorant annatto, and crocetin, the main coloring component of saffron.

Although green leaves contain unesterified hydroxy carotenoids, most carotenols in ripe fruit are esterified with fatty acids. However, the carotenols of a few fruits, particularly those that remain green when ripe, such as kiwi (Gross 1982b), undergo limited or no esterification.

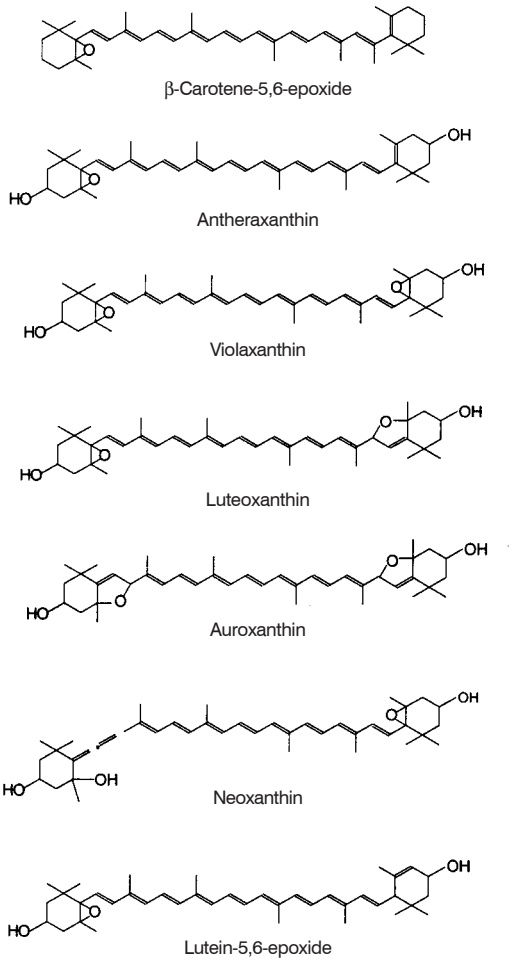


**Figure 5.** Epoxycarotenoids.

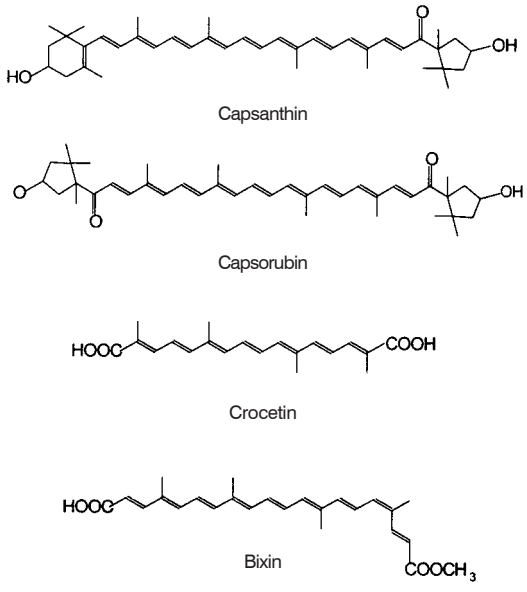


**Figure 6.** Some unique carotenoids.

Astaxanthin (Figure 7) is the principal carotenoid of some fish, such as salmon and trout, and most crustaceans (e.g., shrimp, lobster, and crab). The intermediates in the transformation of dietary carotenoids, such as echinenone and canthaxanthin, are often detected as accompanying minor carotenoids. Tunaxanthin is also a major carotenoid of fish.

Structurally, vitamin A (retinol) is essentially one-half of the molecule of β-carotene with an added molecule of water at the end of the lateral polyene chain. Thus, β-carotene (Figure 3) is a potent provitamin A to which 100% activity is assigned. An unsubstituted β ring with a $C_{11}$ polyene chain is the minimum requirement for vitamin A activity. γ-Carotene, α-carotene (Figure 3), β-cryptoxanthin, α-cryptoxanthin (Figure 4), and β-carotene-5,6-epoxide (Figure 5), all of which have one unsubstituted ring, would have about half the bioactivity of β-carotene. The acyclic carotenoids (Figure 2), which are devoid of β rings, and the xanthophylls other those mentioned above (Figures 4–7), in which the β rings have hydroxy, epoxy, and carbonyl substituents, are not provitamins A.

Other biologic functions or actions attributed to carotenoids (e.g., prevention of certain types of cancer, cardiovascular disease, and macular degeneration) are independent of the provitamin A activity and have been attributed to an antioxidant property of carotenoids through singlet oxygen quenching and deactivation of free radicals (Palozza and Krinsky 1992, Burton 1989, Krinsky 1989). The ability of carotenoids to quench singlet oxygen is related to the conjugated double-bond system, and maximum protection is given by those having nine or more double bonds (Foote et al. 1970). The acyclic lycopene was observed to be more effective than the bicyclic β-carotene (Di Mascio et al. 1989); thus, in recent years studies related to human health have focused on lycopene. Results obtained with a free radical–initiated system also indicated that canthaxanthin and astaxanthin, in both of which the conjugated double-bond system is extended with carbonyl groups, were better antioxidants than β-carotene and zeaxanthin (Terão 1989). Zeaxanthin, along with lutein, is the carotenoid implicated in the prevention of age-related macular degeneration, however.

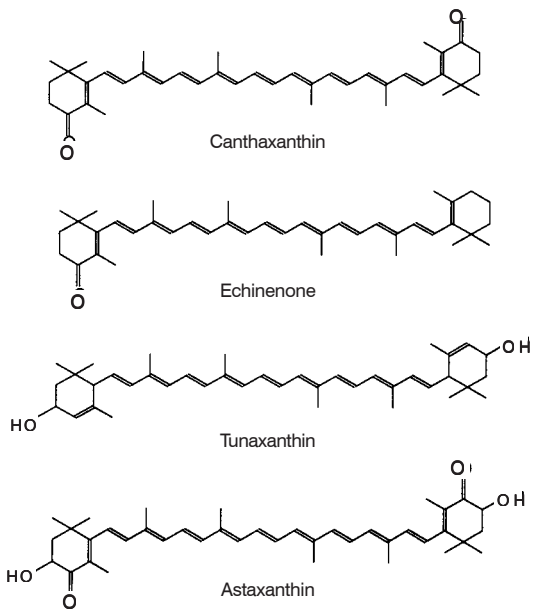


**Figure 7.** Some typical animal carotenoids.

## Composition of Carotenoids in Foods

Most of the papers presenting quantitative data on food carotenoids are limited to provitamin A carotenoids. This monograph will emphasize work that includes at least the major nonprovitamin A carotenoids; provitamin A carotenoids were the focus of two recent reviews (Rodriguez-Amaya 1997, 1996).

Leaves have a strikingly constant carotenoid pattern, often referred to as the chloroplast carotenoid pattern, the main carotenoids being lutein (about 45%), β-carotene (usually 25–30%), violaxanthin (15%), and neoxanthin (15%) (Britton 1991). The absolute concentrations vary considerably (Table 2). α-Carotene, β-cryptoxanthin, α-cryptoxanthin, zeaxanthin, antheraxanthin, and lutein 5,6-epoxide are also reported as minor carotenoids. Lactucaxanthin is a major xanthophyll in a few species, such as lettuce. Other green vegetables, such as broccoli, follow the same pattern as green leafy vegetables (Table 2).

In contrast to leafy and other green vegetables, fruits, including those used as vegetables, are known for their complex and variable carotenoid composition. The major carotenoid composition of some fruits and fruit vegetables are shown in Table 3 to demonstrate the considerable qualitative and quantitative variations. Some palm fruits (e.g., *buriti*) are especially rich in carotenoids, particularly provitamin A carotenes.

**Table 2.** Major provitamin A and nonprovitamin A carotenoids of leafy and nonleafy green vegetables

| Reference, origin of sample, and chromatographic technique[a] | Common English/ Portuguese name, edible portion analyzed, and scientific name | Variety or cultivar and number of sample lots analyzed | Concentration, µg/g edible portion, raw[b] | |
|---|---|---|---|---|
| | | | Provitamin A carotenoids | Nonprovitamin A carotenoids |
| Mercadante and Rodriguez-Amaya (1990) São Paulo, Brazil (OCC) | Beldroega (leaves) *Portulaca oleracea* | Undefined *n*=5 | β-Carotene (30±8) | Neoxanthin (9±2), lutein+ violaxanthin (48±8) |
| Khachik et al. (1992b) Maryland, U.S.A. (HPLC) | Broccoli (flowerets) *Brassica oleracea* | Botrytis *n*=3 | β-Carotene (23±1) | Neoxanthin (6.3±1.0), violaxanthin (14±1), lutein-5,6-epoxide (6.4±1.1), lutein (24±2), cis-lutein (4.4±0.4) |
| Mercadante and Rodriguez-Amaya (1990) São Paulo, Brazil (OCC) | *Caruru* (leaves) *Amaranthus viridis* | Undefined *n*=5 | β-Carotene (110±6), α-cryptoxanthin (1.3±1.2) | Neoxanthin (43±5), lutein+ violaxanthin (237±50), zeaxanthin (8.2±6.5) |
| Wills and Rangga (1996) Sydney, Australia (HPLC) | Chinese cabbage (leaves) *Brassica pekinensis* | Undefined *n*=3 | β-Carotene (22) | Zeaxanthin (2), lutein (27), violaxanthin (3), neoxanthin (2) |
| Wills and Rangga (1996) Sydney, Australia (HPLC) | Chinese spinach (leaves) *Amaranthus tricolor* | Undefined *n*=3 | β-Carotene (20) | Zeaxanthin (6), lutein (29), violaxanthin (19), neoxanthin (13) |
| Mercadante and Rodriguez-Amaya (1990) São Paulo, Brazil (OCC) | *Mentruz* (leaves) *Lepidium pseudodidynum* | Undefined *n*=5 | β-Carotene (85±19) | Neoxanthin (36±6), lutein+ violaxanthin (164±32), zeaxanthin (1.0±2.1) |
| Mercadante and Rodriguez-Amaya (1990) São Paulo, Brazil (OCC) | *Serralha* (leaves) *Sonchus oleraceus* | Undefined *n*=5 | β-Carotene (63±14) | Neoxanthin (29±6), lutein+ violaxanthin (145±52), zeaxanthin (3.1±5.7) |
| Mercadante and Rodriguez-Amaya (1990) São Paulo, Brazil (OCC) | *Taioba* (leaves) *Xanthosoma* spp. | Undefined *n*=5 | β-Carotene (67±21), α-cryptoxanthin (1.0±1.4) | Neoxanthin (40±10), lutein+ violaxanthin (172±38), zeaxanthin (2.7±6.0) |
| Chen and Chen (1992) Taipei, Taiwan (HPLC) | Water convolvulus *Ipomoea aquatica* | Undefined *n*=5 | β-Carotene (100±8), *cis*-β-carotene (6.8±0.8) | Neoxanthin (50±5), violaxanthin (60±5), lutein epoxide (29±3), lutein (78±7), *cis*-lutein (11±1) |
| Wills and Rangga (1996) Sydney, Australia (HPLC) | Water spinach *Ipomoea aquatica* | Undefined *n*=3 | β-Carotene (4) | Neoxanthin (16), violaxanthin (25), zeaxanthin (5), lutein (6) |
| Wills and Rangga (1996) Sydney, Australia (HPLC) | Water cress *Rorippa nasturtium* aquaticum | Undefined *n*=3 | β-Carotene (15) | Neoxanthin (12), violaxanthin (3), zeaxanthin (7), lutein (26) |

[a] OCC, open-column chromatography; HPLC, high-performance liquid chromatography.
[b] Only carotenoids at ≥1 µg/g are included. Unless otherwise stated, the carotenoids are in the *trans* form.

**Table 3.** Major provitamin A and nonprovitamin A carotenoids of fruits and fruit vegetables

| Reference, origin of sample, and chromatographic technique[a] | Common English/ Portuguese name, edible portion analyzed, and scientific name | Variety or cultivar and number of sample lots analyzed | Concentration, µg/g edible portion, raw[b] | |
|---|---|---|---|---|
| | | | Provitamin A carotenoids | Nonprovitamin A carotenoids |
| Khachik et al. (1989) Maryland, U.S.A. (HPLC) | Apricot (pulp) *Prunus armeniaca* L. | Blenum *n*=1 | β-Carotene (64) | |
| Godoy and Rodriguez-Amaya (1995a) Piauí, Brazil (OCC) | *Buriti* (pulp) *Mauritia vinifera* Mart | Undefined *n*=5 | 13-*cis*-β-Carotene (1.5±1.4), α-carotene (80±9), 13-*cis*-β-carotene (3.8±2.9), β-carotene (360±32), β-zeacarotene (5.4±1.5), γ-carotene (37±4) | ζ-Carotene (4.6±0.5), zeaxanthin (20±4) |
| Rodriguez-Amaya and Kimura (1989) Pernambuco, Brazil | *Cajá* (pulp + peel) *Spondias lutea* | Undefined *n*=5 | β-Carotene (1.6±0.2), β-cryptoxanthin (16±2), cryptoflavin (1.8±0.7) | Zeinoxanthin (4.3±0.6) |
| Rodriguez-Amaya et al. (1983) São Paulo, Brazil (OCC) | Fruit of the tree tomato (pulp) *Cyphomandra betacea* | Undefined *n*=3 | β-Carotene (7.9±3.6), β-cryptoxanthin (14±4) | Lutein (1.7±1.1) |
| Rouseff et al. (1992) Florida, U.S.A. (HPLC) | Grapefruit (pulp) *Citrus paradisi* Macf. | Ruby red *n*=6 | β-Carotene (4.2±1.4) | Lycopene (2.2±0.9), phytoene (2.5±0.5), phytofluene (1.8±0.5) |
| | | Flame *n*=3 | β-Carotene (8.6±1.6) | Lycopene (7.9±2.0), phytoene (11±1), phytofluene (6.0±0.6) |
| | | Ray ruby *n*=3 | β-Carotene (7.0±1.7) | Lycopene (21±9), phytoene (5.0±0.4), phytofluene (2.5±0.1) |
| | | Star Ruby *n*=3 | β-Carotene (9.6±1.6) | Lycopene (33±3), phytoene (51±4), phytofluene (17±4) |
| Padula and Rodriguez-Amaya (1986) São Paulo, Brazil (OCC) | Guava (whole fruit) *Psidium guajava* L. | cv. IAC-4 *n*=4 | β-Carotene (3.7±0.7) | Zeinoxanthin (1.0±0.6), lycopene (53±6), trihydroxy-5,8-epoxy-β-carotene (4.0±0.3) |
| Pernambuco, Brazil (OCC) | | Undefined *n*=3 | β-Carotene (12±5) | Zeinoxanthin (1.9±0.7), lycopene (53±14), trihydroxy-5,8-epoxy-β-carotene (2.1±1.9) |
| Godoy and Rodriguez-Amaya (1995b) São Paulo, Brazil (OCC) | Loquat (pulp) *Eriobotrya japonica* Lindl. | Mizuho *n*=6 | β-Carotene (7.8±0.3), β-cryptoxanthin (4.8±0.1) | Neurosporene (1.1±0.3), violaxanthin (1.6±0.1) |
| Mercadante et al. (1998) Bahia, Brazil (HPLC) | Mango (pulp) *Mangifera indica* L. | Keitt *n*=3 | β-Carotene (15±2) | Luteoxanthin isomers (3.8±0.6), violaxanthin (21±3), 9-*cis*-violaxanthin (10±0), 13-*cis*-violaxanthin (1.4±0.1), neoxanthin (2.1±1.3), |

**Table 3.** Major provitamin A and nonprovitamin A carotenoids of fruits and fruit vegetables (continued)

| Reference, origin of sample, and chromatographic technique[a] | Common English/ Portuguese name, edible portion analyzed, and scientific name | Variety or cultivar and number of sample lots analyzed | Concentration, µg/g edible portion, raw[b] | |
|---|---|---|---|---|
| | | | Provitamin A carotenoids | Nonprovitamin A carotenoids |
| Kimura et al. (1991) São Paulo, Brazil (OCC) | Papaya (pulp) *Carica papaya* | Common, orange *n*=5 | β-Carotene (1.2±0.9), β-cryptoxanthin (8.1±1.7), β-cryptoxanthin-5,6-epoxide (2.0±1.1) | |
| Bahia, Brazil (OCC) | | Solo *n*=5 | β-Carotene (2.5±1.0), β-cryptoxanthin (9.1±2.4) | ζ-carotene (1.4±0.8), lycopene (21±16) |
| São Paulo, Brazil (OCC) | | Formosa *n*=5 | β-Carotene (1.4±0.5), β-cryptoxanthin (5.3±1.1), β-cryptoxanthin-5,6-epoxide (3.8±1.3) | ζ-carotene (1.7±0.6), antheraxanthin (1.8±0.1), lycopene (19±4) |
| Bahia, Brazil (OCC) | | Formosa *n*=5 | β-Carotene (6.1±1.4), β-cryptoxanthin (8.6±2.2), β-cryptoxanthin-5,6-epoxide (1.8±0.8) | ζ-carotene (1.5±0.3), antheraxanthin (3.3±0.4), lycopene (26±3) |
| Bahia, Brazil (OCC) | | Tailandia *n*=5 | β-Carotene (2.3±0.7), β-cryptoxanthin (9.7±1.8), β-cryptoxanthin-5,6-epoxide (2.1±0.3) | ζ-carotene (2.0±0.4), antheraxanthin (4.0±2.9), lycopene (40±6) |
| Hart and Scott (1995) Norwich, UK (HPLC) | Pepper, orange *Capsicum annuum* L. | Undefined *n*=4[c] | α-Carotene (6.4), β-carotene (8.9), *cis*-β-carotene (2.4), β-cryptoxanthin (7.8) | Lutein (25), zeaxanthin (85) |
| Cavalcante and Rodriguez-Amaya (1992) Pernambuco, Brazil (OCC) | *Pitanga* (pulp + peel) *Eugenia uniflora* | Undefined *n*=18 | β-Carotene (9.5±2.1), β-cryptoxanthin (47±2), γ-carotene (53±4) | Phytofluene (13±2), ζ-carotene (4.7±1.6), unidentified (3.4±0.4), lycopene (73±1), rubixanthin (23±2) |
| Arima and Rodriguez-Amaya (1988, 1990) São Paulo, Brazil (OCC) | Squash and pumpkin (pulp) *Cucurbita moschata* | Menina verde (immature) *n*=5 | β-Carotene (0.8–2.5) | Lutein (0.7–7.4) |
| | | Menina verde *n*=5 | α-Carotene (8.3–42), β-carotene (14–79), α-cryptoxanthin (tr–2.3) | *cis*-ζ-Carotene (0.9–20), α-zeacarotene (nd–13), lutein (tr–6.4), *cis*-lutein (0.2–3.1) |
| Bahia, Brazil (OCC) | | Baianinha *n*=3 | α-Carotene (17–82), β-carotene (125–294), *cis*-β-carotene (4.9–30), α-cryptoxanthin (2.2–2.8) | *cis*-ζ-Carotene (4.9-30), zeinoxanthin (tr-6.3), lutein (4.8-14) |
| São Paulo, Brazil (OCC) | *Cucurbita maxima* | Exposição *n*=5 | β-carotene (3.1–28), α-cryptoxanthin (ND-3.5) | Lutein (7.2–25), *cis*-lutein (ND–9.7), zeaxanthin (ND–9.7), taraxanthin (ND–3.6), violaxanthin (ND–26), neoxanthin (ND–4.2) |

**Table 3.** Major provitamin A and nonprovitamin A carotenoids of fruits and fruit vegetables (continued)

| Reference, origin of sample, and chromatographic technique[a] | Common English/ Portuguese name, edible portion analyzed, and scientific name | Variety or cultivar and number of sample lots analyzed | Concentration, µg/g edible portion, raw[b] | |
|---|---|---|---|---|
| | | | Provitamin A carotenoids | Nonprovitamin A carotenoids |
| Bahia, Brazil (OCC) | | Jerimum Caboclo *n*=3 | β-Carotene (14–34), *cis*-β-carotene (1.5–2.7), α-cryptoxanthin (tr–6.7) | *cis*-ζ-Carotene (1.5–2.7), α-cryptoxanthin-5,6-epoxide (nd–8.8), lutein (6.4–129), taraxanthin (nd–6.0) |
| São Paulo, Brazil (OCC) | Hybrid | Tetsukabuto *n*=5 | β-Carotene (8.7–18), β-cryptoxanthin (0.8–18) | Neurosporene (nd-5.4), zeinoxanthin (0.6-10), lutein (3.5–34), zeaxanthin (tr–6.5), taraxanthin (nd–8.5), *cis*-violaxanthin (tr–2.7) |
| Hart and Scott (1995) Norwich, UK (HPLC) | Tomato *Lycopersicon esculentum* | Cherry *n*=4 | β-Carotene (4.7) | Lutein (1.0), lycopene (27) |
| | | Flavortop *n*=4 | β-Carotene (4.3) | Lycopene (50) |
| | | Tigerella *n*=4 | β-Carotene (17) | Lutein (1.9), lycopene (12) |
| | | Ida F1 hybrid *n*=4 | β-Carotene (9.6) | Lutein (1.0), lycopene (13) |
| | | Shirley F1 *n*=4 | β-Carotene (7.7) | Lycopene (21) |
| | | Craig *n*=4 | β-Carotene (11) | Lutein (1.5), lycopene (29) |
| | | Moneymaker *n*=4 | β-Carotene (4.3) | Lycopene (35) |
| | | Allicanti *n*=4 | β-Carotene (5.2) | Lycopene (37) |
| | | Beefsteak *n*=4 | β-Carotene (8.8) | Lycopene (27) |
| | | Sungold (yellow) *n*=4 | β-Carotene (22) | Lutein (2.0), lycopene (3.9) |
| Khachik et al. (1992b) Maryland, U.S.A. (HPLC) | | Undefined *n*=3 | β-Carotene, *trans*+*cis* (2.8±0.2) | Lutein (1.3±0.2), lycopene (39±0), neurosporene (3.0±0.1), ζ-carotene (8.4±0.2), phytofluene (5.1±0.8), phytoene (6.0±1.0) |
| Tavares and Rodriguez-Amaya (1994) São Paulo, Brazil (OCC) | | Santa Cruz *n*=10 | β-Carotene (5.1±1.1) | Lycopene (31±20), *cis*-lycopene (3.0±2.4), phytofluene (3.7±4.6) |
| Cavalcante and Rodriguez-Amaya (1992) Pernambuco, Brazil (OCC) | West Indian Cherry (pulp + peel) *Malpighia glabra* | Undefined *n*=18 | β-Carotene (26±4), β-cryptoxanthin (3.6±0.7) | |
| Ceará, Brazil (OCC) | | Undefined *n*=4 | β-Carotene (22±1), β-cryptoxanthin (2.1±0.4) | |
| São Paulo, Brazil (OCC) | | Undefined *n*=4 | β-Carotene (4.0±0.6) | |

[a] OCC, open-column chromatography; HPLC, high-performance liquid chromatography.
[b] Only carotenoids at ≥ 1µg/g are included. Unless otherwise stated, the fruits are ripe and the carotenoids are in the *trans* form. ND, not detected; tr, trace.
[c] Analyzed as one composite sample.

Carotenoids are not widely distributed in root crops. Carrot, in which β-carotene and α-carotene predominate (Table 4), and yellow to orange sweet potatoes, with β-carotene as principal carotenoid, are well-known carotenoid-rich roots. Corn is an example of a carotenogenic seed, although the concentrations are not high.

## Factors Influencing Carotenoid Composition

The carotenoid composition of foods are affected by factors such as cultivar or variety; part of the plant consumed; stage of maturity; climate or geographic site of production; harvesting and postharvest handling; processing and storage (Rodriguez-Amaya 1993, Gross 1991, 1987). A close look at some published values reveals discrepancies that surpass those expected from the effects of these factors, indicating analytic inaccuracy. The analyst must take utmost care to differentiate between natural and analytic variations.

Cultivar or varietal differences can be only in terms of the quantitative composition, because essentially the same carotenoids are found in the different varieties. This is the case with American grapefruit, Brazilian red-fleshed papaya, and British tomato, as shown in Table 3, and Finnish carrot (Table 4). Greater variations, both qualitative and quantitative, can be observed in squash and pumpkin (Table 3), capsicums (Rahman and Buckle 1980), gooseberry (Gross 1982/83), mandarin (Gross 1987), and plums (Gross 1984).

Carotenoids are not evenly distributed in the food itself. Various investigators, for example, found that carotenoids are usually more concentrated in the peel than in the pulp of fruits and fruit vegetables. In the Brazilian *cajá* the total carotenoid content in the deseeded fruit (pulp plus peel) was 26 μg/g whereas that of the pulp alone was 17 μg/g (Rodriguez-Amaya and Kimura 1989). In the *Cucurbita* hybrid *tetsukabuto*, the pulp and the peel had 56 and 642 μg/g total carotenoid, respectively (Arima and Rodriguez-Amaya 1988). This distribution pattern was also noted in kumquat (Huysken et al. 1985), mandarin hybrid (Gross 1987), muskmelon (Flugel and Gross 1982), and persimmon (Gross 1987). An exception to the usual pattern is seen in pink-fleshed guava (Padula and Rodriguez-Amaya 1986) and red pomelo (Gross 1987), in which the high lycopene concentration in the pulp compensates for the greater amounts of other carotenoids in the peel.

**Table 4.** Carotenoids of carrot cultivars from Finland

| | | | Concentration, μg/g | | | |
|---|---|---|---|---|---|---|
| **Cultivar** | **Site[a]** | ***n*[b]** | **α-Carotene** | **β-Carotene** | **γ-Carotene** | **Lutein** |
| Bangor $F_1$ BZ[c] | B | 5 | 25 | 66 | 8 | 1.6 |
| Bergen $F_1$ BZ | B | 3 | 25 | 56 | 12 | 1.3 |
| Berlicum N | C | 3 | 26 | 60 | 8 | 4.0 |
| Berlicum R | C | 3 | 46 | 85 | 24 | 5.6 |
| Casey $F_1$ BZ | B | 3 | 35 | 69 | 12 | 2.2 |
| Chantenay R | C | 5 | 25 | 61 | 6 | 4.5 |
| Flakkeer G | C | 6 | 22 | 55 | 6 | 2.1 |
| Flakkeer R | C | 3 | 27 | 63 | 10 | 3.4 |
| Flaxton $F_1$ BZ | D | 5 | 27 | 56 | 13 | 1.3 |
| Florence $F_1$ BZ | B | 3 | 27 | 46 | 25 | 1.2 |
| Fontana $F_1$ BZ | B | 3 | 30 | 60 | 27 | 1.1 |
| Nairobi $F_1$ BZ | B | 2 | 30 | 60 | 9 | 3.6 |
| Nantes Duke Notabene 370 | A | 2 | 39 | 84 | 17 | 2.0 |
| Nantes Duke Notabene 405 | B | 3 | 42 | 79 | 16 | 1.8 |
| Nantucket $F_1$ BZ | B | 3 | 42 | 74 | 16 | 2.6 |
| Napoli $F_1$ BZ | A | 3 | 36 | 48 | 12 | 1.9 |
| Narbonne $F_1$ BZ | B | 3 | 48 | 103 | 19 | 3.8 |
| Nelson $F_1$ BZ | B | 3 | 49 | 90 | 16 | 2.2 |
| Rondino $F_1$ BZ | B | 3 | 34 | 66 | 12 | 1.5 |

Source: Heinonen (1990).
[a]Growing site of carrot cultivar: A, Pikkiö; B, Hahkiala; C , Jokioinen; and D, Säkylä.
[b]Number of replicate analysis
[c]Hybrid cultivar of Bejo Zaden

In carotenogenic fruits and fruit vegetables, ripening is usually accompanied by enhanced carotenogenesis as chlorophylls decompose and the chloroplasts are transformed into chromoplasts. The simple chloroplast carotenoid pattern gives way to a complex composition, the carotenoids increasing dramatically in number and quantity. This is exemplified by *Cucurbita* menina verde in Table 3.

Increased carotenogenesis with maturation or ripening was also documented in *Momordica charantia* (Rodriguez-Amaya et al. 1976a), yellow Lauffener gooseberry (Gross 1982/1983), red pepper (Rahman and Buckle 1980), badami mango (John et al. 1970), and leaves (Hulshof et al. 1997, Ramos and Rodriguez-Amaya 1987). The one factor that decisively affects the carotenoid content is the maturity of the plant food when harvested and offered for consumption. Squashes and pumpkins showed substantial between-lot variations of the same cultivars so that the ranges rather than the means are presented in Table 3. This variability was attributed to the wide differences in maturity stage, because these fruit vegetables can be harvested over a long period and have a long shelf life during which carotenoid biosynthesis continues.

In fruits in which the color at the ripe stage is due to anthocyanins, such as yellow cherry (Gross 1985), red currant (Gross 1982/1983), strawberry (Gross 1982a), and olive fruit (Minguez-Mosquera and Garrido-Fernandez 1989), and in fruits that retain their green color when ripe, such as kiwi (Gross 1982b), the carotenoid concentrations decrease with ripening. The same trend is seen with some fruits that undergo yellowing simply by unmasking the carotenoids through chlorophyll degradation (Gross 1987).

Carotenogenesis may continue even after harvest as long as the fruit or vegetable remains intact, as shown in tomato (Raymundo et al. 1967) and African mango (Aina 1990). Carotenoid biosynthesis in the flesh of ripening Indian Alphonso mango was observed to be maximal at tropical ambient temperature (28–32 °C) (Thomas and Janave 1975). Storage at 7–20 °C for 16–43 days caused a substantial decrease in total carotenoid content even when the fruits were subsequently ripened at optimal conditions.

Another example of ripening alterations is presented in Table 5 for the mango cultivars Keitt and Tommy Atkins. Because the mangos were analyzed from the mature-green stage (not the immature-green stage) at which the fruits are harvested commercially, the changes were essentially quantitative. Marked increases in all-*trans*-β-carotene, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin occurred during ripening in both cultivars.

Carotenoid losses during postharvest storage were reported in some vegetables, particularly leaves (Kopas-Lane and Warthesen 1995, Simonetti et al. 1991, Takama and Saito 1974, Ezell and Wilcox 1962), especially under conditions favorable to wilting, high temperature, and light exposure. Wu et al. (1992) simulated different retail market conditions in the

**Table 5.** Major carotenoids of ripening mango

| | Concentration, μg/g[a] | | |
|---|---|---|---|
| **Cultivar/carotenoids** | **Mature green** | **Partially ripe** | **Ripe** |
| Cv. Keitt | | | |
| All-*trans*-β-carotene | 1.7±0.3 | 4.2±0.4 | 6.7±1.6 |
| Luteoxanthin isomers | 1.0±0.2 | 1.6±0.4 | 2.7±0.2 |
| All-*trans*-violaxanthin | 5.4±1.7 | 11±2 | 18±4 |
| 9-*cis*-Violaxanthin | 1.7±0.4 | 3.9±0.5 | 7.2±1.4 |
| All-*trans*-neoxanthin | 1.6±0.6 | 1.9±0.5 | 1.9±0.9 |
| Cv. Tommy Atkins | | | |
| All-*trans*-β-carotene | 2.0±0.8 | 4.0±0.8 | 5.8±2.5 |
| Luteoxanthin isomers | 1.3±0.7 | 2.7±1.1 | 2.0±0.6 |
| All-*trans*-Violaxanthin | 6.9±3.0 | 18±7 | 22±9 |
| 9-*cis*-Violaxanthin | 3.3±1.3 | 9.0±3.2 | 14±5 |
| All-*trans*-neoxanthin | 2.6±1.8 | 6.6±5.1 | 4.9±4.5 |

Source: Mercadante and Rodriguez-Amaya (1998).
[a]Mean and standard deviation of three sample lots from São Paulo, Brazil, for each maturity stage. Only carotenoids at ≥1 μg/g are included.

United States for green beans and broccoli and found no statistically significant changes in the β-carotene level. It would be interesting to verify the effect on the other carotenoids.

Temperature and harvest time significantly influenced the carotenoid concentration of tomatoes produced in greenhouse controlled-environment chambers (Koskitalo and Ormrod 1972). At diurnal 17.8/25.6 °C minimum-maximum temperatures, the β-carotene concentration (μg/g) was 2.97, 2.18, and 2.19, respectively, in fruits harvested after 7, 14, and 21 days following the onset of initial coloration. The corresponding levels for lycopene were 43.5, 57.7, and 64.8 μg/g. At 2.8/13.9 °C, β-carotene was found at 3.56, 3.73, and 3.67 μg/g and lycopene at only 9.30, 20.5, and 24.2 μg/g in tomatoes collected after 7, 14, and 21 days following color break.

Geographic effects were shown by Formosa papayas produced in two Brazilian states with different climates. Those from the temperate São Paulo had lower β-carotene, β-cryptoxanthin, and lycopene concentrations than did papayas from the hot state of Bahia (Table 3). Similarly, the β-carotene content of West Indian Cherry from the hot Northeastern states of Pernambuco and Ceará was found to be 5–6 times greater than that of the same fruit from São Paulo (Table 3). All-*trans*-β-carotene was twice as high in Keitt mango from Bahia (Table 3) as in Keitt mango from São Paulo (Table 5), and all-*trans*-violaxanthin and 9-*cis*-violaxanthin were also higher in the Bahian mangos. These differences were greater than those between Keitt and Tommy Atkins mangos from São Paulo, indicating that for carotenoids, climatic effects could surpass cultivar differences. The above studies show that greater exposure to sunlight and elevated temperature heighten carotenoid biosynthesis in fruits.

In kale leaves collected at the same stage of maturity and produced under commercial conditions, the constituent carotenoids were significantly higher in samples from a "natural" farm than in those from a neighboring farm that used agrochemicals (Table 6). In this same study the carotenoids of the two cultivars analyzed showed significant difference only in the summer. The β-carotene, lutein-violaxanthin, and total carotenoid were significantly higher in the winter than in the summer for the Cv. Manteiga, which appears compatible with greater destruction of leaf carotenoids on exposure to higher temperature and greater sunlight (Young and Britton 1990). On the other hand, the neoxanthin content was significantly higher in the summer for the Tronchuda cultivar.

Carotenoids are susceptible to isomerization and oxidation during processing and storage, the practical consequences being loss of color and biologic activity and the formation of volatile compounds that impart desirable or undesirable flavor in some foods. The occurrence of oxidation depends on the presence of oxygen, metals, enzymes, unsaturated lipids, prooxidants, or antioxidants; exposure to light; type and physical state of carotenoid present; severity of the treatment (i.e., destruction of the ultrastructure that protects the carotenoids, increase of surface area, and duration and temperature of heat treatment); packaging material; and storage conditions. Heating promotes *trans-cis* isomerization. Alteration of the carotenoid composition during domestic preparation, industrial processing, and storage, with emphasis on provitamin A carotenoids, was reviewed recently (Rodriguez-Amaya 1997). Some examples of these studies will be cited here.

In guava juice, a significant fivefold increase in *cis*-lycopene (from 1.2 μg/g) was observed on processing (Padula and Rodriguez-Amaya 1987). A slight, statistically insignificant decrease in *trans*-lycopene was also noted. Both isomers decreased during 10 months of storage. The small amount of β-

**Table 6.** Variation of the carotenoid composition (μg/g) of kale in relation to cultivar, season, and type of farm

| Carotenoid | Winter[a] cv. Manteiga, natural farm | cv. Tronchuda, natural farm | Summer[a] cv. Manteiga, natural farm | cv. Tronchuda, natural farm | cv. Manteiga, farm using agrochemicals |
|---|---|---|---|---|---|
| β-Carotene | 54±5[b] | 60±14[b] | 44±3[c] | 57±8[b] | 38±7[d] |
| Lutein plus violaxanthin | 111±16[b] | 114±10[b] | 84±9[c] | 109±10[b] | 71±8[d] |
| Zeaxanthin | 3±2[b] | 2±1[b] | 2±1[b] | 2±1[b] | 1±1[b] |
| Neoxanthin | 18±7[c] | 19±4[c] | 20±3[c] | 26±3[b] | 17±2[d] |
| Total | 187±21[b] | 195±24[b] | 149±10[c] | 194±19[b] | 127±14[d] |

Source: Mercadante and Rodriguez-Amaya (1991).
[a]Each value is the mean and standard deviation of 10 sample lots analyzed individually.
[b,c,d] Values in the same horizontal line with different letters are significantly different.

carotene (2.7 μg/g) was retained on processing and storage.

The carotenoids were essentially retained during the processing of mango slices (Godoy and Rodriguez-Amaya 1987). The only significant change was the increase in luteoxanthin, compatible with the conversion of 5,6- to 5,8-epoxide. More evident changes occurred on processing mango puree. The principal pigment β-carotene decreased 13%; auroxanthin appeared whereas violaxanthin and luteoxanthin decreased. During storage of mango slices in lacquered or plain tin-plate cans, no appreciable loss of β-carotene was noted for 10 months. Between the 10th and 14th months a 50% reduction occurred. Violaxanthin tended to decrease and auroxanthin to increase during storage. β-Carotene showed a greater susceptibility to degrade in bottled mango puree (18% loss after 10 months) than in the canned product. As with the mango slices, however, both bottled and canned puree suffered a 50% loss of β-carotene after the 14th month. Violaxanthin and luteoxanthin tended to decrease whereas auroxanthin maintained a comparatively high level throughout storage. In commercially processed mango juice, processing effects appeared substantial. Violaxanthin, the principal carotenoid of the fresh mango, was not detected; auroxanthin appeared in an appreciable level; and β-carotene became the principal carotenoid (Mercadante and Rodriguez-Amaya, 1998).

Both lycopene (the major pigment) and β-carotene showed no significant change during the processing of papaya puree (Godoy and Rodriguez-Amaya 1991). *cis*-Lycopene increased sevenfold, β-cryptoxanthin decreased 34%, and cryptoflavin appeared. During 14 months of storage, β-carotene, lycopene and *cis*-lycopene remained practically constant. β-Cryptoxanthin did not change significantly during the first 10 months but decreased 27% after 14 months. Auroxanthin and flavoxanthin appeared during storage.

In olives, only β-carotene and lutein resisted the fermentation and brine storage (Minguez-Mosquera et al. 1989). Phytofluene and ζ-carotene disappeared. Violaxanthin, luteoxanthin, and neoxanthin gave rise to auroxanthin and neochrome. The total pigment content did not change.

In carrot juice, canning (121 °C for 30 minutes) resulted in the greatest loss of carotenoids, followed by high-temperature short-time heating at 120 °C for 30 seconds, 110 °C for 30 seconds, acidification plus 105 °C heating for 25 seconds, and acidification (Chen et al. 1995). Heating increased *cis* isomers, 13-*cis*-β-carotene being formed in largest amount, followed by 13-*cis*-lutein and 15-*cis*-α-carotene.

Canning increased the percentage of total *cis* isomers of provitamin A carotenoids in several fruits and vegetables (Lessin et al. 1997). Canning of sweet potatoes caused the largest increase (39%), followed by processing of carrots (33%), tomato juice (20%), collards (19%), tomatoes (18%), spinach (13%), and peaches (10%).

# SOME PHYSICOCHEMICAL PROPERTIES OF CAROTENOIDS

A good understanding of some of the physical and chemical properties of carotenoids allows analysts to determine carotenoids with greater ease and reliability.

## Solubility

With very few exceptions, carotenoids are lipophilic. They are insoluble in water and soluble in organic solvents, such as acetone, alcohol, ethyl ether, chloroform, and ethyl acetate. Carotenes are readily soluble in petroleum ether, hexane, and toluene; xanthophylls dissolve better in methanol and ethanol. Crystalline carotenoids may be difficult to dissolve in the above solvents but do dissolve in benzene and dichloromethane (Schiedt and Liaaen-Jensen 1995). Solubility of both ß-carotene and the xanthophyll lutein in tetrahydrofuran was shown to be excellent (Craft and Soares 1992).

## Light Absorption

The conjugated double-bond system constitutes the light-absorbing chromophore that gives carotenoids their attractive color and provides the visible absorption spectrum that serves as a basis for their identification and quantification. The color enables analysts to monitor the different steps of carotenoid analysis. Loss or change of color at any time during the analysis gives an immediate indication of degradation or structural modification. The color permits visual monitoring of the separation of carotenoids in open-column chromatography, and mainly for this reason this classical technique is still a viable option for quantitative analysis of carotenoids.

The ultraviolet and visible spectrum is the first diagnostic tool for the identification of carotenoids. The wavelength of maximum absorption (λmax) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore. The structure-spectrum relationship has been extensively discussed. The λmax values of common carotenoids, taken mainly from Britton's (1995) compilation, are shown in Table 7 and will be discussed in relation to the structures by using the absorption in petroleum ether.

Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra (Figure 8). The greater the number of conjugated double bonds, the higher the λmax values. Thus, the most unsaturated acyclic carotenoid lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λmax at 444, 470, and 502 nm) (Figure 8). At least 7 conjugated double bonds are needed for a carotenoid to have perceptible color. Thus, ζ-carotene is light yellow. Being also acyclic, its spectrum has three well-defined peaks, but these are at wavelengths much lower than those of lycopene (λmax at 378, 400, and 425 nm), commensurate with

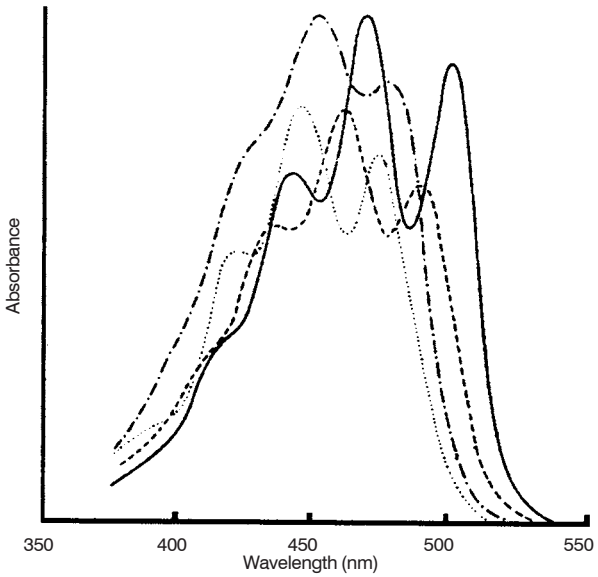


**Figure 8.** Visible absorption spectra of lycopene (—), γ-carotene (- - -), β-carotene (-.-.-.), and α-carotene (....) in petroleum ether.

**Table 7.** Ultraviolet and visible absorption data for common food carotenoids

| Carotenoid | Solvent | λmax, nm[a] | | | %III/II[b] |
|---|---|---|---|---|---|
| Antheraxanthin | Chloroform | 430 | 456 | 484 | |
| | Ethanol | 422 | 444 | 472 | 55 |
| | Hexane, petroleum ether | 422 | 445 | 472 | 60 |
| Astaxanthin | Acetone | | 480 | | 0 |
| | Benzene, chloroform | | 485 | | 0 |
| | Ethanol | | 478 | | 0 |
| | Petroleum ether | | 468 | | 0 |
| Auroxanthin | Acetone | 381 | 402 | 427 | 101 |
| | Chloroform | 385 | 413 | 438 | |
| | Ethanol, petroleum ether | 380 | 400 | 425 | 102 |
| Bixin | Chloroform | 433 | 470 | 502 | |
| | Ethanol | 429 | 457 | 484 | |
| | Petroleum ether | 432 | 456 | 490 | |
| Canthaxanthin | Chloroform | | 482 | | 0 |
| | Ethanol | | 474 | | 0 |
| | Petroleum ether | | 466 | | 0 |
| Capsanthin | Ethanol | | 476 | | |
| | Petroleum ether | (450) | 475 | 505 | |
| Capsorubin | Petroleum ether | (455) | 479 | 510 | |
| α-Carotene | Acetone | 424 | 448 | 476 | 55 |
| | Chloroform | 433 | 457 | 484 | |
| | Ethanol | 423 | 444 | 473 | |
| | Hexane, petroleum ether | 422 | 445 | 473 | 55 |
| β-Carotene | Acetone | (429) | 452 | 478 | 15 |
| | Chloroform | (435) | 461 | 485 | |
| | Ethanol | (425) | 450 | 478 | 25 |
| | Hexane, petroleum ether | (425) | 450 | 477 | 25 |
| β-Carotene-5,6-epoxide | Ethanol | 423 | 445 | 474 | |
| β-Carotene-5,8-epoxide | Ethanol | 407 | 428 | 452 | |
| β-Carotene-5,6,5′,6′-diepoxide | Ethanol | 417 | 440 | 468 | |
| β-Carotene-5,8,5′8′-diepoxide | Ethanol | 388 | 400 | 425 | |
| δ-Carotene | Chloroform | 440 | 470 | 503 | |
| | Petroleum ether | 431 | 456 | 489 | 85 |
| γ-Carotene | Acetone | 439 | 461 | 491 | |
| | Chloroform | 446 | 475 | 509 | |
| | Ethanol | 440 | 460 | 489 | 35 |
| | Hexane, petroleum ether | 437 | 462 | 494 | 40 |
| ζ-Carotene | Ethanol | 377 | 399 | 425 | |
| | Hexane, petroleum ether | 378 | 400 | 425 | 103 |
| Crocetin | Chloroform | 413 | 435 | 462 | |
| | Ethanol | 401 | 423 | 447 | |
| | Petroleum ether | 400 | 422 | 450 | |
| α-Cryptoxanthin/Zeinoxanthin | Chloroform | 435 | 459 | 487 | |
| | Ethanol | 423 | 446 | 473 | 60 |
| | Hexane | 421 | 445 | 475 | 60 |
| β-Cryptoxanthin | Chloroform | (435) | 459 | 485 | |
| | Ethanol | (428) | 450 | 478 | 27 |
| | Petroleum ether | (425) | 449 | 476 | 25 |
| Echinenone | Acetone | | 460 | | 0 |
| | Chloroform | | 471 | | |
| | Ethanol | | 461 | | 0 |
| | Petroleum ether | | 458 | (482) | |

**Table 7.** Ultraviolet and visible absorption data for common food carotenoids (continued)

| Carotenoid | Solvent | λmax, nm[a] | | | %III/II[b] |
|---|---|---|---|---|---|
| Lutein | Chloroform | 435 | 458 | 485 | |
| | Ethanol | 422 | 445 | 474 | 60 |
| | Petroleum ether | 421 | 445 | 474 | 60 |
| Lutein-5,6-epoxide | Chloroform | 433 | 453 | 483 | |
| | Ethanol | 420 | 441 | 470 | 85 |
| | Hexane, petroleum ether | 420 | 440 | 470 | 85 |
| Lycopene | Acetone | 448 | 474 | 505 | |
| | Chloroform | 458 | 484 | 518 | |
| | Ethanol | 446 | 472 | 503 | 65 |
| | Petroleum ether | 444 | 470 | 502 | 65 |
| Mutatoxanthin | Chloroform | | 437 | 468 | |
| | Ethanol | 409 | 427 | 457 | 50 |
| | Petroleum ether | 407 | 426 | 456 | 45 |
| Neoxanthin | Acetone | 416 | 440 | 470 | 85 |
| | Chloroform | 423 | 448 | 476 | |
| | Ethanol | 415 | 439 | 467 | 80 |
| | Petroleum ether | 416 | 438 | 467 | 87 |
| Neurosporene | Chloroform | 424 | 451 | 480 | |
| | Ethanol, hexane | 416 | 440 | 470 | |
| | Petroleum ether | 414 | 439 | 467 | 100 |
| Phytoene | Hexane, petroleum ether | (276) | 286 | (297) | 10 |
| Phytofluene | Hexane, petroleum ether | 331 | 348 | 367 | 90 |
| Rubixanthin | Chloroform | 439 | 474 | 509 | |
| | Ethanol | 433 | 463 | 496 | 40 |
| | Petroleum ether | 434 | 460 | 490 | |
| Violaxanthin | Chloroform | 426 | 449 | 478 | |
| | Ethanol | 419 | 440 | 470 | 95 |
| | Petroleum ether | 416 | 440 | 465 | 98 |
| α-Zeacarotene | Hexane | 398 | 421 | 449 | |
| β-Zeacarotene | Ethanol, hexane, petroleum ether | 406 | 428 | 454 | |
| Zeaxanthin | Acetone | (430) | 452 | 479 | |
| | Chloroform | (433) | 462 | 493 | |
| | Ethanol | (428) | 450 | 478 | 26 |
| | Petroleum ether | (424) | 449 | 476 | 25 |

Source: Britton (1995) and Davies (1976).
[a] Parentheses indicate a shoulder.
[b] Ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100.

its conjugated system of 7 double bonds (Figure 9).

The two carotenoids that precede ζ-carotene in the desaturation biosynthetic pathway, phytoene (3 conjugated double bonds) and phytofluene (5 conjugated double bonds), are colorless and absorb maximally at 276, 286, and 297 nm and at 331, 348, and 367 nm, respectively (Figure 9). The degree of spectral fine structure is small for phytoene, increases through phytofluene and ζ-carotene, then decreases again as the chromophore is extended. Neurosporene, which has a structure intermediate between ζ-carotene and lycopene (9 conjugated double bonds), exhibits maximum absorption at 414, 439, and 467 nm.

Cyclization results in steric hindrance between the ring methyl group at C-5 and the hydrogen atom at C-8 of the polyene chain, taking the π electrons of the ring double bond out of plane with those of the chain. Consequently, a hypsochromic shift (displacement of λmax to lower wavelength), hypochromic effect (decrease in absorbance), and loss of fine structure (spectrum with less-defined peaks) are observed. Thus, bicyclic β-carotene, although possessing the same number of conjugated double bonds as lycopene, is yellow orange and has λmax at 450 and 477 nm and a mere inflection (shoulder) at 425 nm (Figure 8). Monocyclic γ-carotene is red orange and exhibits a spectrum intermediate between those of lycopene and β-carotene in λmax and shape, reflecting a structure that is intermediate between the other two carotenoids. The double bond in the ε ring of α-

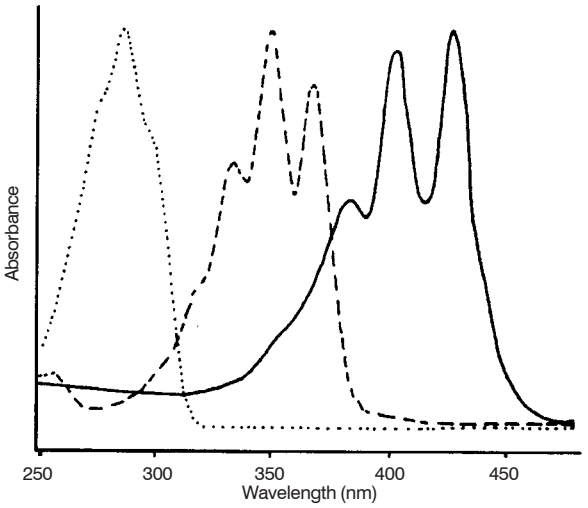


**Figure 9.** Photodiode array spectra of ζ-carotene (——), phytofluene (– – –) and phytoene (....). Mobile phase: acetonitrile–ethyl acetate–methanol (85:10:5).

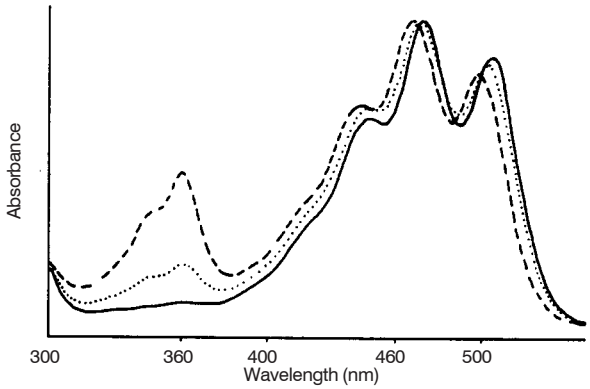


**Figure 10.** Photodiode array spectra of all-*trans*-lycopene (——), 15-*cis*-lycopene (- - -), and 13-*cis*-lycopene (....). Mobile phase: acetonitrile–ethyl acetate–methanol (85:10:5).

carotene is out of conjugation, leaving 10 conjugated double bonds (9 in the polyene chain and 1 in the β ring); thus, this carotenoid is light yellow and its absorption spectrum is more defined with λmax at slightly shorter wavelengths (422, 445, and 473 nm) than those of β-carotene.

An isolated carbonyl group, which is not in conjugation with the chromophore, does not alter the spectrum. A carbonyl group in conjugation with the series of conjugated double bonds extends the chromophore. This results in a bathochromic shift (displacement to higher wavelengths) and loss of spectral fine structure, to the extent that the three-maxima spectrum is replaced by a single broad curve, unsymmetrical with λmax at 458 and a shoulder at 482 nm for echinenone (orange) and symmetrical with the λmax at 466 nm for canthaxanthin (red orange) (Table 7).

The introduction of hydroxy and methoxy substituents in the carotenoid molecule does not affect the chromophore and therefore has virtually no effect on the absorption spectrum. Thus, the spectra of lutein, zeinoxanthin, and α-cryptoxanthin resemble that of α-carotene, and those of β-cryptoxanthin and zeaxanthin are identical to that of β-carotene.

*Cis*-isomerization of a chromophore's double bond causes a slight loss in color, small hypsochromic shift (usually 2 to 6 nm for mono-*cis*), and hypochromic effect, accompanied by the appearance of a *cis* peak in or near the ultraviolet region (Figure 10). The intensity of the *cis* band is greater as the *cis* double bond is nearer the center of the molecule. Thus, the 15-*cis* isomer, in which the *cis* double bond is in the center of the molecule, has an intense *cis* peak.

The 5,6-monoepoxide and 5,6,5′,6′-diepoxides of cyclic carotenoids, having lost one and two ring double bonds, respectively, absorb maximally at wavelengths some 5 and 10 nm lower (Table 7) and are lighter colored than the parent compounds. When a 5,6-epoxide is rearranged to the 5,8-epoxide (furanoid), an additional double bond (this time from the polyene chain) is lost. Thus, the λmax of the 5,8-monoepoxide and 5,8,5′,8′-diepoxide are 20–25 and 50 nm lower, respectively, than those of the parent carotenoids. Because only the polyene chain conjugated double bonds remain, the degree of spectral fine structure increases, resembling that of acyclic carotenoids.

Slightly different λmax values are reported in the literature, which is understandable considering that the reproducibility of recording spectrophotometer in the 400–500 nm region is about ±1–2 nm. Instrumental errors should be kept at a minimum by calibrating the instruments (e.g., using a holmium oxide filter and recording the spectra of authentic carotenoid standards).

The absorption spectra of carotenoids are markedly solvent dependent (Table 7). This has to be remembered when spectra are taken by the photodiode array detector in high-performance liquid chromatography (HPLC), in which the spectra are taken in mixed solvents in isocratic elution and in varying mixed solvents in gradient elution. The λmax values relative to hexane and petroleum ether are practically the same in diethyl ether, methanol, ethanol, and acetonitrile and 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).

The absorption spectra are now rarely presented in published papers. To give an idea of the spectral fine structure, the %III/II (Figure 11) can be presented, along with the λmax values. The %III/II is the ratio of the height of the longest-wavelength ab-

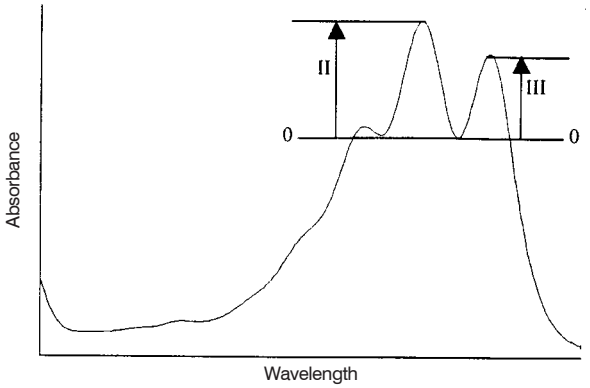


**Figure 11.** Calculation of %III/II as indication of spectral fine structure (%III/II = III/II × 100).

sorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100 (Britton 1995). In a few cases, such as ζ-carotene, III is greater than II, thus the %III/II value is slightly higher than 100 (Table 7). For conjugated ketocarotenoids, such as canthaxanthin and echinenone, the spectrum consists of a broad single maximum, having no defined fine structure, thus %III/II is 0.

The absorption coefficient $A^{1\%}_{1cm}$ of a carotenoid (absorbance at a given wavelength of a 1% solution in spectrophotometer cuvette with a 1-cm light path) used in the calculation of the concentration also varies pronouncedly in different solvents (Table 8).

Carotenoids in solution obey the Beer-Lambert law—their absorbance is directly proportional to the concentration. Thus, carotenoids are quantified spectrophotometrically. This quantification, however, depends on the availability of accurate absorption coefficients, which are difficult to obtain. The procedure normally involves weighing a small amount of the carotenoid, typically 1 to 2 mg, with an accuracy of ±0.001 mg (Britton 1995). An accurate and sensitive balance is required and the carotenoid should be absolutely free from all contaminants, including residual solvent. Moreover, complete dissolution of the carotenoids in the desired solvent can be difficult. Thus, some published values may have some significant level of error or uncertainty (Britton 1995), and some discrepancies can be noted in the values presented in Table 8. Because authors choose different coefficients for some carotenoids (in the same solvents), this alone can account for a good part of the variations in analytic results. For the accuracy of both open-column chromatography and HPLC methods , reassessment of the absorption coefficients is warranted.

## Adsorption and Partition Properties

The chromatographic behavior of carotenoids bears a definite relationship with their structures. However, chromatographic data cannot be used as sole criteria for carotenoid's identity. Nevertheless, these data serve as useful complementary information. In normal-phase open-column chromatography, the adsorption affinity depends on the number of conjugated double bonds, cyclization, and the presence of oxygen substituents.

The influence of the double bonds is best illustrated by the adsorption affinities of the acyclic carotenoids, which elute in the sequence phytoene, phytofluene, ζ-carotene, neurosporene, and lycopene. Comparing monocyclic and bicyclic carotenes, δ-carotene elutes before γ-carotene, and α-carotene elutes before β-carotene.

Cyclization decreases the adsorption affinity. Thus, β-carotene is much more weakly adsorbed than γ-carotene, which in turn elutes before lycopene.

The presence of oxygen substituents increases adsorption, the extent of such increase depending on the type, number, and location of the functions. This is demonstrated in a silica thin layer developed with 3% methanol in benzene or 5% methanol in toluene, in which all carotenes elute with the solvent front and the xanthophylls distributed in the plate according to the number and type of substituents present (Figure 12).

The hydroxyl group exerts a great influence on adsorption; methylation, acetylation, and silylation markedly reduce this effect. The adsorption affinity of a carbonyl group is less than that of a free hydroxyl substituent. The contribution of the functional groups on adsorption affinity increases in the sequence (Davies 1976)

-OR < -C=O < 2 [-C=O] < -OH < -COOH

The 5,8-epoxide is more strongly adsorbed than is the corresponding 5,6-epoxide. Thus, the adsorption affinity of β-carotene and its epoxides on an alumina thin layer increases in the following order (El-Tinay and Chichester 1970):

β-carotene < 5,6-epoxide < 5,6,5′,6′-diepoxide < 5,8-epoxide < 5,6,5′,8′-diepoxide < 5,8,5′,8′-diepoxide

The effects of more than one oxygen substituent are not always additive; a second substituent in the same end group tends to have less influence than the first.

The order of elution of carotenoids in a given adsorbent-solvent system does not vary but the order may differ in different adsorbents. For example, β-

**Table 8.** Absorption coefficients ($A^{1\%}_{1cm}$) of common food carotenoids

| Carotenoid | Solvent | λmax, nm | $A^{1\%}_{1cm}$ |
|---|---|---|---|
| Antheraxanthin | Ethanol | 446 | 2350 |
| Astaxanthin | Hexane | 470 | 2100 |
| Auroxanthin | Ethanol | 400 | 1850 |
| Bixin | Petroleum ether | 456 | 4200 |
| Canthaxanthin | Petroleum ether | 466 | 2200 |
| Capsanthin | Benzene | 483 | 2072 |
| Capsorubin | Benzene | 489 | 2200 |
| α-Carotene | Petroleum ether | 444 | 2800 |
| | Hexane | 445 | 2710 |
| β-Carotene | Petroleum ether | 450 | 2592 |
| | Ethanol | 450 | 2620 |
| | Chloroform | 465 | 2396 |
| β-Carotene-5,6-epoxide | Hexane | 444 | 2590 |
| β-Carotene-5,6,5′,6′-diepoxide | Hexane | 440 | 2690 |
| δ-Carotene | Petroleum ether | 456 | 3290 |
| γ-Carotene | Petroleum ether | 462 | 3100 |
| | Hexane | 462 | 2760 |
| ζ-Carotene | Hexane | 400 | 2555 |
| Crocetin | Petroleum ether | 422 | 4320 |
| α-Cryptoxanthin/zeinoxanthin | Hexane | 445 | 2636 |
| β-Cryptoxanthin | Petroleum ether | 449 | 2386 |
| | Hexane | 450 | 2460 |
| Echinenone | Petroleum ether | 458 | 2158 |
| Lutein | Ethanol | 445 | 2550 |
| | Diethyl ether | 445 | 2480 |
| | Diethyl ether | 445 | 2600 |
| Lutein-5,6-epoxide | Ethanol | 441 | 2400 |
| | Ethanol | 441 | 2800 |
| Lycopene | Petroleum ether | 470 | 3450 |
| Lycoxanthin | Acetone | 474 | 3080 |
| Mutatochrome | Diethyl ether | 428 | 2260 |
| Neoxanthin | Ethanol | 438 | 2470 |
| | Ethanol | 439 | 2243 |
| Neurosporene | Hexane | 440 | 2918 |
| Phytoene | Petroleum ether | 286 | 1250 |
| Phytofluene | Petroleum ether | 348 | 1350 |
| | Hexane | 348 | 1577 |
| Rubixanthin | Petroleum ether | 460 | 2750 |
| Violaxanthin | Ethanol | 440 | 2550 |
| | Acetone | 442 | 2400 |
| α-Zeacarotene | Petroleum ether | 421 | 2450 |
| | Hexane | 421 | 1850 |
| β-Zeacarotene | Petroleum ether | 428 | 2520 |
| | Hexane | 427 | 1940 |
| Zeaxanthin | Petroleum ether | 449 | 2348 |
| | Ethanol | 450 | 2480 |
| | Ethanol | 450 | 2540 |
| | Acetone | 452 | 2340 |

Source: Britton (1995).

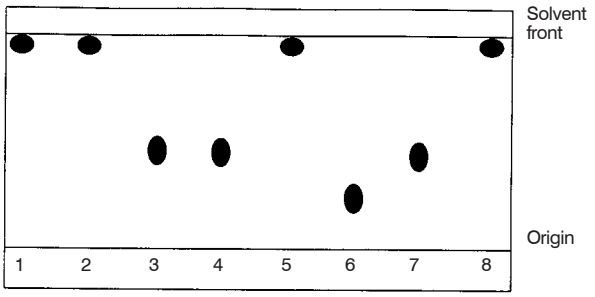


**Figure 12.** Thin-layer silica-gel chromatogram of carotenoids and reaction products, developed with 5% methanol in toluene. 1) β-Carotene, 2) lycopene, 3) β-cryptoxanthin, 4) β-cryptoxanthin methylated with acidic methanol—negative response, 5) β-cryptoxanthin acetylated with acetic anhydride, 6) lutein, 7) lutein methylated with acidic methanol, and 8) lutein acetylated with acetic anhydride.

cryptoxanthin elutes before and after lycopene in magnesium oxide–Hyflosupercel and alumina columns, respectively (Table 9). This indicates that the influence of cyclization is greater than that of the presence of hydroxyl substituents in the magnesium oxide–Hyflosupercel column.

In the current widely used reversed-phase HPLC, in which partition is the major chromatographic mode, the order is more or less the reverse of that seen in normal-phase adsorption open-column chromatography. The more polar xanthophylls (the trihydroxy-5,6-epoxy neoxanthin, the dihydroxy-5,6,5′,6′-diepoxy violaxanthin, and the dihydroxy lutein and zeaxanthin) elute well before the carotenes (Figure 13); the monohydroxy carotenoids elute between these two groups. Elution of carotenes does not always follow the expected pattern and differs with the type of column (monomeric or polymeric) and the mobile phase, with β-carotene eluting after (Figure 14) or before lycopene. α-Carotene usually elutes before β-carotene as in normal phase chromatography (Figure 15).

## Isomerization and Oxidation

The highly unsaturated carotenoid is prone to isomerization and oxidation. Heat, light, acids, and adsorption on an active surface (e.g., alumina) promote isomerization of *trans* carotenoids, their usual configuration, to the *cis* forms. This results in some loss of color and provitamin A activity. Oxidative degradation, the principal cause of extensive losses of carotenoids, depends on the availability of oxygen and is stimulated by light, enzymes, metals, and co-oxidation with lipid hydroperoxides. Carotenoids appear to have different susceptibilities to oxidation, ζ-carotene, lutein, and violaxanthin being cited as more labile. Formation of epoxides and apocarotenoids (carotenoids with shortened carbon skeleton) appears to be the initial step (Figure 16). Subsequent fragmentations yield a series of low-molecular-weight compounds similar to those produced in fatty acid oxidation. Thus, total loss of color and biologic activities are the final consequences.

Conditions necessary for isomerization and oxi-

**Table 9.** Elution pattern of some carotenoids in magnesium oxide–Hyflosupercel and alumina columns[a]

| Magnesium oxide–Hyflosupercel | Alumina |
|---|---|
| Phytoene | Phytoene |
| Phytofluene | Phytofluene |
| α-Carotene | α-Carotene |
| β-Carotene | β-Carotene |
| ζ-Carotene | ζ-Carotene |
| δ-Carotene | δ-Carotene |
| Zeinoxanthin/α-cryptoxanthin | γ-Carotene |
| γ-Carotene | Lycopene |
| β-Cryptoxanthin | Zeinoxanthin/α-cryptoxanthin |
| Lycopene | ß-Cryptoxanthin |
| Rubixanthin | Rubixanthin |
| Lutein | Lutein |
| Zeaxanthin | Zeaxanthin |

Source: Rodriguez-Amaya et al. (1976a, 1975).
[a]Columns developed with petroleum ether containing increasing amounts of ethyl ether and then acetone. Carotenoids listed according to order of elution.

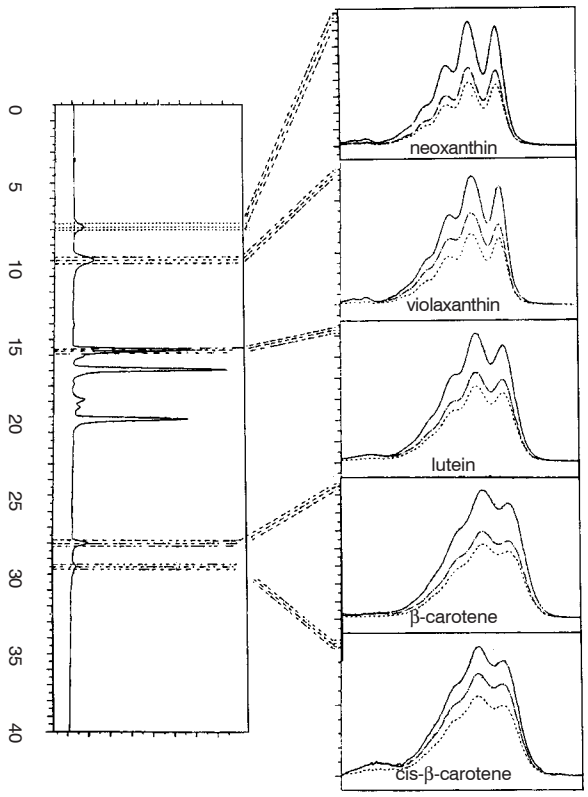


**Figure 13.** HPLC chromatogram and photodiode array spectra of the carotenoids of water cress. Column: polymeric $C_{18}$ Vydac 218TP54, 4.6 x 250 mm, 5 µm; mobile phase: gradient of 10% $H_2O$ to 10% tetrahydrofuran in methanol. The other major peaks are of chlorophylls.

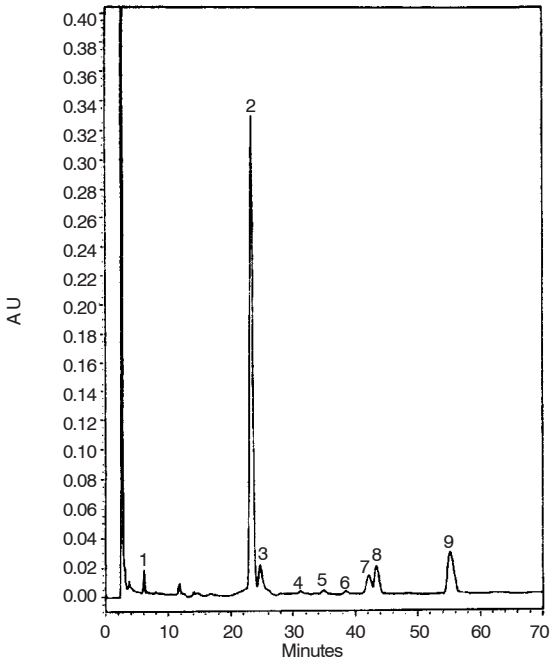


**Figure 14.** HPLC chromatogram of the carotenoids of tomato. Column: Spherisorb S5 ODS2, 2.0 × 250 mm, 5 µm; mobile phase: acetonitrile–methanol–ethyl acetate (73:20:7). 1) lutein, 2) lycopene, 3) *cis*-lycopene, 4) γ-carotene, 5) *cis*-ζ-carotene, 6) ζ-carotene, 7) β-carotene, 8) phytofluene, and 9) phytoene.

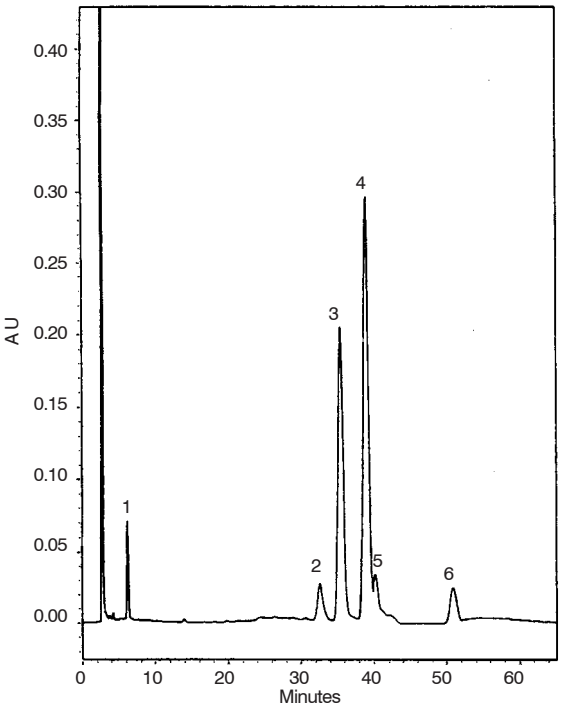


**Figure 15.** HPLC chromatogram of the carotenoids of carrot. Column: Spherisorb S5 ODS2, 2.0 × 250 mm, 5 µm; mobile phase: acetonitrile–methanol–ethyl acetate (73:20:7). 1) lutein, 2) *cis*-ζ-carotene, 3) α-carotene, 4) β-carotene, 5) phytofluene, and 6) phytoene. Detection at wavelengths of maximum absorption (max plot).

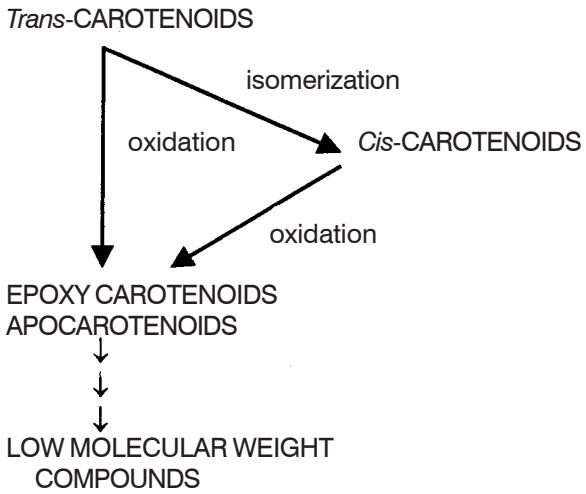


**Figure 16.** Possible scheme for the degradation of carotenoids.

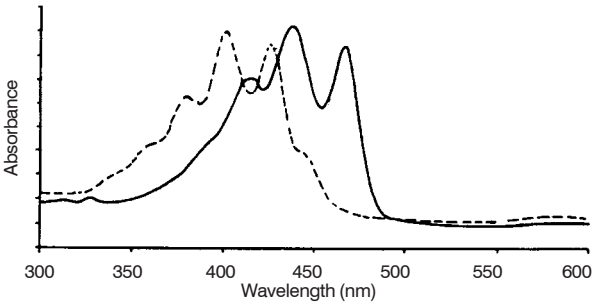


**Figure 17.** Visible absorption spectra of violaxanthin (—) and its furanoid derivative (– – –).

dation of carotenoids exist during preparation, processing, and storage of food. Thus, retention of naturally occurring or added carotenoids in prepared, processed, and stored foods is an important consideration. Carotenoids are also subject to isomerization and oxidation during analysis, and preventative measures must be taken to guarantee the reliability of analytic results.

## Chemical Reactions of Functional Groups

Xanthophylls undergo group chemical reactions that can serve as simple chemical tests in the identification of carotenoids. Many of the chemical reactions, in extensive use in the late 1960s and early 1970s, have now been supplanted by mass and nuclear magnetic resonance spectrometry. However, some reactions remain useful and can be performed quickly with only a small amount of the test carotenoid and are amenable to rapid monitoring by ultraviolet or visible spectrometry, thin-layer chromatography, or HPLC.

For example, primary and secondary hydroxy groups are acetylated by acetic anhydride in pyridine. Allylic hydroxyls, isolated or allylic to the chromophore, are methylated with acidic methanol. In both reactions a positive response is shown by an increase in the silica thin-layer chromatography $R_F$ value or the retention time in reversed-phase HPLC, the extent of the increase depending on the number of hydroxy substituents.

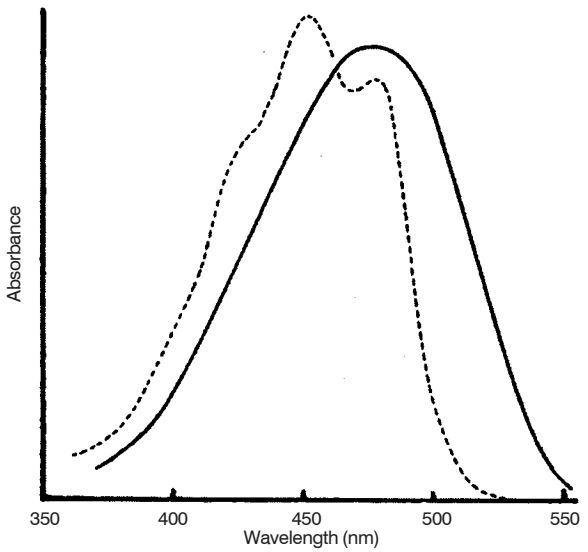


**Figure 18.** Visible absorption spectra of canthaxanthin (—) and its reduction product (– – –).

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 or 50 nm, respectively (Figure 17).

Ketocarotenoids, such as echinenone and canthaxanthin, and apocarotenals undergo reduction with $LiAlH_4$ or $NaBH_4$, manifested by the appearance of the three-maxima spectra of the resulting hydroxycarotenoid (Figure 18).

# GENERAL PROCEDURE AND SOURCES OF ERRORS IN CAROTENOID ANALYSIS

Trends in the analysis of carotenoids have mirrored not only advances in analytic instrumentation, but more importantly the perception of the changing or widening role attributed to these compounds from their coloring properties to their provitamin A activity and their potential protective effect against degenerative diseases. Determination of the total carotenoid content, through the visible absorption at the λmax of the principal carotenoid, although still done and attractive for its simplicity, yields insufficient information and is considered inadequate except as an estimate of the total pigment content. This type of work has given way to the determination of individual carotenoids because of their differing physicochemical properties and bioactivities.

Analyzing individual carotenoids, however, is inherently difficult because of several factors (Rodriguez-Amaya and Amaya-Farfan 1992, Rodriguez-Amaya 1990, 1989):

- There are many naturally occurring carotenoids. More than 600 natural carotenoids are now known, including the enormous variety of carotenoids in algae, fungi, and bacteria. The number of carotenoids found in foods is much less but the food carotenoid composition can still be very complex.
- The carotenoid composition of foods varies qualitatively and quantitatively. Thus, the analytic procedure, principally the chromatographic step, has to be adapted to the carotenoid composition of each type of food sample. The identification of the carotenoids in every food has to be done carefully and, in fact, inconclusive or incorrect identification appears to be a common flaw encountered in the literature.
- The carotenoid concentrations in any given food vary over a wide range. Typically, one to four principal carotenoids are present, with a series of carotenoids at low or trace levels. The separation, identification, and quantification of these minor carotenoids are a formidable challenge to food analysts.
- The highly unsaturated carotenoid molecule is susceptible to isomerization and oxidation, reactions that can easily occur during analysis.

Because of these confounding factors, the reliability of a substantial part of current data on food carotenoids still appears to be questionable.

## Special Precautions in Carotenoid Work

The main problem in carotenoid analysis arises from their instability. Thus, whatever the analytic method chosen, precautionary measures to avoid formation of artifacts and quantitative losses should be standard practice in the laboratory. These include completion of the analysis within the shortest possible time, exclusion of oxygen, protection from light, avoiding high temperature, avoiding contact with acid, and use of high purity solvents that are free from harmful impurities (Schiedt and Liaaen-Jensen 1995, Britton 1991, Davies 1976).

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 even in crude extracts of carotenoids can rapidly lead to bleaching 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 (e.g., butylated hydroxytoluene, pyrogallol, and ascorbyl palmitate) may also be used, especially when the analysis is prolonged. They can be added during sample disintegration or saponification or added to solvents (e.g., tetrahydrofuran), standard solutions, and isolates.

Exposure to light, especially direct sunlight or ultraviolet light, induces *trans-cis* photoisomerization and photodestruction of carotenoids. Thus, carotenoid work must be done under subdued light. Open col-

umns and vessels containing carotenoids should be wrapped with aluminum foil, and thin-layer chromatography development tanks should be kept in the dark or covered with dark cloth or aluminum foil. Polycarbonate shields are now available for fluorescent lights, which are notorious for emission of high-energy, short-wavelength radiation. Absorbing radiation of 375–390 nm and shorter wavelengths, these shields allow the use of full, usual light in laboratories. However, this should not preclude covering flasks, columns, etc., whenever possible.

Speed of manipulation and shielding from light are especially important in extracts containing chlorophylls (e.g., extracts of green leafy or nonleafy vegetables) or other potential sensitizers. In the presence of these sensitizers, photodegradation and isomerization occur very rapidly, even with brief exposure to light.

Because of the thermolability of carotenoids, heating should be done only when absolutely necessary. Carotenoid extracts or solution should be concentrated in a rotary evaporator at reduced pressure and a temperature below 40 °C, and the evaporation of solvent should be finished with nitrogen or argon. Care should be taken to prevent the extract from going to complete dryness in the rotary evaporator because this may result in degradation of carotenoids, especially lycopene (Tonucci et al. 1995). Additionally, part of the carotenoids (especially the more polar ones), may adhere strongly to the glass walls, precluding quantitative removal from the flask.

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

Reagent-grade, ultraviolet-and-visible-grade, or HPLC-grade solvents should be used. If only technical-grade solvents are available, these should be purified, dried, and freshly distilled before being used for extraction or chromatography. Diethyl ether and tetrahydrofuran should be tested for peroxides, which can be removed by distillation over reduced iron powder or calcium hydride. Because it easily accumulates peroxides, tetrahydrofuran is usually supplied stabilized with the antioxidant butylated hydroxytoluene, but there is a time limit to its use.

Chloroform is best avoided because it is difficult to remove all traces of hydrochloric acid. In addition, it is generally stabilized with 1% ethanol, which can affect its properties as a solvent for chromatography. Benzene, although an excellent solvent, should also be avoided because of its toxicity. Chloroform can be replaced by dichloromethane and benzene by toluene.

Fractions or isolates should be kept dry under nitrogen or argon or dissolved in a hydrocarbon solvent, 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. In our laboratory, carotenoids extracted with acetone are immediately transferred to petroleum ether.

It must also be remembered that storing carotenoids in flammable volatile solvents, such as ether, in a refrigerator is a safety hazard and should be avoided. An explosion-proof refrigerator is also recommended.

## General Analytic Procedure

Carotenoid analysis usually consists of

- sampling and sample preparation,
- extraction,
- partition to a solvent compatible with the subsequent chromatographic step,
- saponification and washing,
- concentration or evaporation of solvent,
- chromatographic separation, and
- identification and quantification.

Errors can be introduced in each of these steps. Common sources of error in carotenoid analysis are samples not representing the food lots under investigation; incomplete extraction; physical losses during the different steps, such as incomplete transfer of carotenoids from one solvent to the other when partition is carried out, loss of carotenoids in the washing water, and partial recovery of carotenoids adhering to walls of containers when carotenoid solution are brought to dryness; incomplete chromatographic separation; misidentification; faulty quantification and calculation; and isomerization and oxidation of carotenoids during analysis or storage of food samples before analysis. A good understanding of the purpose of each step and the possible errors is therefore warranted.

Because of the various factors that affect the carotenoid composition of foods as discussed previously, proper sampling and sample preparation to obtain representative and homogeneous samples for analysis are of paramount importance. In addition, results should be accompanied by pertinent informa-

tion, such as the variety, stage of maturity, season, geographic origin, and part of the plant analyzed. Errors incurred in sampling and sample preparation can easily surpass those of the analysis per se.

Laboratory work should be planned so that the samples are analyzed as soon as they are collected because it is difficult to store samples without changing their carotenoid composition, even at very low temperature. Because carotenoid concentration is expressed per unit weight of sample, changes in the food's weight during storage also affect the final result. The Scientific Committee on Oceanic Research (Mantoura et al. 1997) does not recommend storage of filtered microalgae at –20 °C for longer than 1 week.

When storage is absolutely unavoidable, tissue disintegration should be postponed until after storage (at –20 °C or even lower for longer periods) and then carried out immediately before or simultaneously with extraction. Degradative enzymatic reactions during thawing can be minimized by allowing the sample to thaw in the refrigerator (4–6 °C) (Schiedt and Liaaen-Jensen 1995).

Lyophilization is considered by many workers as the appropriate way of preserving biologic samples that have to be stored before carotenoid analysis. However, degradation of carotenoids occurs during this processing (Craft et al. 1993, Ramos and Rodriguez-Amaya 1993, Park 1987), which also makes the sample more porous, thus increasing exposure of carotenoids to oxygen during storage. Because lyophilization causes rapid loss and extensive degradation of chlorophylls and carotenoids, it is not recommended for storage and transport of filtered samples of microalgae or phytoplankton (Mantoura et al. 1997).

To prepare a homogeneous, representative sample for analysis and to facilitate the extraction, samples are cut into small pieces or minced. Once this is done, extraction should immediately follow because tissue disruption releases enzymes (e.g., lipoxygenase) that catalyze substantial carotenoid oxidation and acids that promote *trans-cis* isomerization. In fact, sample maceration, homogenization, and extraction with an organic solvent are usually carried out simultaneously.

## Prechromatographic Steps

Sampling, sample preparation, and the steps preceding chromatography, which are often given only cursory attention, can introduce considerable errors that cannot be compensated for in the measurement steps regardless of how modern or sophisticated the analytic instrumentation is. A good extraction procedure should release all the carotenoids from the food matrix and bring them into solution without causing any change in them. Because carotenoids are found in a variety of foods, the extraction procedure should be adapted to suit the food being analyzed. The solvent chosen should efficiently extract the range of carotenoids present in the sample.

Carotenoids are usually extracted from biologic samples, which contain large amounts of water, with water-miscible organic solvent, such as acetone, methanol, ethanol, or mixtures thereof, to allow better solvent penetration. Dried materials can be extracted with water-immiscible solvents, but extraction is usually more efficient if the samples are rehydrated first and then extracted with water-miscible solvents. Acetone has been frequently used, but with the advent of HPLC, tetrahydrofuran has also become a popular extracting solvent.

The sample is generally homogenized with celite (or Hyflosupercel) and the solvent in a suitable electric blender for 1-2 minutes or with a mortar and pestle. A mechanical blender is fast and efficient for mechanical disruption and homogenization of soft fruits and juice. For samples such as fresh leaves, the simple mortar and pestle is better because small pieces of leaves, which can escape the blender blades, can be well ground. A combination of the two can also be used, starting with the blender and finishing with the mortar and pestle. In fact, leaves and other difficult-to-extract matrices may also need previous soaking in the extracting solvent (about 15 minutes for leaves) to soften the cell wall. Prolonged soaking should, however, be avoided to prevent isomerization and degradation of the carotenoids. Celite facilitates both tissue disintegration and filtration. After filtration the solid residue is returned to the blender and reextracted with fresh solvent. Extraction and filtration are repeated until the residue is colorless (three extractions are usually sufficient).

Oxidation can be reduced by directing nitrogen into the blending vessel or by adding dry ice before homogenization. This will, however, increase the cost of analysis. In our experience, using cold acetone (left in the refrigerator for a short time before use) and doing the extraction rapidly are sufficient to prevent errors in this step.

Filtration can be done with a sintered glass funnel (porosity 3; pore size 20–30 μm) or with a Buchner funnel. The latter is less expensive and does not have the problem of clogged pores. The filter paper should, however, be properly fitted so that celite and the fine sample particles do not pass through.

Extraction and open-column chromatography

(OCC) should be carried out under a fume hood to protect the analyst from inhaling solvent vapor. Breathing hexane, for example, should be avoided because of reported neurotoxicity of some of its oxidative metabolites (Schiedt and Liaaen-Jensen 1995).

The extract usually contains a substantial amount of water, which can be removed by partition to hexane, petroleum ether, diethyl ether, or dichloromethane or mixtures of these solvents. 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 during partition with pure hexane or petroleum ether. Partition is an integral part of open-column methods so that chromatography can be started at low mobile-phase polarity, the polarity being increased during the separation process. In HPLC methods the extract is often evaporated to dryness and then dissolved in the mobile phase or a solvent compatible with the mobile phase.

Partition is best done by adding small portions of the acetone extract to petroleum ether or another appropriate solvent in a separatory funnel. 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 acetone) is discarded. When the entire extract has been added, the petroleum ether phase is washed 4 or 5 times with water to remove residual acetone.

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 an 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 reextracted with fresh petroleum ether. The combined petroleum ether solution is then washed 5 times with water. In our experience, the first procedure is more efficient and emulsions are less likely to form.

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

Saponification is an effective means of removing chlorophylls and unwanted lipids, which may interfere with the chromatographic separation and shorten the column's life in HPLC. In samples such as fruits, saponification hydrolyzes the carotenol esters. This simplifies the chromatographic separation, identification, and quantification because the free carotenols are analyzed instead of the carotenol esters, which usually occur as a difficult-to-separate mixture of esters with a variety of fatty acids. However, saponification extends the analysis time, and may provoke artifact formation and degradation of carotenoids. The extent of degradation depends on the conditions used, being greater with higher concentration of alkali and on hot saponification (Kimura et al. 1990).

Although provitamin A carotenoids (α-carotene, β-carotene, γ-carotene, and β-cryptoxanthin) can resist saponification (Kimura et al. 1990, Rodriguez-Amaya et al. 1988), lutein, violaxanthin, and other dihydroxy-, trihydroxy-, and epoxycarotenoids are reduced considerably during saponification and the subsequent washing step (Riso and Porrini 1997, Kimura et al. 1990, Khachik et al. 1986). Saponification should therefore be omitted from the analytic procedure whenever possible. 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 coextracted with carotenoids from leaves can be separated during chromatography.

When indispensable, saponification is best done after transferring 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 5 times with water to remove the alkali. To avoid losing carotenoids with the washing water, especially the more polar ones, this step should be done in the same manner as in partition, described earlier. When apocarotenals (e.g., β-citraurin in citrus) are present in the sample, all traces of acetone must be removed before saponification to avoid facile aldol condensation between the apocarotenals and acetone.

For high-lipid samples, such as red palm oil, a better procedure for eliminating lipids is being pursued. Using the nonspecific *Candida cylindracea* lipase, complete hydrolysis of red palm oil was achieved after four hours at 35 °C under a nitrogen atmosphere (Lietz and Henry 1997). This mild hydrolysis allowed quantitative analysis of carotenoids without isomerization and degradation. Unfortunately, production of the *C. cylindracea* lipase has been discontinued. Lietz (personal communication) now injects the red palm oil samples without fat elimination. The oil (about 0.05 g) is dissolved first with 20% dichloromethane and then with 80% acetone in a 5-

mL flask. An injection volume of around 20 µL and the acetonitrile-based mobile phase of Hart and Scott (1995) are used. A guard column is required and it should be changed when the pressure increases to 3300 psi.

In our laboratory, the palm oil sample is dissolved in acetone and left in a freezer (–15 °C) for 4–5 hours to solidify the lipids (Trujillo et al., unpublished). The lipids are then separated by filtration with a sintered glass funnel, the operation being carried out in the freezer compartment to maintain the low temperature. About 90% of the lipids is removed in this process. After partition to petroleum ether, the carotenoid solution is saponified with equal volume of 20% potassium hydroxide in methanol overnight at room temperature with the addition of butylated hydroxytoluene. Concern about the possible negative effects of saponification has recently led researchers to shorten the duration of ambient temperature saponification (e.g., 1 or 2 hours). In our experience, however, carotenol esters of papaya were completely hydrolyzed only after overnight saponification (Kimura and Rodriguez-Amaya, unpublished).

To follow the chromatography rule that the sample be introduced in the chromatographic system in the smallest volume possible, the carotenoid solution, after partition in unsaponified sample or after washing in saponified sample, is dried with sodium sulfate and then concentrated for OCC or evaporated to dryness to be taken up in the mobile phase or another appropriate solvent for HPLC.

## Chromatographic Separation

The extent to which chromatographic separation is carried out in carotenoid analysis depends on the information desired. A perusal of current literature shows that this type of analysis is carried out to determine only the provitamin A carotenoids, major provitamin A and nonprovitamin A carotenoids, *cis* and *trans* isomers of provitamin A carotenoids, and complete carotenoid composition. Considering the much greater complexity and added cost of complete carotenoid analysis and the very low levels of minor carotenoids, on one hand, and the importance of both provitamin A and nonprovitamin A carotenoids, on the other hand, the second approach appears to be the most suitable for gathering data for food composition tables. For research, however, the complete carotenoid composition gives valuable, detailed information. For the first and second approach, classical OCC can do as well as HPLC (Adewusi and Bradbury 1993, Wilberg and Rodriguez-Amaya 1995, Carvalho et al. 1992) provided that optimum conditions are used for both techniques. The advantage of HPLC becomes evident when the analysis is aimed at the full range of carotenoids.

Carotenoids are found in nature primarily in the more stable *trans* configuration, but small amounts of *cis* isomers are increasingly being encountered. Because *cis* isomers have different biologic potency from that of their *trans* counterparts (e.g., lower provitamin A activity), the necessity of separating and quantifying *cis* isomers apart from the *trans* carotenoids, has been raised. This appears particularly important in green vegetables (Godoy and Rodriguez-Amaya 1998) and in thermally processed or cooked food (Lessin et al. 1997, Chen et al. 1995, Rodriguez-Amaya and Tavares 1992, Chandler and Schwartz 1988, Sweeney and Marsh 1971). This level of detail, however, makes the analysis even more complicated.

Food samples typically contain both the apolar carotenes and the more polar xanthophylls. Whatever the method used, the chromatographic process should be able to cope with this polarity range.

Chromatography in descending, gravity-flow (often with slight pressure provided by a water aspirator) columns, currently referred to as OCC, is the classical method of separating carotenoids for quantitative analysis. It is also useful in separating and purifying carotenoids to be used as standards for HPLC. Separation of the carotenoid pigments is followed visually. Low pressure may also be applied at the top of the column (e.g., with nitrogen gas), this technique being called flash column chromatography.

Thin-layer chromatography, although efficient in monitoring the progress of chemical tests for identification purposes, is not adequate for quantitative analysis because of the danger of degradation and isomerization on a highly exposed plate (Taylor 1983, Liaaen-Jensen 1971). Carotenoids are particularly prone to oxidation by air when adsorbed on the thin layer. Additionally, it is not easy to quantitatively apply the sample on the plate and quantitatively recover the separated carotenoids from the plate for measurement. Gas chromatography is also inappropriate because of the thermal lability and low volatility of carotenoids.

A major advantage of OCC is the simple and inexpensive column (i.e., glass column packed with the adsorbent). However, reproducibility and efficiency of the separation of carotenoids depend on the skill, patience, and experience of the analyst, particularly in packing the column and adjusting the volumes and proportions of the eluting solvent, as well as the analyst's acuity for detecting the separation.

The possibility of degradation varies with different stationary phases (adsorbents) and increases as the chromatographic process is prolonged. Rechromatography of an impure fraction may sometimes be necessary, extending the analysis time and increasing the danger of carotenoid decomposition.

Common adsorbents are magnesium oxide–Hyflosupercel, activated or not, and in different proportions (e.g., 1:1 or 1:2), and deactivated, neutral alumina. Magnesium oxide was found to be least likely to cause carotenoid alteration (Tanaka et al. 1981, Rodriguez-Amaya et al. 1976b), although the contrary was observed with magnesium oxide activated according to the Association of Official Analytical Chemists (Rouchaud et al. 1984). Isomerization, degradation, or both are more likely to happen in an alumina column, so magnesium oxide–Hyflosupercel should be the first choice. Magnesium oxide is usually diluted with celite or Hyflosupercel to lower adsorption affinity and thus prevent irreversible adsorption of polar carotenoids. Also, when used alone, magnesium oxide is sufficiently basic to catalyze aldol condensation and cause polymerization of acetone. Silica gel is not a popular adsorbent because its inherent acidity may cause carotenoid isomerization and degradation (Taylor 1983, Tanaka et al. 1981, Rodriguez-Amaya et al. 1976b). Many solvent combinations have been tried, but the most common is petroleum ether or hexane containing increasing amount of diethyl ether and acetone.

Commercially available adsorbents are known to vary in their adsorptive properties, and even minute amounts of impurities, especially polar substances, alter the solvent's eluting strength. Although variations are greater between brands, lot-to-lot differences also exist, and these variations tend to be greater in developing countries where quality control of laboratory materials may not be as rigorous. Therefore, a laboratory's first attempt may not duplicate reported separation, and adjustment of the chromatographic conditions may be necessary.

The adsorption capacity can be increased by activating the adsorbent for 4 hours at 110 °C or decreased by increasing the proportion of Hyflosupercel (e.g., magnesium oxide–Hyflosupercel, 1:2). The composition and volumes of the eluting solvents should also be optimized. For example, to increase the separation of α- and β-carotene, activated magnesium oxide–Hyflosupercel (1:1) can be used, the volumes of the initial solvents (i.e., petroleum ether and 1% ether in petroleum ether) can be increased, or both can be done. Because carotenoids are colored, alterations in the eluting solvents can be made without resorting to the collection and scanning of numerous fractions, which would be necessary for colorless compounds.

To separate *cis* and *trans* isomers by OCC, especially of the provitamin A carotenoids, each fraction separated by the magnesium oxide–Hyflosupercel column is rechromatographed on a smaller (dimensions depend on the amount of carotenoid) calcium hydroxide column, using 0%, 2%, and 4% ethyl ether in petroleum ether to elute the isomers of β-carotene and 10% and 20% acetone in petroleum ether to elute the isomers of β-cryptoxanthin (Godoy and Rodriguez-Amaya 1998, 1994, Rodriguez-Amaya and Tavares 1992, Bickoff et al. 1949). Although quite laborious, this traditional method is still considered the most effective and practical way of separating isomeric mixtures of carotenoids in quantity (Tsukida 1992).

In OCC, a column has to be packed for each analysis. A definite improvement in HPLC is the possibility of reproducible separations with a reusable column, under controlled conditions, without undue exposure to air or light. Reversed-phase HPLC on $C_{18}$ columns is clearly the preferred mode. Reasons for the popularity of the $C_{18}$ column are its weak hydrophobic interactions with the analytes (thus it is expected to be less destructive than the polar forces in normal-phase OCC), compatibility with most carotenoid solvents and the polarity range of carotenoids, and wide commercial availability.

Many different $C_{18}$ reversed-phase materials are available from different manufacturers and vary in the degree of carbon loading, end capping, and the nature of the bonded phase (i.e., monomeric or polymeric). Lack of reproducibility is a persisting problem. The properties and quality of the same kind of column differ considerably between brands, between batches of the same brand, and even within the same batch (Pfander and Riesen 1995). Thus, some adjustments are often needed when published methods are adapted.

Most carotenoid separations have been carried out with 5-μm $C_{18}$ spherical particles packed in a 250 × 4.6 mm column. Some laboratories are already using shorter and narrower (narrow bore) columns, smaller particles, and a $C_{30}$ stationary phase. Most commercial reversed-phase columns are now end capped to minimize polar interaction of the silanol residues with the analytes and thus diminish tailing and improve column reproducibility.

Monomeric phases are simpler to use and more reproducible. Polymeric $C_{18}$ phases, on the other hand, have been found to have excellent selectivity for structurally similar carotenoids, as in the difficult separation of geometric isomers of β-carotene (Craft et al.

1990, Lesellier et al. 1989, Quackenbush and Smallidge 1986, Bushway 1985) and of lutein and zeaxanthin (Epler et al. 1992). However, the total carbon load is lower in the wide-pore polymeric phases, resulting in weak retention of the carotenoids (Craft 1992). Additionally, the peaks tend to be broader and columns from different production lots are more variable than with monomeric columns.

Guard columns, which should be changed frequently, are needed for food samples to prevent particulate material and impurities from entering the analytic column, thus prolonging the column's life. It can, however, increase band broadening, and the possibility that part of the carotenoid can be retained in it cannot be overlooked.

Metal surfaces, particularly stainless steel frits in the guard and analytic columns, were reported to be damaging to carotenoids (Scott 1992). Thus, the use of metal-free columns (e.g., with "biocompatible" Teflon frits) (Craft et al. 1992) and poly ether ether ketone (PEEK) tubing for column connections (Hart and Scott 1995) has been recommended.

The most important properties to be considered in selecting the mobile phase are polarity, viscosity, volatility, and toxicity. In addition, it must be inert with respect to the carotenoids. Many solvent systems have been suggested as mobile phases for carotenoids, but the primary solvents are acetonitrile and methanol, and most systems are actually slight modifications of some basic combinations (Craft 1992). Acetonitrile has been widely used because of its lower viscosity and slightly better selectivity for xanthophylls when monomeric $C_{18}$ column is used (Khachik et al. 1986). Epler et al. (1992) reported, however, that with methanol-based solvents, higher recoveries of carotenoids occurred in almost all of 65 columns tested. Methanol is also more available, less expensive, and less toxic than acetonitrile.

Recovery of carotenoids from the column was improved when ammonium acetate was added to acetonitrile-based solvents. Addition of triethylamine to the mobile phase containing ammonium acetate further increased recovery, from around 60% to over 90% (Hart and Scott 1995).

Small amounts of other solvents are added to obtain the desired retention, increase solubility, and improve resolution. Frequently used for this purpose are chlorinated solvents (e.g., chloroform and dichloromethane) because of their good solvent properties and effects on selectivity, although these solvents can be contaminated with traces of hydrochloric acid. Other solvents used as modifiers are tetrahydrofuran, ethyl acetate, hexane, acetone, and water. Some methanol has also been added to acetonitrile-based mobile phase. Craft et al. (1992) investigated nine solvent modifiers and found tetrahydrofuran to be the most beneficial modifier of methanol. There is a tendency to use mixtures of three or more solvents. Craft (1992) cautioned against this practice because it can make the method more complicated, enhance demixing, and result in different evaporation rates, causing variation in the retention times during the course of the day.

Nelis and Leenheer (1983) advocated the use of nonaqueous reversed-phase liquid chromatography for the separation of complex carotenoid mixtures, citing optimal sample solubility hence minimum risk of sample precipitation on the column, increased sample capacity, excellent chromatographic efficiency, and prolonged column life. Many workers, however, use solvent mixtures containing water. For example, when using the efficient Vydac columns, a small amount of water may be needed to resolve the early eluting free xanthophylls, such as those in leaves.

Gradient elution should only be used when the analysis cannot be done isocratically. Isocratic separation is rapid, can be performed with simple equipment (with a single high-pressure pump and premixed solvent), and results in stable baseline and more reproducible retention times. It is usually sufficient for the determination of provitamin A carotenoids or the principal carotenoids of food samples.

Gradient elution has the advantage 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: 1) increased complexity, 2) requirement for more sophisticated and expensive equipment, 3) need for column reequilibration between runs, 4) greater differential detector's response (i.e., different detector's signals for the same concentration of different compounds), and 5) often poor reproducibility. The column must be brought back to the starting solvent and equilibrated for 10–30 minutes in this solvent before a new run is commenced. Good solvent miscibility is required to prevent baseline disturbance resulting from outgassing and refractive index effects (Craft 1992).

Because of the qualitative and quantitative variation of the carotenoid composition of foods, it is doubtful that a single chromatographic system can be established that can be applicable to the different foods. At least some modification of the mobile phase is needed when changing from one food to another.

The injection solvent must be compatible with the HPLC mobile phase. If the carotenoids are much more soluble in the injection solvent than in the mobile phase, and especially if the solution is nearly satu-

rated, the carotenoids will precipitate on injection, leading to peak tailing, or they will remain in the injection solvent while passing though the column, resulting in broad bands and doubled peaks (Craft 1992). On the other hand, the sample will not dissolve completely if the solvent is too weak. Samples can be injected in the mobile phase to avoid this problem of incompatibility. However, because of the solubility range of carotenoids in food samples, another solvent may be preferred for solubilization and injection. Porsch (1993) suggested that sample solvent–mobile phase viscosity should be kept fairly below 2, and the much higher dissolving power of the injection solvent should be decreased by mixing with the mobile phase before injection.

Khachik et al. (1988) observed peak splitting when *trans* carotenoids were injected in dichloromethane, chloroform, tetrahydrofuran, benzene, or toluene, the mobile phase being a mixture of methanol, acetonitrile, dichloromethane, and hexane. No such splitting occurred when the injection solvent was acetone, acetonitrile, methanol, or hexane. In our experience (Kimura and Rodriguez-Amaya, unpublished) and that of other authors (Lietz and Henry 1997), acetone is a good injection solvent because it has similar polarity and solubility properties to those of the mobile phase. Zapata and co-workers (Zapata and Garrido 1991, Zapata et al. 1987) reported severe peak distortion when acetone extracts were injected into their reversed-phase HPLC system with a linear gradient from 100% methanol–1M ammonium acetate (8:2) to 100% methanol-acetone (8:2). Because peak splitting depends on the chromatographic system and reported results do not seem to be consistent, analysts should test their own systems. Khachik et al. (1988) also showed the importance of injection volume, demonstrating that HPLC peak distortions resulting from the injection solvents mentioned above can be eliminated if the injection volume is reduced to 5 or 10 μL.

Temperature regulation is recommended to maintain within- day and day-to-day reproducibility. Variations in column temperature results in substantial fluctuation of the carotenoids' retention times. Temperature also influences selectivity. With a monomeric $C_{18}$ column and acetonitrile-dichloromethane-methanol (70:20:10) as mobile phase, no separation of lutein and zeaxanthin, and 9-*cis*- and *trans*-β-carotene occurred at ambient (30 °C) temperature (Sander and Craft 1990). At subambient temperature (–13 °C), good separation of lutein and zeaxanthin and baseline separation of 9-*cis*- and *trans*-β-carotene were achieved. In a Vydac 201TP54 (polymeric) column with acetonitrile-methanol-dichloromethane (75:20:5) as mobile phase, optimum resolution of lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene was achieved at 20–22.5 °C (Scott and Hart 1993). Also with a Vydac 201TP column and 5% tetrahydrofuran in methanol as mobile phase, resolution of lutein and zeaxanthin and of β-carotene and lycopene was better at lower temperature, while the separation of echinenone and α-carotene improved as the temperature increased (Craft et al. 1992). The latter system was optimized at a column temperature of 20 °C. In addition to good separation, recovery of seven carotenoids was found to be nearly 100%.

One difficult separation that has been pursued in earnest is the resolution of *cis* and *trans* isomers. At present the best column for this purpose is a polymeric $C_{30}$ column developed at the National Institute of Standards and Technology for optimum separation of carotenoids (Sander et al. 1994). It was designed to have high absolute retention, enhanced shape recognition of isomers, and moderate silanol activity. These qualities were achieved by $C_{30}$ polymeric surface modification of a moderate pore size, moderate surface area silica, and without subsequent end capping.

The $C_{30}$ column has been shown to provide excellent resolution of photoisomerized standards of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene (Emenhiser et al. 1996a, 1995), as well as geometric isomers of β-carotene, α-carotene, lutein, and lycopene in extracts of biologic samples (Emenhiser et al. 1996b). With an isocratic solvent system consisting of methanol–methyl *tert*-butyl ether (89:11), this column was recently used for the quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables (Lessin et al. 1997).

Previous to the introduction of the $C_{30}$ column, the laboratory-packed calcium hydroxide column (Schmitz et al. 1995; Petterson and Jonsson 1990; Chandler and Schwartz 1988, 1987; Tsukida et al. 1982), and the polymeric $C_{18}$ Vydac 201TP column (Craft et al. 1990, Quackenbush and Smallidge 1986) were considered the most efficient (O'Neil et al. 1991). Chromatographic analysis of *cis-trans* carotenoid isomers was reviewed by O'Neil and Schwartz in 1992.

Recovery from the HPLC column varies with different carotenoids (Hart and Scott 1995, Epler et al. 1992). Special attention must be given to lycopene. Distinctly higher intralaboratory (Hart and Scott 1995) and interlaboratory (Scott et al. 1996) coefficients of variation and a lower range of linearity (Riso and Porrini 1997) were found for this carotenoid. Konings and Roomans (1997) observed a consider-

able loss of about 40% of lycopene even when the biocompatible hastalloy frit was used. This problem was solved by changing to PAT (PEEK alloyed with Teflon) frits.

## Identification and Quantification

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. The relationship between these two characteristics of the spectrum and structural features of carotenoids is discussed in the section on physicochemical properties.

In HPLC the availability of the photodiode array detector allows the acquisition of the spectra on-line, making the use of this criterion easier. Spectra can be taken, stored, and subsequently compared with those of standards. Spectra taken at points across the peak provide a means of verifying peak purity (i.e., absence of interfering substances). On the other hand, in OCC enough isolated carotenoids are collected to submit to chemical tests.

Identification of carotenoids based solely on the retention times and the absorption spectra may lead to erroneous conclusions. Retention times are difficult to reproduce even within a laboratory and may vary during the course of a day. Even when carotenoid standards are available and co-chromatography (i.e., spiking) can be done, identification is still not conclusive because different carotenoids can have the same retention time in a given chromatographic system. By the same token, different carotenoids may have the same chromophore, thus presenting the same absorption spectrum. Because of the widespread use of these two parameters as the only criteria, cases of misidentification can be discerned in the literature. Thus, it is now recommended that the following minimum criteria be fulfilled for identification (Schiedt and Liaaen-Jensen 1995, Pfander et al. 1994):

- the visible (or ultraviolet for shorter chromophores) absorption spectrum (λmax and fine structure) in at least two different solvents must be in agreement with the chromophore suggested;
- 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; and
- a mass spectrum should be obtained, which allows at least confirmation of the molecular mass.

The requirement of a mass spectrum, however, would limit carotenoid analysis to a very few laboratories around the world, precluding its execution in areas where it is very much needed. Moreover, common, major carotenoids can be conclusively identified by the judicious and combined use of chromatographic data, absorption spectra, and specific chemical reactions, the latter to confirm the type, location, and number of functional groups.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are, however, indispensable in the elucidation of unknown or inconclusive structures of carotenoids. MS has been extensively used to elucidate the structures of carotenoids in algae, fungi, and bacteria but had been used only in a few publications on food carotenoids, such as some papers by Gross and co-workers in the late 1970s and 1980s. Increased use of this technique has been evident in recent years.

Although newer mass spectrometry ionization techniques have been used, such as fast atom bombardment (van Breemen et al. 1995, Schmitz et al. 1992) and chemical ionization (CI) (Khachik et al. 1992a, 1989, 1986), electron impact is still the most commonly used technique. Almost all carotenoids give good molecular ions and, in addition, many fragmentations have been identified that are diagnostic of structural features. Electron impact–MS was used recently to confirm the identity of carotenoids from mango and yellow passion fruit (Mercadante et al. 1998, 1997a). A recent chapter on MS presents tabulated data for 170 different carotenoid end-group fragmentations (Enzell and Back 1995). HPLC-MS is being applied to carotenoids and is considered particularly important for those coming from natural sources, which are usually present in trace quantities and often contaminated with interfering compounds (van Breemen 1996). When not coupled with HPLC, MS (as well as NMR) requires rigorous isolation and purification procedures by OCC, TLC, and preparative HPLC.

NMR analysis may prove a carotenoid structure unequivocally, including the geometry of the double bonds. With refinements in instrumentation in the past decade, NMR has become an even more efficient spectroscopic tool for the structural elucidation of carotenoids, and comprehensive $^{1}$H-NMR and $^{13}$C-NMR chemical shift data for carotenoids are available (Englert 1995). Although still limited, it is increasingly used in food carotenoids, such as in the structural elucidation of the β-cryptoxanthin monoepoxide (5,6-epoxy-5,6-dihydro-β,β-caroten-3-ol) in papaya (Godoy et al. 1990), *cis*-isomers of β-carotene (Tsukida et al. 1981) and α-carotene (Emenhiser et al. 1996a), new apocarotenoids from annatto

(Mercadante et al. 1997b, 1996), carotenoids from guava (Mercadante et al. 1999), and crocetin derivatives from saffron and gardenia (Van Calsteren et al. 1997).

The quantification step in OCC methods is fairly straightforward. The separated carotenoid fractions are collected and quantified spectrophotometrically through the use of tabulated absorption coefficients. In quantitative analysis by HPLC, the following facts should be considered: carotenoids absorb maximally at different wavelengths and have different absorption coefficients; solvent effects on absorption are substantial (tabulated absorption coefficients and λmax values refer to single solvents, mobile phase in HPLC isocratic elution is usually a mixture, and in gradient elution the mixture's composition varies during the chromatographic process); and obtaining and maintaining carotenoid standards, which are required for calibration, are difficult.

Modern liquid chromatographs allow measurement of carotenoids at the wavelengths of maximum absorption. In older chromatographs, more than one injection for the same sample may be necessary for samples containing phytoene, phytofluene, or ζ-carotene along with other carotenoids.

HPLC quantification is carried out by means of internal or external calibration for which the concentrations of the standards are also determined spectrophotometrically as in OCC. Both OCC and HPLC methods assume that tabulated absorption coefficients are accurate, which is not the case for all carotenoids included in the tables.

A constant supply of carotenoid standards is needed in HPLC methods, especially when external standardization is used. The accuracy of the analytic results depends on how accurately the concentrations of the standard solutions are known. Unfortunately, only a few carotenoid standards (e.g., α-carotene, β-carotene, and lycopene) are available commercially. Moreover, commercial β-carotene standards have been shown to have widely varied purity (Craft et al. 1990, Quackenbush and Smallidge 1986). Other carotenoids have to be isolated and purified from natural sources by the analyst. This can be done by OCC or by accumulating separated fractions from several HPLC runs. Both procedures are time consuming and tedious and require experience and patience.

An ideal commercially available internal standard has not been encountered. It is not easy to find a readily available and stable compound that has chemical and spectral properties similar to those of the carotenoids. The stable Sudan I has been used for the determination of provitamin A carotenoids (Quackenbush and Smallidge 1986), but it can coelute with major nonprovitamin A carotenoids. Synthetic carotenoids, such as β-apo-carotenal, canthaxanthin, and echinenone, which are not found in the samples being analyzed, have also been used but are subject to instability problems as the sample's carotenoids.

The instability of carotenoid standards is a serious problem. 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. The analyst has to prepare solutions of various concentrations, inject each of these solutions, and construct the curve. Inaccuracies in the preparation of the solutions, determination of the concentrations, and construction of the calibrating curves will affect the reliability of the results.

Khachik et al. (1992a) cited the following parameters for evaluating the validity of the standards and the instrumentation: the correlation coefficient should be greater than 0.9, the intercept should be very close to zero, and the relative standard deviation of the regression (standard error of the estimate divided by average concentration of standards multiplied by 100) should be less than 5%. If any of these parameters is out of range, the standard as well as the HPLC instrumentation should be carefully examined and the standard curve should be rerun.

The wide concentration range of carotenoids in any given food is more of a problem in HPLC than in OCC, in which each fraction is simply diluted or concentrated to have an adequate volume for spectrometric reading.

In both OCC and HPLC, accurate quantification requires conclusive identification and optimal separation of the carotenoids. Numerous papers have been dedicated to the separation of carotenoids by HPLC. Only a few involved quantification of the carotenoids, but this number has increased notably in recent years. Especially in earlier studies, and despite the repeatedly cited excellent resolving potential of HPLC, highly overlapping peaks have been quantified without mention of or allusion to the error involved in such a practice. It is obvious that in quantitative HPLC analysis the accuracy is dictated by how accurately the peak areas are determined. The continued improvement in column efficiency resulting in chromatograms with well-resolved peaks is reassuring, indicating that this source of error is ceasing to be a serious problem in carotenoid analysis.

Aside from the internal standard for calibration, standards also termed internal standards have been added at the beginning of the analysis to appraise losses of carotenoids during extraction and the entire work-up procedures. Given the differing stability of carotenoids and the standards themselves, it is questionable whether recovery percentages of the standards do indicate true losses of the carotenoids. Additionally, use of these standards does not evaluate the extraction step because they are not intimately linked with the food matrices and, therefore, are more easily extracted than are the endogenous carotenoids.

Notwithstanding the inherent difficulties and the many possible errors, reliable analytic data on food carotenoids can be obtained in the hands of careful and well-informed analysts. The satisfaction from accomplishing such a daunting task is immeasurable.

# DO'S AND DON'TS IN CAROTENOID ANALYSIS

For emphasis, the necessary measures that should be taken to ensure the reliability of carotenoid data are summarized below.

## Do's

- Before starting any analytic work, familiarize yourself with the nature of food carotenoids and the physicochemical properties of these compounds. With this background information, you will save time and money and prevent analytic errors.
- If you have the opportunity, undergo training for at least 15 days in an experienced carotenoid laboratory. This will put you ahead in your work much faster.
- If you are going to use a high-performance liquid chromatographic (HPLC) method, be sure that you know how to use this chromatographic technique very well. It is very easy to make errors with this technique, and because the results are reproducible (high precision), erroneous data are not easily perceived.
- Search the literature for previous carotenoid work with the food samples you intend to analyze. This will give you an idea of what to expect, but remember that natural variations exist among samples of the same food (as a function of factors such as stage of maturity, variety, climate or season, and portion taken as edible) and analytic errors persist in the literature.
- Work under subdued artificial light from the extraction step on. If the laboratory is used by other analysts for other types of analyses (as is usually the case in developing countries) and it is not possible to put the whole laboratory under dim light, choose the part of the laboratory most protected from sunlight, turn off the lights in this area, and shield carotenoids from diffuse light by covering vessels and equipment (e.g., open columns and thin-layer chromatography development tanks) with aluminum foil or black cloth. It may also be necessary to put blinds on windows, use tinted windows, or cover some windows with aluminum foil or other appropriate material.
- Test your selected analytic method exhaustively before you use it to gather data. The time, resources, and effort you spend in testing will be more than compensated by avoidance of erroneous or meaningless results.
- Use reagent-grade solvents and reagents for the prechromatographic steps and for open-column chromatography. If only technical-grade solvents are available, distill them before use. Use HPLC-grade solvents for HPLC. Purification of solvents for HPLC in the laboratory may be time consuming and, in the end, may be more costly.
- Test ethyl ether and tetrahydrofuran for peroxides; if present remove peroxides, for example, by distillation over reduced iron powder. Stabilize tetrahydrofuran with an antioxidant (e.g., butylated hydroxytoluene BHT); if stabilized tetrahydrofuran is bought, observe the time limit for its use.
- Carry out all operations as rapidly as possible, especially the steps that introduce risks of oxidation and isomerization. Take advantage of the color of carotenoids and monitor carotenoid losses in the different steps by carefully observing color changes.
- Exclude oxygen as much as possible. Store carotenoids in air-tight containers under vacuum or nitrogen.
- Obtain and prepare samples for analysis according to an adequate plan that meets the objectives of your work and submits homogeneous and representative samples for analysis. Unless sampling and sample preparation are done properly, it is useless to spend time and effort in carrying out the analysis per se with great accuracy.
- Plan your work so that samples are analyzed immediately after collection. Changes are difficult to prevent during storage of samples, even at low temperature. If you have to store samples, store them

intact at –20 °C or lower for the shortest possible time.

- While recognizing that carotenoid analysis is inherently complicated, minimize this complexity and keep the number of steps to a minimum—without jeopardizing the analysis—to limit the opportunities for errors.
- Protect yourself from solvent fumes by working in a well-ventilated laboratory, preferably under a fume hood.
- Use amber glassware or cover ampoules, vials, and flasks with aluminum foil. The latter has the advantage that one can visually verify the complete removal of the carotenoids when transferring to another container.
- Make sure that the extraction is complete by using solvents capable of penetrating the food matrix and dissolving the range of carotenoids present in the sample without altering or degrading them while posing minimal or no toxic effects to you, the analyst.
- If partitioning is part of your analytic method, test and improve your operational skill so that carotenoids are not lost with the phase to be discarded.
- Dry carotenoid solution by simply adding a small amount of anhydrous sodium sulfate (until a few crystals appear loose). Passing the solution though a column of sodium sulfate extends the drying time, some carotenoids may be retained in the column without being perceived, and more sodium sulfate than is necessary may be used.
- Concentrate carotenoid solutions in a rotary evaporator, not letting the temperature exceed 40 °C (we use 35 °C). Avoid bringing carotenoids to complete dryness. If it is necessary to remove the solvent totally, then evaporate just to dryness. Prolonged drying increases the possibility of degradation and may leave the carotenoids tightly adhered to the glass walls, making removal from the flask difficult. If your budget permits, finish solvent evaporation or concentrate small volumes of carotenoid with nitrogen.
- Before applying the sample, test the open column by passing petroleum ether or hexane, adjusting the solvent flow, and verifying that the flow is even and the adsorbent smooth, indicating uniform packing. Apply the sample in the smallest volume possible to minimize band broadening.
- Calibrate your ultraviolet-visible spectrophotometer with a holmium wavelength calibration standard. Because of the variation in reported λmax values, these values should be used with caution and compared with spectra recorded in the laboratory with carotenoid standards. Calibrate absorbance by using potassium dichromate. As with any spectrophotometric determination, scan carotenoid solution at concentrations corresponding to absorbances between 0.2 and 1.0.
- Remember that the HPLC injection solvent should be compatible with the mobile phase in eluting and dissolving strength and viscosity to avoid peak distortion and splitting.
- To maximize the lifetime of your column, follow closely the manufacturer's instructions on caring for, cleaning and regenerating, and storing the column.
- Use guard and analytic columns with metal-free frits (e.g., Teflon frits) because metal surfaces may react with carotenoids. Use a polymeric, small-bore tubing such as poly ether ether ketone to connect components through which carotenoids traverse (i.e. injector, column, and detector).
- Check the purity of carotenoid standards on arrival. Divide pure standards into several portions. Use one portion for preparing stock and working solution for current analyses. Store the other portions in sealed ampoules under nitrogen or argon at –20 °C or at an even lower temperature for future use. Verify the time it takes for carotenoids in your stock and working solutions to start degrading. Remember that the accuracy of your result cannot be better than the accuracy of the concentrations of your standard solutions.
- Identify the carotenoids in your sample with the combined use of chromatographic data (TLC $R_F$, HPLC $t_R$), cochromatography, ultraviolet and visible spectra, and chemical reactions. Mass spectrometry is required for carotenoids that cannot be conclusively identified by the other parameters cited.
- In HPLC quantification remember that the standard curve should be linear, pass through the origin, and bracket the expected concentrations of your samples. It should be constructed with at least three, preferably five, different concentrations of the standards measured in triplicate.
- Use the least amount of solvents, reagents, and other materials to carry the different steps efficiently and recycle used materials (e.g., distill used petroleum ether and reuse aluminum foil). Think of the cost and resources spent in the manufacture of these materials and the ecologic problem of waste disposal.
- Keep all glassware extra clean. To avoid contamination with carotenoids of previous analyses, rinse glassware with acetone before and after the usual laboratory washing.

## Don'ts

- Do not leave or store carotenoids in dichloromethane, ethyl ether, or acetone because they degrade faster in these solvents. When not in use, carotenoids should be left in petroleum ether or hexane or dry, under vacuum or nitrogen.
- Do not use blenders with plastic cup. Acetone corrodes plastic, and carotenoids adhere to plastic easily and tightly.
- Do not saponify, unless it is absolutely necessary, to avoid structural changes and degradation of carotenoids. If needed, the mildest conditions that can accomplish its purpose should be used (e.g., saponifying carotenoids dissolved in petroleum ether with equal volume of 10% methanolic potassium hydroxide, at room temperature in the dark overnight).
- Do not shake the separatory funnel during partition to avoid forming an emulsion.
- Do not let extracts containing chlorophyll (e.g., extracts of green vegetables) or other potential sensitizers stand even for a brief period, especially when exposed to light. Photodegradation and isomerization occur very rapidly under such conditions.
- Do not quantify overlapping peaks in the HPLC chromatogram. The accuracy of the quantitative data depends directly on how accurately the peak areas are known. Efficient columns are now available, and in the right combination with the mobile phase, give baseline or near baseline separations of at least the major carotenoids.

# SAMPLING AND SAMPLE PREPARATION

To provide meaningful and reliable analytic data, the sample must be representative of the entire lot under investigation and adequately prepared for analysis. Recent years have witnessed growing recognition of the importance and concern about the propriety of sampling and sample preparation in food analysis. Contrary to this general trend, these two initial steps in the analytic process have received little attention in the carotenoid field. Most carotenoid papers do not include a description of the sampling procedure, and the sample treatment is not or is only superficially discussed. Thus, this section of the monograph will be drawn from consensual tendencies in general food analysis, although some carotenoid papers that did touch on these two topics will be cited.

Concepts diverge somewhat in relation to when sampling ends and sample preparation (sometimes called sample processing) begins and when preparation ends and the analysis per se commences. According to Kratochvil and Taylor (1981), the major steps in sampling are

- identification of the population from which the sample is to be obtained,
- selection and withdrawal of valid gross samples of this population, and
- reduction of each gross sample to a sample suitable for the analytic technique to be used.

Horwitz (1988) defined anything sent to the laboratory as a laboratory sample and also considered reduction of the laboratory sample to a test sample for analysis as part of the sampling process. To Pomeranz and Meloan (1994), the aim of sampling is to secure a portion of the material that satisfactorily represents the whole and the purpose of sample preparation is to homogenize the large sample in the laboratory and subsequently reduce it in size and amount for analysis. As far as the Committee on Environmental Improvement of the American Chemical Society (Keith et al. 1983, ACS-CEI 1980) is concerned, sample pretreatment begins after a sample is received in the laboratory.

In this monograph the second concept will be adopted, as shown in Figure 19, which illustrates the total error of carotenoid analysis as the sum total of the errors of sampling, sample preparation, the analysis itself, and interpretation.

Many papers involving chromatographic methods extend sample preparation to obtaining the final extract to be injected into the chromatograph. Following the general opinion in food analysis, sample preparation in this monograph includes all operations from the receipt of the laboratory sample that precede the weighing of the sample to be submitted to analysis.

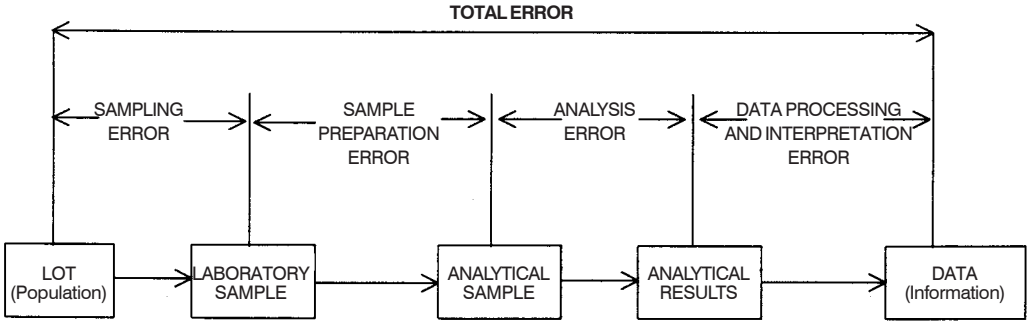


**Figure 19.** Illustration of the total error in carotenoid analysis.

## Sampling

In designing a sampling plan, the following factors should be considered (Kratochvil and Taylor 1981, Kramer and Twigg 1970):

- the purpose of the analysis (information sought),
- the nature of the population to be studied,
- the nature of the analyte (substance to be measured),
- the distribution of the analyte within the population,
- the desired accuracy and precision of the analytic results, and
- the analysis to be performed.

The more heterogenous the material, the greater the difficulties and effort required to obtain a representative sample; the more sensitive modern methods become, the smaller the portions of the original lots that are subject to actual analysis, making it more challenging to minimize sampling errors. Because food samples are typically heterogeneous, a large number of samples should ideally be analyzed. In practice, however, the sampling procedure adopted is usually a compromise between heterogeneity considerations and the cost of the operation. It is worthwhile for analysts to consult statisticians to arrive at a feasible but sound sampling protocol.

An acceptable sampling program should at least include a sampling plan that takes into account the goals of the studies and the expected uncertainties associated with the number of samples collected and the population variability; instructions for sample collection, labeling, preservation, and transport to the analytic facility; and the training of personnel in the sampling techniques and procedures specified (Keith et al. 1983).

The program should consider the reasons for choosing sampling sites, number of samples, timing of sample acquisition, and expected levels of fluctuation resulting from heterogeneity. Once the sampling site and time of collection are decided, the following questions should be addressed (Kratochvil and Taylor 1981):

- How many samples should be taken?
- How large should each sample be?
- From where in the bulk material (population) and how should the samples be taken?
- Should individual samples be analyzed or should a composite be prepared?

A statistical approach to determine the number of samples to be taken is possible when the standard deviation of the population is known or can be reasonably estimated. A relationship that may be used for a given standard deviation and for a given acceptable error is (Keith et al. 1983, Walpole and Myers 1972)

$$N_S = (z\sigma_p / e)^2$$

where $N_S$ is the number of samples, $z$ is the value of the standard normal variate (from tables) based on the level of confidence desired, $\sigma_p$ is the standard deviation of the sample population, and $e$ is the tolerable error in the estimate of the mean for the characteristic of interest.

Often, however, the data needed to calculate the minimum number are not available and empirical approaches are used. Kratochvil and Taylor (1981) suggested that, in this case, a small number of samples (as representative as possible of the population) should be collected and analyzed. The sampling plan can then be developed by using this preliminary information.

If an average compositional value is desired, a large number of randomly selected samples can be obtained, combined, and blended to obtain a reasonably homogeneous composite, subsamples of which may be analyzed (Keith et al. 1983, ACS-CEI 1980).

Random sampling involves drawing samples from different parts of the entire lot, each part of the lot having an equal chance of being collected. It is not as simple as it seems. Samples selected haphazardly may not constitute a representative sample, but collection cannot be so defined that the protocol may reflect bias (Kratochvil and Taylor 1981).

To evaluate changes in composition with time, temperature, location, etc., systematic sampling should be used and the results should be statistically analyzed.

## Sample Preparation

The sample that is brought to the laboratory is usually too large, both in bulk and particle size, for direct analysis. It must be transformed into a homogeneous, small sample for analysis while maintaining its representativeness. Homogenization and subsampling may be done simultaneously or sequentially in an interchangeable order. 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. Because the food product is usually analyzed in the form in which it is commonly used, inedible portions (i.e., peel, seed, shell, etc.) are initially removed.

The problems encountered by analysts in the preparation of samples for analyses include (Pomeranz and Meloan 1994):

- difficulty in obtaining representative small samples from large samples;
- loss of plant material;
- difficulty in removal of extraneous material from plants without removal of plant constituents, including the analyte;
- enzymatic changes before and during analysis;
- compositional changes during grinding; and
- changes in unstable components.

The sample preparation procedure should be adapted to the nature of the food, analyte, and analytic method as well as the distribution of the analyte in the food.

## Sampling and Sample Preparation in Food Carotenoid Analysis

To obtain representative data for provitamin A carotenoids in fresh fruits and vegetables available to consumers across the United States, Bureau and Bushway (1986) collected samples from five cities (Los Angeles, Dallas, Chicago, Miami, and Boston), 3 times during a year (November, March, and July). The foods were shipped by air to the laboratory (one crate per item). All foods were removed from the containers and sampled 3 times. These samples (1–2 kg each) were chopped into small pieces and a 10-g subsample was removed from each of the three samples. This extensive sampling procedure was designed to account for geographic, seasonal, cultivar, and handling conditions. It was therefore not surprising that the ranges of the results were very wide and, apparently because of the very high standard deviations, no statistically significant differences were observed among locations or months of analysis.

In light of Bureau and Bushway's results, our investigations, and those of others on the effects of various factors and the fact that very wide natural variability overshadows refinements in analytic methods, it appears that some delimitation of natural variation should be made. For example, samples of different cultivars and samples from regions of different climates should not be mixed, and the cultivar and region of origin should be specified in composition tables. Mature or ripe samples should also be presented separately from immature or unripe samples.

In our laboratory, the sampling and sample preparation scheme depends on the food under investigation and is described in each paper. Sampling for average carotenoid composition is done in distribution centers, supermarkets, groceries, and other retail outlets so as to represent the composition of foods as offered to the consumers. Perennial crops are sampled at different times during the year and seasonal produce is sampled at different times during the season. For each laboratory sample, several increments are taken at random from different parts of a big lot at the retail outlet. Depending on the food under investigation, 200 g to 1 kg are usually taken to the laboratory. At the laboratory inedible portions are removed. For small fruits or fruit vegetables, several fruits are taken at random from the laboratory sample and homogenized in a mechanical blender; duplicate portions are then weighed and extracted immediately to avoid enzymatic oxidation. Big fruits or fruit vegetables, also taken at random from the laboratory sample, are quartered longitudinally and opposite sections from each fruit are combined and homogenized in a mechanical blender. Vegetables such as leafy vegetables and green beans are cut into small pieces and mixed. For headed vegetables, such as cabbage, and bunches, such as unheaded lettuce, the head or bunch is opened at the center and a proportional number of young and mature leaves are taken from each side before cutting. For mature squashes and pumpkins, especially the cultivar menina verde, which can weigh up to 7 kg, a different subsampling procedure was used (Arima and Rodriguez-Amaya 1988). For commercial processed products, which normally would undergo homogenization during processing, at least two units are taken randomly from the same production lot and mixed before weighing the sample for analysis.

Lin and Chen (1995) showed that the concentrations of the carotenoids in orange juice of two cultivars changed during the harvesting season, which justifies the collection of samples at different times during the season.

In measuring the carotenoid content of vegetables and fruits commonly consumed in the United Kingdom, Hart and Scott (1995) purchased four individual samples of each item from various retail outlets in the Norwich area between April and October 1993. After removal of the outer leaves, peeling, coring, etc., large items, such as cabbage, were quartered, cut, and mixed. Small items were cut and mixed, and frozen or canned items were mixed. Subsamples of 100 g were taken from each of the four individual samples of each food item and bulked to give a composite sample of 400 g. Because of the high level of carotenoid contribution and frequency of consumption, the four individual samples of frozen peas, frozen and fresh carrots, and fresh tomatoes were analyzed individually.

To determine the carotenoid content of thermally processed tomato-based food products, Tonucci et al. (1995) purchased frequently consumed products from three U.S. cities (New York, Chicago, and San

Francisco), which were chosen to represent three distinct regions (northeast, north central, and west). For each tomato product, analyses were performed on at least one name-brand and one store-brand product from each of the three cities. For tomato soup, for example, at least two units of a name brand (having the same lot number) and two units of a store brand (having the same lot number) were obtained from each of the three cities. Name-brand or store-brand products having the same lot number were combined. Aliquots from the resulting six samples were then analyzed.

To investigate the effects of influencing factors, sampling has to be so designed that variables are controlled. For example, to verify cultivar differences and seasonal variation in the carotenoid composition of kale, mature leaves of each of two cultivars were hand-picked at random from the same farm, thus stage of maturity and environmental conditions were the same for both cultivars (Mercadante and Rodriguez-Amaya 1991). Ten sample lots (laboratory sample: about 250 g each) were collected and analyzed individually for each cultivar for each season, the lots being harvested at the same time (morning) at different days during the season. One sample lot each of the two cultivars was collected at each sampling day. At the laboratory, the leaves were finely cut and mixed, and 4–5g subsamples were immediately taken for analysis. To verify the possible effects of agrochemicals, 10 sample lots of mature kale were collected during the same time period from neighboring farms, one natural and the other using agrochemicals.

# OPEN-COLUMN METHOD

Analytic methods for carotenoids are classified by chromatographic technique into open-column (OCC) and high-performance liquid chromatography (HPLC) methods. The OCC method herein described was put together in 1976 (Rodriguez-Amaya et al. 1976a) from the best procedure for each step available at the time. It has been used in several laboratories in Brazil, evaluated in terms of repeatability for provitamin A carotenoids in different food samples, along with the assessment of the extraction and saponification steps (Rodriguez-Amaya et al. 1988), and compared with HPLC methods in terms of provitamin A carotenoids in different foods (Carvalho et al. 1992) and of major carotenoids in cassava leaves (Adewusi and Bradbury 1993). Additionally, results obtained with this method compare well with those obtained by some HPLC methods, as shown for β-carotene and lycopene in tomatoes (Table 3), α-carotene and β-carotene in carrot (Rodriguez-Amaya 1989), and major carotenoids of mango, guava, and papaya (Wilberg and Rodriguez-Amaya 1995). In fact, there seems to be more coherence between the results from this method and those of some HPLC methods than among results of HPLC methods.

The method as described below may appear tedious because it has the capacity to identify and quantify the range of carotenoids in the sample, including minor carotenoids. If the objective is to determine only major provitamin A and nonprovitamin A carotenoids, the analysis is much simpler because only one to five carotenoids need to be separated, identified, and quantified.

This method is also efficient for isolating carotenoids that are not available synthetically or commercially to serve as standards for HPLC.

## Precautions

Review the precautionary measures discussed in "General Procedure and Sources of Errors in Carotenoid Analysis" and put them into practice.

## Reagents and Apparatus

- Acetone, reagent-grade or distilled, left in the refrigerator for about 1 hour.
- Mechanical blender with stainless steel or glass cup, or mortar and pestle.
- Vacuum filtration device—Buchner funnel or sintered glass funnel (porosity 3), suction flask, and water aspirator.
- Separatory funnel with Teflon stopcock.
- Potassium hydroxide solution—10% in methanol. Dissolve 10 g reagent-grade potassium hydroxide in 100 mL methanol, mix, and cool.
- Chromatographic glass tube—2.5 cm i.d. × 30 cm, tapering at the bottom.
- Adsorbent—Mix magnesium oxide and Hyflosupercel (diatomaceous earth) (1:1 or 1:2 w/w) by shaking until well mixed. This can be done manually by shaking the adsorbent in a container big enough to allow thorough mixing. If needed, activate the adsorbent already mixed by heating in an oven at 110 °C for 4 hours. After cooling in a desiccator, store in tightly closed container. When alumina (activity grade III) is used, mix neutral alumina (activity grade I) with 6% water thoroughly (until no lumps can be observed). This is best done using a rotary evaporator flask without applying vacuum. Store in tightly closed container.
- Eluting solvent—Petroleum ether, reagent grade or distilled (b.p. 30–60 °C), with increasing percentage of diethyl ether (peroxide-free), acetone, and water (v/v).
- Anhydrous sodium sulfate.
- Rotary evaporator.
- Ultraviolet-visible recording spectrophotometer.
- Volumetric flasks, beakers, Erlenmeyer flasks, and other common laboratory glassware.
- Silica gel thin-layer chromatography (TLC) plates and TLC development tank.
- TLC developing solvent—3% methanol in benzene. (Because of the high toxicity of benzene, this de-

veloping solvent has been replaced in our laboratory with 5% methanol in toluene, the chromatographic behavior being equivalent.)
- Reagents and solvents for chemical tests—pyridine, acetic anhydride, hydrochloric acid, sodium borohydride, 95% ethanol, and iodine.

## Extraction

Weigh a portion of the homogeneous, representative sample. The weight depends on the carotenoid content of the sample, varying from 2 g of dark green leafy vegetable to 100 g of low-carotenoid fruit or vegetable. Blend in a mechanical blender for 30–60 seconds with enough cold acetone to cover and celite or Hyflosupercel. Alternatively, grind sample with a mortar and pestle, with enough cold acetone to cover and celite or Hyflosupercel. Filter with suction through a Buchner funnel or sintered glass funnel. Wash the blender or mortar, funnel, and residue with small amounts of acetone, receiving the washings in the suction flask with the extract. Return the residue to the blender or mortar, add fresh acetone, and macerate again. Filter and wash as before. Repeat the extraction and filtration until the residue is devoid of any color and washings are colorless (usually 3 times is enough).

## Partitioning to Petroleum Ether

Put about 100 mL of petroleum ether in a separatory funnel and add a small portion of the acetone extract. Add distilled water slowly, letting it flow along the walls of the funnel. To avoid formation of an emulsion, do not shake. (Once formed, an emulsion can be broken by adding acetone or saturated salt solution and swirling the funnel to mix. When an emulsion is difficult to break, it is better to start the analysis over rather than proceed with an analysis that can give an erroneous result.) Let the two phases separate and discard the lower aqueous-acetone phase. Add another portion of the acetone extract and repeat the operation until all of the extract has been transferred to petroleum ether, then wash 4-5 times with water to remove residual acetone. Collect the petroleum ether phase and dry with sodium sulfate (add sodium sulfate until some crystals become loose). If saponification will be carried out, skip this drying step. If at any time during this process the lower phase appears colored, collect it and add it back in portions to the upper phase.

The partitioning to petroleum ether is more difficult with the extract obtained from the first extraction, which contains water, lipids, and other components of the sample. You may want to separate this first extract so that you can partition it more carefully. The carotenoids of the succeeding extracts are easily transferred to petroleum ether.

If your sample has appreciable amount of xanthophylls, add some diethyl ether to petroleum ether to facilitate the transfer of these xanthophylls from acetone to petroleum ether.

## Saponification

Do this step only when absolutely necessary (see "General Procedure and Sources of Errors in Carotenoid Analysis"). To the carotenoid solution in petroleum ether, add butylated hydroxytoluene (0.1% in petroleum ether) and an equal volume of 10% potassium hydroxide in methanol. Flush with nitrogen before stoppering the flask. Let stand in the dark at room temperature overnight (up to about 16 hours). Wash with distilled water to remove the potassium hydroxide. Do this as in the partition procedure by adding portions to a separatory funnel, each addition followed by washing with water (adding water, allowing the phases to separate, and discarding the lower aqueous phase). When all of the carotenoid has been added to the funnel, wash an additional 4-5 times with water to get rid of the potassium hydroxide. Collect the carotenoid phase and dry with sodium sulfate.

## Concentration

Concentrate carotenoid solution so that it can be introduced into the chromatographic column in the smallest volume possible. Decant solution to a round-bottom flask and rinse the receiving flask and sodium sulfate with small amounts of petroleum ether, combining washings with the carotenoid solution. If difficult-to-wash xanthophylls are present, rinse also with a small amount of ethyl ether. Connect the flask to the rotary evaporator and concentrate the solution to about 10–20 mL, the temperature not exceeding 40 °C.

## Chromatographic Separation

### *Preparing the Column*

Mount a chromatographic glass tube on a suction flask. Place a small glass wool plug at the bottom of the chromatographic tube. Add adsorbent loosely up to a height of 20 cm and apply a moderate vacuum from a water aspirator (the vacuum should be continuously applied from this point on). Use a flat instrument (such as an inverted cork mounted on a rod or a tampering rod, the diameter just a little bit smaller

than that of the glass tube so that it fits snugly into the tube) to press down the adsorbent and flatten the surface (the packed column should about 12 cm high). Top the column with a 1-cm layer of anhydrous sodium sulfate to ensure that no residual water gets into the adsorbent. Test the column with petroleum ether. Pass about one bed volume of petroleum ether through the column (the adsorbent surface must be smooth and the solvent flow even) and adjust the vacuum so that the solvent flow is about 2–3 drops per second. Once petroleum ether is added to the column, keep the top of column covered with solvent until the chromatography is completed.

### *Developing the Column*

Pour the carotenoid solution into the column and let the sample layer go down almost to the surface of the sodium sulfate layer before adding the rinse (petroleum ether) from the round-bottom flask. The objective is to keep the carotenoids in as small a volume as possible to diminish band broadening and to prevent the separation from initiating before the entire carotenoid sample has reached the adsorbent top.

Once the petroleum ether rinse almost reaches the surface of the sodium sulfate layer, develop the magnesium oxide–Hyflosupercel (1:2) column successively with 50 mL each of 1%, 2%, and 5% ethyl ether and then 2%, 5%, 8%, 10%, 15%, and 20% acetone in petroleum ether. Depending on the carotenoid composition, the amount of acetone can thereafter be increased by 10% and then 20% up to pure acetone. Tightly adsorbed carotenoids can be eluted by 5% and then 10% water in acetone. Hexane may be used instead of petroleum ether.

For the activated magnesium oxide–Hyflosupercel (1:1) column, start development with 1% acetone and continue increasing the acetone percentage as described above.

For succeeding analyses modify the above development pattern according to the carotenoid composition of the sample to obtain the best separation within the shortest possible time. Some of the solvent mixtures may be deleted or the volume reduced, whereas others may have to be increased. With experience it may not be necessary to develop the column with the entire series of solvents, even when a commodity is being analyzed for the first time. In this case, add only a small amount of the eluting solvent. If no separation is observed, go to the next solvent. If, by bypassing a solvent, two bands appear to fuse together, go back to the previous solvent. Examples of separation patterns are shown in Figure 20.

Monitor separation of the carotenoids visually and collect each separated fraction as it leaves the column. Change suction flasks rapidly but gently so that breaking the vacuum will not perturb the adsorbent column. Alternatively, when all of the carotenoids have been separated, let the column dry, remove the adsorbent from the glass tube by tapping the inverted column gently, cut the remaining bands, and extract them with acetone or acetone with 5–10% water for the more polar carotenoids. Dilute or concentrate fractions eluted with petroleum ether or petroleum ether containing small amount of diethyl ether to a suitable volume for spectrophotometric reading.

Because acetone affects the absorption of carotenoids in petroleum ether, remove acetone from fractions eluted with petroleum ether containing acetone or transfer carotenoids eluted with acetone or acetone with water to petroleum ether. To do this, put the fraction in the separatory funnel (there is no danger of emulsion formation at this point) all at once, add petroleum ether if necessary, and then add distilled water. After phase separation, discard the lower phase. Wash with water an additional 3–4 times, collect the petroleum ether phase, dry with sodium sulfate, transfer to a suitable volumetric flask, and adjust the volume with petroleum ether.

Record the spectrum of all carotenoids in a 1-cm cuvette from 330 nm (lower for phytoene and phytofluene) to 550 nm.

For some food samples, better separation is achieved with an alumina column or unresolved fractions from the magnesium oxide–Hyflosupercel column are rechromatographed on another magnesium oxide–Hyflosupercel column or on an alumina column. Pack the alumina column by filling the chromatographic tube (a smaller tube than that used for the first separation is usually used for rechromatography) with the adsorbent (usually neutral alumina, activity III) and tapping gently to accommodate the adsorbent better in the tube. Application of a vacuum is not required, but the column is also developed with petroleum ether or hexane containing an increasing amount of diethyl ether and then acetone.

### Thin-layer Chromatography

After recording the spectra, concentrate all fractions and apply on a silica gel thin layer. Develop the thin-layer gel with 5% methanol in toluene. All carotenes will run with the solvent front. Xanthophylls will be distributed in the chromatogram according to the type and number of substituents present. For example, monohydroxy carotenoids will be situated in the middle, trihydroxy carotenoids will remain in the origin, and

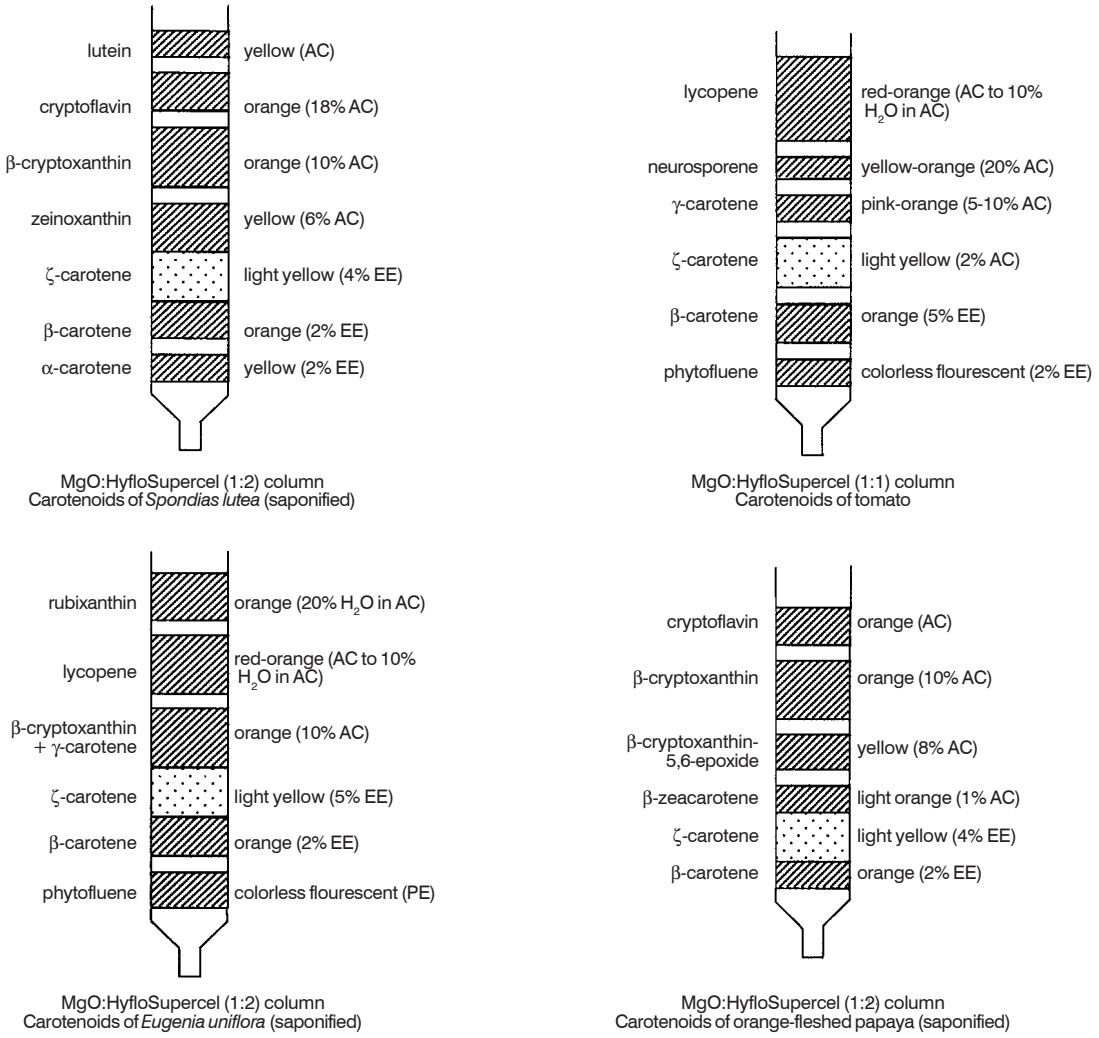


**Figure 20.** Examples of separation patterns on a magnesium oxide–Hyflosupercel column. Unless otherwise stated, elution solvents presented in parentheses are in petroleum ether (PE). EE, ethyl ether; AC, acetone. ζ-Carotene appears as a diffuse light yellow band. γ-Carotene and β-cryptoxanthin of *Eugenia uniflora* are separated by rechromatography on an alumina column.

dihydroxy carotenoids will be located between the other two groups.

After noting the carotenoid spots, expose the thin-layer plate to hydrogen chloride gas. Place a beaker with small amount of concentrated hydrochloric acid in the development tank and leave it for a while with the lid on. Then put the thin-layer plate briefly in the tank. Epoxy carotenoids will turn green (usually indicating a monoepoxide) or blue (usually indicating a diepoxide).

## Chemical Tests

To verify the type and position of substituents in xanthophylls, perform the appropriate chemical reactions (Eugster 1995, Davies 1976, Rodriguez-Amaya et al. 1976a, 1973). To verify geometric configuration, carry out iodine-catalyzed isomerization.

### *Acetylation of Primary and Secondary Hydroxyl Group*

Dissolve the carotenoid (about 0.1 mg; if the carotenoid is dissolved in another solvent, evaporate solvent first) in 2 mL pyridine and add 0.2 mL acetic anhydride. Leave the reaction mixture in the dark at room temperature for 21 hours. Then transfer carotenoid to petroleum ether in a separatory funnel with the addition of water. Wash with water, collect, dry with sodium sulfate, concentrate, and apply on a silica thin-layer plate next to unreacted carotenoid. Develop with 5% methanol in toluene. The reaction is positive if the resulting product has an $R_F$ much higher than that of the original carotenoid (Figure 12).

### Methylation of Allylic Hydroxyl Groups

Dissolve the carotenoid (about 0.1 mg) in 5 mL methanol. Add a few drops of 0.2 N hydrochloric acid. Allow the reaction to proceed at room temperature in the dark for 3 hours. Transfer the carotenoid to petroleum ether and submit to thin-layer chromatography as described above. A positive reaction is also shown by an increase in $R_F$ (Figure 12). Both the acetylated and methylated products have unchanged ultraviolet and visible spectra but are less polar than the original carotenoids.

### Epoxide-furanoid Rearrangement

Dissolve the carotenoid in ethanol and record the spectrum. Add a few drops of 0.1N hydrochloric acid. Record spectrum again after 3 minutes. A hypsochromic shift of 20–25 nm indicates the transformation of a 5,6-epoxide to a 5,8-epoxide.

### Reduction of a Conjugated Carbonyl Group

Dissolve the carotenoid in 95% ethanol and record the spectrum. Add a few crystals of sodium borohydride. Let the reaction mixture stand for at least 3 hours in the refrigerator. Record the spectrum. If the reaction is positive, the single broad band of a ketocarotenoid or an apocarotenal is transformed into the three-peak spectrum of the resulting hydroxycarotenoid (Figure 18).

### Iodine-catalyzed cis-trans Isomerization

Dissolve a few crystals of iodine in petroleum ether. Record spectrum of the carotenoid dissolved in petroleum ether and add a drop of the iodine solution. Take spectrum after 1–5 minutes of exposure to light. This reaction can be done directly in the spectrophotometer cuvette. The λmax values of *trans* carotenoids will shift 3–5 nm to a lower wavelength whereas those of *cis* carotenoids (such as 15-*cis*- and 13-*cis*-β-carotene) will shift by the same amount to longer wavelengths. 9-*cis*-β-Carotene does not change λmax.

## Identification

Identify the separated carotenoids by the combined use of the following parameters as discussed in "Conclusive Identification":

- order of elution from the column,
- $R_F$ values and co-chromatography on thin-layer chromatography,
- ultraviolet and visible spectrum, and
- chemical tests.

## Calculation of the Concentration

Calculate the concentration of each identified carotenoid according to the following formulas:

$$x\ (\mu g) = \frac{A \cdot y\ (mL) \cdot 10^6}{A^{1\%}_{1cm} \cdot 100}$$

$$x\ (\mu g/g) = \frac{x\ (\mu g)}{\text{weight of sample (g)}}$$

where x is the weight or concentration of the carotenoid, y is the volume of the solution that gives an absorbance of A at a specified wavelength, $A^{1\%}_{1cm}$ is the absorption coefficient of the carotenoid in the solvent used, given in Table 8. For example, for β-carotene in petroleum ether, the absorbance (A) at 450 nm and an $A^{1\%}_{1cm}$ of 2592 should be used.

# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS

Several high-performance liquid chromatographic (HPLC) methods were selected for presentation here so that analysts can choose the one that suits their objectives and laboratory resources. No HPLC method has been generally adopted, although some trends can be perceived. The methods are presented as described by the authors responsible for their development. Mention of a brand of material or equipment should not be considered as endorsement. When some modifications have been made, the latest version of the method is presented.

## Method of Bushway et al. (Bureau and Bushway 1986, Bushway 1985, Bushway and Wilson 1982)

Developed for the determination of provitamin A carotenoids, particularly α-carotene, β-carotene, and β-cryptoxanthin, this method has been used by various laboratories in different countries. Mean recovery of β-carotene added at 0.25 and 0.75 mg/100 g of carrots was 100%. For several samples tested for repeatability, the coefficient of variation (CV) ranged from 1.1% to 14.2% for α-carotene and from 1.3% to 8.6% for β-carotene (Bushway and Wilson 1982). Identification was based on retention times compared with those of standards, ultraviolet and visible spectra, and ratios of absorbance at 470 vs. 450 nm.

### *Reagents and Apparatus*

- Tetrahydrofuran (THF) stabilized with butylated hydroxytoluene (BHT)
- Anhydrous sodium sulfate
- Magnesium carbonate
- Mechanical blender
- Buchner funnel, Whatman no. 42 filter paper
- Rotary evaporator
- α-Carotene, β-carotene, and β-cryptoxanthin standards
- Low-actinic volumetric flasks, round-bottom flasks, and other glassware
- HPLC equipment with a variable wavelength detector; a Partisil 5 ODS column, 250 mm × 4.6 mm i.d.; and acetonitrile-THF-water (85:12.5:2.5 v/v/v) as the mobile phase pumped at a flow rate of 2.0 mL/minute
- Ultraviolet-visible light recording spectrophotometer

### *Analysis*

Weigh 10 g of the fruit or vegetable. Extract with 125 mL THF, 5.0 g anhydrous sodium sulfate, and 1.0 g magnesium carbonate in a 1-L mechanical blender at a moderate speed for 5 minutes. Vacuum-filter through a Buchner funnel fitted with Whatman no. 42 filter paper. Reextract the filter cake to remove all carotenoids. Combine the filtrates and bring to a 500-mL volume with THF. Transfer a 100-mL aliquot to a 250-mL round-bottom flask and evaporate to dryness, using a rotary evaporator at 40 °C. Redissolve in 10 mL THF with the aid of sonication and inject 10–25 μL of each sample extract into the HPLC equipment. Quantify using the peak height with the detection set at 470 nm.

If the sample contains low levels of carotenoids, evaporate the entire filtrate to dryness. Redissolve in 10 mL THF and inject 10–25 μL into the chromatograph.

### *Preparation of Standards*

Prepare stock solutions of α- and β-carotene by weighing 25 mg of each into separate 100-mL low-actinic volumetric flasks. Bring to volume with stabilized THF. Take aliquots of 0.5, 1.0, 1.5, and 2.0 mL from each and bring to volume in separate 50-mL low-actinic volumetric flasks.

To prepare a stock solution of β-cryptoxanthin, weigh 12 mg into a 100-mL low-actinic volumetric flask and bring to volume with stabilized THF. Take aliquots of 1.0, 2.0, 2.5, and 3.0 mL; place in four 50-mL low-actinic flasks; and bring these working stan-

dards to volume with stabilized THF. Calibrate HPLC daily by injecting 10 µL of each working standard in duplicate.

Determine the purity of standards spectrophotometrically by using $A^{1\%}_{1cm}$ of 2800 at 444 nm in petroleum ether for α-carotene and of 2396 at 465 nm in chloroform for β-carotene.

## Method of Heinonen et al. (Heinonen 1990, Heinonen et al. 1989)

The most extensive work of carotenoid analysis in foods in Europe has been carried out by this group. Recovery of β-carotene in tomato, carrot, and broccoli was 101% (*n*=8); lycopene in tomato was 103% (*n*=4);, and lutein in broccoli was 103% (*n*=2) (Heinonen et al. 1989). The mean within-laboratory CV was 10%, ranging from 0.8% to 44%, depending on the amount and complexity of the carotenoids in the food item. Identification was based on comparison of retention times with those of standards, and for some samples the ultraviolet and visible spectra were obtained with a photodiode array detector. The method was used to determine α-carotene, β-carotene, γ-carotene, cryptoxanthin, lutein, and lycopene in Finnish vegetables, fruits, and berries (Heinonen et al. 1989) and in carrot cultivars (Heinonen 1990).

### Reagents and Apparatus

- Acetone
- Mechanical blender
- Sodium sulfate
- BHT
- Vacuum filtration device
- Potassium hydroxide solution—100 g potassium hydroxide + 100 mL $H_2O$
- Ethanol
- Ascorbic acid
- Sodium chloride—10% in $H_2O$
- *n*-Hexane-diethyl ether (70:30 v/v)
- HPLC equipment with an ultraviolet and visible light detector; a guard column, 50 × 4.6 mm i.d. packed with Bondapack Ax/Corasil, 37–50 µm; analytic columns, Zorbax ODS, 250 × 4.6 mm i.d., 5-6 µm; and acetonitrile-dichloromethane-methanol (70:20:10 v/v/v) as the mobile phase at a flow rate of 1 mL/min.
- Ultraviolet-visible recording spectrophotometer

### Analysis

Homogenize the sample in a blender. Extract 3–10 g of the sample homogenate with 100 mL acetone in a mechanical blender, using 20 g sodium sulfate as desiccant and BHT (0.1% in acetone) as antioxidant. Vacuum-filter the mixture. Extract the sample 2 to 3 times to remove all color. Concentrate the extract before saponification. Saponify the extract overnight at room temperature with 1 or 5 mL (for green vegetables) of potassium hydroxide in a mixture of 50 mL ethanol and 20 mL water. Add ascorbic acid (1g /20 mL $H_2O$) as antioxidant. Before solvent extraction, dilute the saponification extract with sodium chloride solution (10% in $H_2O$). Extract the carotenoids with *n*-hexane-diethyl ether ( 70:30) with BHT (0.1% in hexane) as antioxidant.

Inject both sample and standard via a full loop, approximately 55 µL. Quantify by using the external standard method, the calibration curves being determined daily over the range 50–3600 ng/mL. Use an $A^{1\%}_{1cm}$ of 2550 (445 nm) for lutein in ethanol; 2480 (452 nm) for zeaxanthin in benzene; and 2592 (453 nm), 2725 (446 nm), 2720 (461 nm), 2470 (452 nm), and 3450 (472 nm) for all-*trans*-β-carotene (and 15-*cis*-β-carotene), α-carotene, γ-carotene, cryptoxanthin, and lycopene in hexane, respectively.

## Method of Hart and Scott (Scott et al. 1996, Hart and Scott 1995)

This method (without the saponification step) was evaluated in the proponents' laboratory by using a candidate vegetable reference material. Short-term repeatability, measured by analyzing 20 samples of the vegetable mix in duplicate over 7 days, showed CVs ranging from 5.6% for lutein to 11.8% for lycopene (average 8.3%) (Hart and Scott 1995). Long-term repeatability, measured by the analysis of an additional 20 samples over 12 months, showed CVs ranging from 4.9% for lutein to 10.8% for lycopene(average 7.8%). The method was used to determine lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene in vegetables commonly consumed in the United Kingdom.

Evaluation of reproducibility (results of 6–11 participating laboratories), using the same mixed vegetable reference material, resulted in CVs ranging from 11% for total α-carotene to 40% for total lycopene (average 24%). Before or along with this interlaboratory analyses, several measures were taken: evaluation of the homogeneity and stability of the reference material; spectrophotometer calibration with circulated holmium reference solution and circulated β-carotene solution; analysis of circulated extract of the reference material; and use by the different laboratories of the same absorption coefficients and absorption maxima and of peak area rather than the peak height.

### Reagents and Apparatus

- Extracting solvent—THF-methanol (1:1 v/v)
- Internal standard—β-apo-8′-carotenal or echinenone
- Ultra-turrax homogenizer
- Buchner funnel with glass fiber filter pad
- Separatory funnel
- Petroleum ether—40–60° fraction, containing 0.1% BHT
- Rotary evaporator
- Sodium chloride solution—10%
- Dichloromethane (DCM), chloroform, hexane
- Potassium hydroxide solution—10% potassium hydroxide in methanol
- Standard lutein, lycopene, α-carotene, β-carotene (Sigma Chemicals), β-apo-8′-carotenal (Fluka Chemicals), zeaxanthin, β-cryptoxanthin, and echinenone (Hoffman La Roche).
- HPLC equipment with an ultraviolet and visible light variable wavelength detector; a photodiode array detector; a chromatographic data handling system; a 5-µm ODS 2 metal-free guard column, 10 mm; a 5-µm ODS 2 metal-free column, 100 × 4.6 mm; a 5-µm Vydac 201TP54 analytic column, 250 × 4.6 mm, modified by the replacement of metal frits with biocompatible Teflon frits; column connections made with poly ether ether ketone tubing; a mobile phase consisting of acetonitrile-methanol-dichloromethane (75:20:5 v/v/v) containing 0.1% BHT and 0.05% triethylamine (methanol contains 0.05 M ammonium acetate); a thermostatically controlled circulating water bath, maintaining the column temperature at 22.5 ± 0.1 °C; and a Rheodyne syringe loading sample injector fitted with a 50-µL loop.
- Ultraviolet-visible recording spectrophotometer.

### Analysis

Place a 10-g aliquot of the ground sample in conical flask together with 1 g magnesium carbonate. Add 50 mL of THF-methanol (1:1 v/v) along with an internal standard (β-apo-8′-carotenal or echinenone) appropriate for the type of sample. Extract carotenoids from the food matrix by homogenizing for 1 minute using an ultra-turrax homogenizer. Filter through a glass fiber filter pad in a Buchner funnel under vacuum. Wash the flask and homogenizer with 50 mL THF-methanol and filter the washing. Wash filter pad with two further 50-mL aliquots of THF-methanol. Transfer the combined THF-methanol filtrates to a separatory funnel. Add 50 mL petroleum ether (containing 0.1% BHT) and 50 mL 10% sodium chloride solution and mix by careful shaking. Draw off the THF-methanol aqueous phase and transfer the upper petroleum ether phase to a 250-mL evaporating flask. Extract the THF-methanol aqueous phase twice more with 50-mL aliquots of petroleum ether. Combine petroleum ether phases in the flask and evaporate at 35 °C in a rotary evaporator to near dryness. Add 10–15 mL petroleum ether, redissolve the residue by ultrasonic agitation, transfer to a 25-mL evaporating flask, and evaporate just to dryness. Redissolve the residue by ultrasonic agitation to a final volume of 5 mL in DCM. If necessary, saponify this extract as described below. Otherwise, dilute with the mobile phase to a suitable concentration for HPLC analysis and filter through a 0.45-µm PVDF (polyvinylidene diflouride) syringe filter. Perform all manipulations under gold lighting.

Saponify samples such as pepper and fruit. Saponify 4 mL of the DCM extract with an equal volume of 10% potassium hydroxide in methanol (under nitrogen in the dark) for 1 hour at room temperature. Extract carotenoids from the potassium hydroxide–methanol phase by careful shaking with 20 mL petroleum ether and 20 mL 10% sodium chloride solution in a separatory funnel. Remove the lower potassium hydroxide–methanol aqueous phase to another separatory funnel and extract twice more with 20-mL aliquots of petroleum ether. Combine the petroleum ether phases in a separatory funnel and wash with water until washings are neutral on pH paper. Transfer the petroleum ether phase to a 100-mL round-bottom flask and dry in a rotary evaporator at 35 °C. Add 10–15 mL of petroleum ether, redissolve the residue by ultrasonic agitation, transfer to a 25-mL evaporating flask, and evaporate just to dryness. Redissolve the residue by ultrasonic agitation in 4 mL DCM, dilute with mobile phase to a suitable concentration for HPLC analysis, and filter through a 0.4-µm PVDF syringe filter. Perform all manipulations under gold fluorescent lighting.

### Internal Standards

Use internal standard to assess losses during extraction. Use β-apo-8′-carotenal except for green vegetables, for which echinenone should be used because β-apo-8′-carotenal co-elutes with chlorophyll b.

### Preparation of Standard Carotenoid Solution and Calibration

Dissolve lutein, α-carotene, β-carotene, and β-apo-8′-carotenal in chloroform and bring to volume with hexane to give a final solvent ratio of 1:9 (v/v). Dissolve echinenone and β-cryptoxanthin in chloroform-hexane (1:1 v/v). Dissolve zeaxanthin and lycopene

in chloroform. Add BHT at 0.1% to all solvents. Except for lycopene, store all solutions in air-tight screw-topped brown bottles under nitrogen at –18 °C. To avoid degradation, divide lycopene solution into 1-mL aliquots in brown glass vials, dry under nitrogen, and seal before storing at –18 °C. When required for use, add 1 mL chloroform to redissolve.

Before measuring absorbance, bring the stock solutions to room temperature and filter through a 0.45-µm PVDF syringe filter. Evaporate an aliquot of the solution under nitrogen and dilute in appropriate solvent to give an approximate absorbance reading of 0.5 AU. Measure exact absorbance and calculate the concentration by using appropriate absorption coefficients (lutein in ethanol at 445 nm, 2550; zeaxanthin in ethanol at 452 nm, 2480; β-cryptoxanthin in hexane at 451 nm, 2460; lycopene in hexane at 472 nm, 3450; α-carotene in hexane at 444 nm, 2800; β-carotene in hexane at 450, 2592).

Prepare individual working solutions of around 0.5–1.0 µg/mL from stock solutions by evaporating an aliquot under nitrogen and bringing it to volume with the mobile phase. Assess purity by HPLC. Express the purity of a carotenoid as the peak area of that carotenoid as a percentage of the total area of the chromatogram and calculate concentration from the absorbance reading corrected accordingly. Measure the concentrations and purity of the stock standard solutions each time a new working standard is prepared.

### *Calculation of Carotenoid Concentration*

Calculate concentrations of carotenoids (µg/100 g) by using response factors relative to β-cryptoxanthin. Analyze a working solution of β-cryptoxanthin with each batch of samples on the day of analysis (Equation 1).

## Method of Khachik et al. (Tonucci et al. 1995, Khachik et al. 1992b, 1986)

This method comes from the laboratory that carried out much of the work done on food carotenoids in the United States in recent years. It has been used to determine the whole range of carotenoids in the food sample. Percentage recovery of the internal standard (β-apo-8´-carotenal) was at least 90% for all extractions (Tonucci et al. 1995). The CV of the carotenoid concentrations for a vegetables juice used as control sample ranged from 4% for phytoene to 13% for lycopene-5,6-diol. Aliquots from a single large pool of the vegetable juice control sample (stored at –60 °C until needed) was extracted and analyzed at the beginning, after every 10 sample extractions, and at the end of the study to indicate repeatability over time. Vegetable juice was considered to be a suitable control sample because it contained all the carotenoids of interest in concentrations that can be easily measured. Identification was based on the comparison of retention times, ultraviolet and visible spectra obtained by a photodiode array detector, and mass spectra (desorption chemical ionization, ammonia; negative-ion electron capture, methane).

### *Reagents and Apparatus*

- β-Apo-8´-carotenal (internal standard)
- THF stabilized with 0.01% BHT
- Dichloromethane with 0.1% N,N´-diisopropylethylamine
- Celite
- Omni mixer
- Buchner funnel
- Separatory funnel
- Rotary evaporator
- Anhydrous sodium sulfate
- HPLC equipment with a photodiode array detec-

**Equation 1.**

Calculate relative response factor (RF) as follows:

$$RF = \frac{\text{Peak area of carotenoid working solution} \equiv 1\ \mu g/mL}{\text{Peak area of } \beta\text{-cryptoxanthin working solution} \equiv 1\ \mu g/mL}$$

Calculate carotenoid concentration in sample as follows:

$$\text{Carotenoid A } (\mu g/mL \text{ of extract}) = \frac{\text{area of peak A of diluted extract}}{\text{area of } \beta\text{-cryptoxanthin} \equiv 1 \mu g/mL} \bullet \text{RF (A)} \bullet \text{dilution of extract} \bullet \frac{100}{\%\text{ recovery of internal standard}}$$

$$\text{Concentration of carotenoid A } (\mu g/100\ g) = \frac{\text{concentration of carotenoid A } (\mu g/mL \text{ of extract})}{\text{concentration of food sample in extract } (g/mL)} \bullet 100$$

tor; Microsorb-MV $C_{18}$ column, 250 × 4.6 mm i.d., 5 µm; column inlet filter, 0.5 µm, 3 mm i.d.; Brownlee $C_{18}$ guard column; and printer-plotter. An isocratic mixture of acetonitrile (85%), methanol (10%), dichloromethane (2.5%), and hexane (2.5%) at time 0 is followed by a linear gradient beginning at 10 minutes and completed at 40 minutes. The final composition of the gradient mixture is acetonitrile (45%), methanol (10%), dichloromethane (22.5%), and hexane (22.5%).
- Ultraviolet-visible recording spectrophotometer

### Analysis

Carry out extraction at 0 °C by immersing the mixer in an ice bath, under gold fluorescent lights. Add an appropriate amount of β-apo-8´-carotenal as the internal standard to each food sample (e.g., 0.85–1.15 mg β-apo-8´-carotenal to 50–300 g tomato-based product) before extraction to indicate the extent of losses as a result of extraction and chromatography. Add magnesium carbonate and celite as a filter aid (each at 10% of the weight of the sample) and blend the sample for 20 minutes in an Omni mixer with THF. Filter through Whatman no. 1 filter paper on a Buchner funnel. Extract solid material 2 or 3 more times until it is devoid of color after filtering and the filtrate is colorless. Combine THF extracts and reduce the volume by about two-thirds under vacuum at 35 °C on a rotary evaporator. Partition components of the combined extract into dichloromethane (250 mL) and salt water (150 mL) in a separatory funnel. Remove the organic layer and wash with water (3x150 mL) containing sodium chloride. If color remains in the water layer, wash it with dichloromethane until carotenoids are completely removed. Dry dichloromethane layer containing carotenoids over anhydrous sodium sulfate (powder) and filter through Whatman no. 42 filter paper on a Buchner funnel. Reduce the volume of the filtrate under vacuum to approximately 10 mL. (Preliminary studies demonstrated that lycopene was lost if the solution was permitted to go to complete dryness.) Filter 10 mL of concentrated solution quantitatively through a 0.45-µm filter and bring the volume to 50 mL in dichloromethane in a volumetric flask. Make appropriate dilutions by transferring aliquots of this solution into HPLC injection solvent (acetonitrile, 40%; methanol, 20%; dichloromethane, 20%; hexane, 20%). Inject 20 µL of the final dilution onto the HPLC column.

Establish calibration curves based on peak area for the internal standard and for each carotenoid and use these curves to determine concentrations. The wavelength for calibration of standards and integration of peaks from sample extract depend on the wavelength of optimum absorption for each carotenoid. Quantify all carotenoids at 450 nm except for the following: lycopene at 470 nm, β-cryptoxanthin at 445 nm, ζ-carotene at 400 nm, phytofluene at 350 nm, and phytoene at 290 nm.

# CONCLUSIVE IDENTIFICATION

Conclusive identification is obviously a requirement for the accurate determination of the carotenoid composition of foods. Ideally, the identification procedure should include mass spectrometry (MS), especially electron-impact MS, which gives the molecular ion and fragments characteristic of structural features of the carotenoid molecule. However, availability of the MS equipment is highly limited and well-known, principal carotenoids of foods can be identified by the judicious combined use of chromatographic data, ultraviolet-visible spectra, and chemical reactions. The identification of some carotenoids will be discussed to illustrate this point.

Because the order of elution from open-column chromatography (OCC; normal phase) is more definitive, it will be given more emphasis than the order of elution in reverse-phase high-performance liquid chromatography (HPLC). Because of the weaker interactions of carotenoids with the stationary phase in HPLC, inversion of the elution order is common and co-chromatography is needed in this technique.

For the application of MS to food carotenoids, the readers are referred to Khachik et al. (1992b, 1989, 1986) and Mercadante et al. (1998, 1997a), the latter interpreting characteristic fragmentation. It must be remembered that even sophisticated MS cannot be used as the sole criterion for identification of carotenoids.

## Phytoene

This carotenoid absorbs maximally at 286 nm with shoulders at 276 and 297 nm in petroleum ether (Table 7), commensurate with a conjugated double-bond system of only three double bonds (Figure 2), the spectrum having little fine structure (%III/II = 10). The limited number of conjugated double bonds also explains the lack of color and elution as the first carotenoid in OCC with magnesium oxide–Hyflosupercel or alumina.

## Phytofluene

With five conjugated double bonds, phytofluene (Figure 2) exhibits a spectrum with well-defined peaks (%III/II = 90) at 331, 348, and 367 nm in petroleum ether (Table 7). It elutes as a fluorescent band after phytoene in OCC.

## α-Carotene

Having nine conjugated double bonds in the polyene chain and one conjugated double bond in the β ring (Figure 3), α-carotene has a spectrum with λmax at 422, 445, and 473 nm in petroleum ether (Table 7). With one of the conjugated double bonds in a ring, the spectrum loses fine structure (%III/II = 55) (Figure 8). The absence of substituents can be shown by silica gel thin-layer chromatography (TLC), developed with 5% methanol in toluene, in which, as a carotene, it runs with the solvent front. Co-chromatography can be done with TLC and HPLC by using a commercial α-carotene standard or α-carotene isolated from carrot by OCC.

## β-Carotene

With 11 conjugated double bonds, two of which are located in β rings (Figure 3), β-carotene has λmax at (425), 450, and 477 nm in petroleum ether (Table 7) with little fine structure (%III/II = 25), the absorption at 425 appearing as a mere shoulder (Figure 8). It runs with the solvent front in the TLC system, described above for α-carotene, which reflects the absence of functional groups. For TLC or HPLC co-chromatography, commercial β-carotene standard or β-carotene isolated from carrot can be used.

## ζ-Carotene

Appearing as a diffuse faint yellow band in the magnesium oxide–Hyflosupercel column after β-carotene, ζ-carotene presents a spectrum with well defined

peaks (%III/II = 103), at 378, 400, and 425 nm in petroleum ether (Table 7). Its carotene nature can be shown, as with α- and β-carotene, with TLC. ζ-Carotene is not available commercially; in Brazil it can be isolated by OCC from passion fruit, in which it is the principal carotenoid.

## β-Carotene-5,6-epoxide

With one of the ring double bonds eliminated by epoxidation (Figure 5), this carotenoid absorbs at wavelengths slightly lower than those of β-carotene (Table 7). On silica gel TLC it runs slightly below the carotenes, and its yellow color turns greenish-blue on exposure of the plate to hydrogen chloride gas. Addition of dilute hydrochloric acid to an ethanolic solution of the carotenoid results in a hypsochromic shift of about 20 nm resulting from the rearrangement of the 5,6-epoxy group to a 5,8-epoxide.

## β-Carotene-5,8-epoxide

The introduction of a 5,8-epoxy group eliminates from the β-carotene molecule a double bond from the polyene chain, in addition to the double bond of the β ring, resulting in λmax values about 20 nm lower than those of β-carotene (Table 7). This carotenoid runs lower than β-carotene-5,6-epoxide on the TLC gel and turns greenish-blue on exposure to hydrogen chloride gas.

## δ-Carotene

This carotenoid absorbs maximally at 431, 456, and 489 nm in petroleum ether (%III/II = 85) (Table 7), reflecting the 10 conjugated double bonds in the polyene chain (Figure 3). As a carotene it runs with the solvent front in the silica TLC plate developed with 5% methanol in toluene. Not available commercially, δ-carotene can be isolated from high-δ-carotene tomato or peach palm for co-chromatography.

## γ-Carotene

This bright orange monocyclic carotenoid has 11 conjugated double bonds, one of which is in a β ring (Figure 3). Thus, its adsorption spectrum has λmax values at 437, 462, and 494 nm with fine structure (Figure 8) (%III/II = 40) between β-carotene and lycopene (Table 7). As with other carotenes, it runs with the solvent front on TLC. For co-chromatography, it can be isolated from rose hips (or *Eugenia uniflora* in Brazil).

## Zeinoxanthin

This monohydroxy derivative of α-carotene has the same chromophore (Figure 4) and, therefore, the same absorption spectrum as α-carotene. The presence of the single hydroxyl group, already indicated by the order of elution on OCC and $R_F$ on TLC (around 0.56), is confirmed by acetylation with acetic anhydride, resulting in a product that behaves almost like a carotene on TLC. Because the hydroxyl group is not in the allylic position, response to methylation with acidified methanol is negative.

## α-Cryptoxanthin

Having the same chromophore, α-cryptoxanthin (Figure 4) has the same absorption spectrum as zeinoxanthin and α-carotene (Table 7). It also has the same chromatographic behavior as zeinoxanthin. The only difference in relation to zeinoxanthin is the location of the hydroxyl group in the ε rather than the β ring, placing this group in an allylic position. Thus, α-cryptoxanthin responds positively not only to acetylation but also to methylation.

## β-Cryptoxanthin

With the same chromophore as β-carotene (Figure 4), this xanthophyll presents a visible spectrum resembling that of β-carotene (Table 7). The presence of the hydroxyl group, manifested in the chromatographic behavior on OCC and TLC ($R_F$ around 0.44) is confirmed by the positive response to acetylation with acetic anhydride. That the hydroxyl is not in allylic position is demonstrated by the negative response to methylation with acidified methanol. β-Cryptoxanthin for co-chromatography can be isolated by OCC from papaya.

## Neurosporene

This carotene is acyclic and has nine conjugated double bonds (Figure 2); thus, its visible spectrum has well defined peaks at 414, 439, and 467 nm in petroleum ether (%III/II = 100) (Table 7). It behaves like the other carotenes in the silica TLC plate developed with 5% methanol in toluene, showing the absence of substituents.

## Lycopene

The red lycopene absorbs maximally at 444, 470, and 502 nm in petroleum ether with defined fine structure (%III/II = 65) (Figure 8), in agreement with 11 conjugated double bonds in an acyclic structure (Figure 2). The absence of functional groups is shown by its behavior on TLC. Lycopene standard is available commercially but can also be isolated by OCC from tomato for co-chromatography.

## Zeaxanthin

The visible spectrum of this derivative of β-carotene resembles that of β-carotene (Table 7). That it is a dihydroxy carotenoid is reflected in its behavior on OCC and TLC ($R_F$ is around 0.19 on the silica TLC gel developed with 5% methanol in toluene). The presence and nonallylic position of these groups are shown by the positive response to acetylation with acetic anhydride and negative response to methylation with acidified methanol, respectively. Partial acetylation will yield two acetylated products, one near the solvent front and the other at the middle of the silica plate developed with 5% methanol in toluene, the latter corresponding to the acetylation of only one of the hydroxyl groups. Complete acetylation yields one product with both hydroxyls acetylated, running near the solvent front on TLC.

## Lutein

This carotenoid has the same chromophore and, consequently, the same spectrum as its parent carotenoid, α-carotene (Figure 4, Table 7). Differing only in the location of one of the terminal double bonds, it is difficult but possible to separate from zeaxanthin chromatographically. It exhibits multizoning on TLC, appearing as two spots, with the principal spot having an $R_F$ of around 0.21. The presence of two hydroxy groups can be confirmed by acetylation, as with zeaxanthin. The allylic position of one of the hydroxyls is verified by the positive response to methylation with acidic methanol, producing a compound that behaves like a monohydroxy carotenoid on TLC. For co-chromatography, lutein, violaxanthin, and neoxanthin can be isolated from leaves.

## Violaxanthin

Having two epoxy groups at the 5,6 and 5′,6′-positions (Figure 5), this carotenoid has an absorption spectrum with λmax values about 10 nm lower than those of β-carotene and much greater fine structure (%III/II = 98) (Table 7) because the conjugated double bonds remaining after epoxidation are of the polyene chain. The presence of two hydroxy and two epoxy groups is indicated on TLC ($R_F$ lower than zeaxanthin and yellow color turning blue on exposure of the TLC plate to hydrogen chloride gas). The presence of the hydroxy groups can be confirmed by acetylation. A hypsochromic shift of about 40 nm on addition of dilute hydrochloric acid to an ethanolic solution of this carotenoid confirms the presence and position of the epoxy substituents, the displacement of the λmax being a consequence of the epoxide-furanoid rearrangement of violaxanthin to auroxanthin (Figure 17).

## Neoxanthin

Among the food carotenoids, neoxanthin is unique because it has an allenic group in the polyene chain (Figure 5). The spectrum is close to that of violaxanthin (Table 7). On TLC (silica gel with 5% methanol in toluene), this carotenoid stays near the origin because of the three hydroxy groups and it turns green on exposure of the plate to hydrogen chloride gas because of the presence of an epoxide. Acetylation, partial or complete, can confirm the presence of the three hydroxyls. A shift of the λmax values to lower wavelengths by about 20 nm shows that a 5,6-epoxy group is part of the molecule.

## Canthaxanthin

This symmetrical ketocarotenoid with two conjugated carbonyl groups (Figure 7), has a spectrum consisting of a broad symmetrical peak with the maximum at 466 nm in petroleum ether (%III/II = 0). On reduction with sodium borohydride, this single maximum transforms into the three-peak spectrum of the resulting dihydroxycarotenoid (isozeaxanthin), similar to that of β-carotene (Figure 18). On TLC the red-orange spot of canthaxanthin, which elutes at an $R_F$ of about 0.51, transforms into the yellow spot of isozeaxanthin with an $R_F$ of about 0.2.

## Echinenone

Also a ketocarotenoid with one conjugated carbonyl group (Figure 7), echinenone gives a spectrum having an unsymmetrical broad peak at 458 nm and a shoulder at 482 nm in petroleum ether (Table 7). Reduction with sodium borohydride transforms the spectrum into the typical three-peak spectrum of the resulting monohydroxy isocryptoxanthin. On the silica TLC plate, the orange spot of echinenone, which runs at an $R_F$ of about 0.72, turns to yellow with an $R_F$ of a monohydroxy carotenoid.

## Rubixanthin

A derivative of γ-carotene, rubixanthin has the same spectrum as γ-carotene (Table 7). The presence of the single hydroxyl group, which is indicated by its behavior on OCC (Table 9) and on TLC (eluting at a lower $R_F$ than β-cryptoxanthin), can be confirmed by a positive response to acetylation. The nonallylic position is shown by the negative reaction to methylation with acidic methanol.

# METHOD VALIDATION AND QUALITY ASSURANCE

It is now widely recognized that the acquisition of reliable analytic data requires representative samples, validated analytic methods, quality assurance, adequately trained personnel, and ancillary support staff and facilities.

The representativeness of the sample submitted to analysis should be ensured, a properly validated method should be chosen, and the good performance of the method in the laboratory should be demonstrated. Frequently, the question is not so much how good the method is but how well it is being used in the laboratory. Thus, method validation and quality assurance should always go hand in hand. Obviously, the first three requirements cited above can only be fulfilled if the laboratory is run by a quality technical staff supported by the necessary back-up staff, resources, and infrastructure.

## Validation of Methods

Development and validation of an analytic method usually take place in three stages (Conacher 1990):

- evaluation of performance parameters within a laboratory,
- demonstration of successful performance in limited interlaboratory studies, and
- demonstration of successful performance in a recognized collaborative study.

The degree of confidence that can be attributed to the validity of the method increases as the validation process progresses from the first to the third stage. The third stage, conducted according to the guidelines of the Association of Official Analytical Chemists (AOAC) or similar organization, is generally accepted as the highest degree of method validation. The main performance parameters that should be taken into account in assessing any analytic method are

- accuracy,
- precision,
- specificity,
- limit of detection,
- limit of determination,
- linear range, and
- scope.

*Accuracy* is the degree of agreement or closeness between the determined value and the true value. To simulate the actual analysis as closely as possible, certified reference materials should be analyzed. Ideally, a standard reference material should be a matrix similar to the actual samples to be analyzed, available at analyte concentrations comparable with those expected of the real samples, homogeneous, and stable in terms of both the analyte and the matrix.

Unfortunately, the few certified reference materials available are usually of high cost and are at only one concentration of the analyte. For carotenoids, a major concern is instability of the reference material. Because of these limitations, recovery of added analyte over an appropriate concentration range is commonly carried out and taken as the indication of accuracy. The concentration range should, as much as possible, bracket the levels expected in the samples to be analyzed; to evaluate the performance of the entire method, addition of the analyte should be as early as possible in the analytic process. It must be recognized, however, that an analyte added to a substrate may behave differently from an endogenous analyte. On one hand, the added analyte, not being intimately linked with the sample matrix, may be more easily extracted, giving an artificially high recovery. On the other hand, added analyte (especially polar compounds) may tightly adsorb to the glass walls of the container, particularly at high concentration, yielding low recovery. To approach the real situation, analytes should be added to the substrate with care (with contact with glass walls avoided) and left for a reasonable holding time before analysis to allow for bonding with the sample matrix.

The accuracy of a particular method may also

be assessed by comparison with a method already known to be accurate.

A standard reference material is now available for carotenoids from the U.S. National Institute of Standards and Technology: baby food composite SRM 2383. In Europe a candidate reference material was prepared from selected vegetables (sweet corn, tomatoes, and carrots), which were size reduced, mixed, pureed, and lyophilized. This mixed vegetable material was used in an interlaboratory study involving 17 European laboratories (Scott et al. 1996).

Some authors have resorted to in-house reference or control materials, such as a homogenized baby food containing carrots, peas, low-fat milk, and parsley (Hulshof et al. 1997). This homogeneous, well-mixed material is usually divided and dispensed into small sealed bottles, stored under freezing condition, and analyzed periodically along with analytic samples. In-house reference materials are valuable in evaluating precision, especially coherence of results over time, but do not assess accuracy.

Although it is appropriate to use processed food as a reference material so that enzymatic oxidation will not be a problem and homogeneity and stability of the matrix are better, it must be remembered that these materials do not represent raw commodities, which are much more difficult to sample and extract.

*Precision* evaluates how well a method performs under different conditions of repeated use. It is the degree of agreement between determined values and is generally expressed in terms of standard deviation or coefficients of variation (CVs) (also called relative standard deviation, RSD). Repeatability (within-laboratory precision) of a method may be measured by multiple analyses of identical samples at different analyte levels, performed on the same day by a single analyst using the same apparatus. Even better, it may be determined by multiple analyses over different days. More important is reproducibility (between-laboratory precision), which shows the variability of results produced by different laboratories. Repeatability is generally one-half to one-third of reproducibility. In the harmonization of protocols, repeatability and reproducibility limits and intra- and interlaboratory relative standard deviations have been designated r, R, $RSD_r$, and $RSD_R$, respectively (Poncklington 1990).

Although there are exceptions, CV values tend to be greater at low analyte concentrations and smaller at higher analyte concentrations (Lynch 1998). Over a reasonable range of concentrations, however, CV is usually independent of analyte concentration.

*Specificity* is the ability of a method to measure exclusively the element or compound of interest (analyte). Ideally, to verify the identity and amount of an analyte, two entirely different analytic principles should be used. For organic analytes, the following are resorted to: mass spectrometric confirmation, use of different detectors operating under different principles, chromatography using different systems, and chemical reactions (e.g., derivatization of a functional group followed by analysis) (Conacher 1990).

In any method, it is absolutely necessary to run reagent and substrate blanks to ensure that interfering compounds are not being measured together with the analytes. Substrate blanks should be run for each commodity examined.

Because carotenoids are measured by light absorption in the visible region, few noncarotenoid interferences occur. Anthocyanins are soluble in water and are either not coextracted with carotenoids or are removed during partition. Chlorophylls are eliminated by saponification; if saponification is not carried out, the chromatographic step must be able to separate chlorophylls from carotenoids. More common problems are the interference of one carotenoid with another during measurement or the erroneous identification of one carotenoid as another.

The *limit of detection* is the lowest concentration of an analyte that the analytic process can reliably differentiate from background levels. It has been given somewhat different definitions but in general is defined as the (background) level measured in the substrate blank plus 3 standard deviations of this baseline level. The *limit of determination* is the lowest concentration of an analyte that can be measured with a stated degree of confidence. It has been defined as the level measured in the substrate blank plus 10 standard deviations. Notwithstanding the definitions, it is recommended that limits of detection and determination be established in practice from the results of repeated analyses of spiked or endogenous samples.

For analysis of major carotenoids, the limits of detection and determination do not have much use. These limits become important when the whole range of carotenoids in a sample is determined, particularly for the minor or trace carotenoids.

*Method sensitivity* is defined as the ratio of the change in instrument signal to the change in analyte concentration (i.e., the slope of the standard curve) and should not be confused with limit of detection.

The *linear range* is generally taken as the range over which the method has been demonstrated to give a linear detector or instrument response. Because carotenoids are usually present in a specified food at widely differing concentrations, the linear range should be carefully assessed for the different carotenoids being quantified.

*Scope* refers to the number of different substrates to which the method can be successfully applied. Validated methods can be considered valid only for the commodities that were successfully included in the validation study. Applicability of the method to other foods should be demonstrated, both in terms of the matrix and the analyte concentration.

Horwitz (1982) insists that methods must be evaluated with their purpose in mind and that evaluation must balance between the level of scientific requirements and practical considerations of cost, time, and level of training required. Analysts must always strive to achieve the best accuracy and precision possible but must not lose sight of the fact that normal (natural) variability of the commodity and the inherent limitations of bulk sampling may render a very high degree of accuracy and precision in the method meaningless.

In carotenoid analysis, validation of methods has not been strongly advocated, even with the introduction of high-performance liquid chromatography (HPLC), because the emphasis has been on chromatographic separation. In the few papers involving quantification, validation consisted mainly of recovery tests and determination of repeatability. Recovery of added carotenoids, however, should be considered with caution. It indicates losses during the steps after extraction but does not truly evaluate the extraction. Added carotenoids are more easily extracted than endogenous carotenoids, which are protected by the plant's ultrastructure or are bound to the matrix, and adsorption to the glass walls of the container can occur. Internal standards (e.g., carotenoids not found in the sample, available in synthetic form) are sometimes used to correct for losses during analysis. It must be remembered, however, that different carotenoids have different stability, and the recovery percentage of the internal standard may not represent percentage retention of the sample's carotenoids during analysis. In our laboratory, comparison of the results obtained by open-column chromatography and HPLC methods gives a better appraisal of the reliability of the results. As with other types of analysis, repeatability results of various laboratories demonstrate that the CV increases as the carotenoid concentration decreases.

Interlaboratory studies strengthens confidence in a method and laboratory and often pinpoint the critical or error-prone steps in the analytic process. Two approaches can be discerned in the literature: 1) an interlaboratory study in which the participating laboratories analyze the same homogeneous sample, using their own methods of choice, thus evaluating the laboratories' performance, including their ability to choose the right method, and 2) a collaborative interlaboratory study, conducted according to the guidelines of AOAC, in which the same method is used by the participating laboratories, thus evaluating the method's performance in an interlaboratory setting.

AOAC has set the following minimum requirements for a collaborative study (Cunniff 1997): a minimum of five materials; a minimum of eight laboratories reporting valid data for each material; and a minimum of one replicate if within-laboratory repeatability parameters are not desired and two replicates if these parameters are required. Replication should ordinarily be attained by blind replicates or split levels (Youden pairs).

Official AOAC methods are available only for carotenes in fresh plant materials and silage and for carotenes and xanthophylls in dried plant materials and mixed feeds. Both methods are considered inadequate to meet the current need for individual carotenoid determination.

The only published interlaboratory studies involving individual carotenoid analysis were those conducted by Scott et al. (1996). Seventeen European laboratories assessed the accuracy of HPLC procedures for the determination of lutein, zeaxanthin, lycopene, $\alpha$-carotene, and $\beta$-carotene in a vegetable mix. Possible problem areas were investigated, including chromatographic systems, standardization of carotenoid stock solutions, extraction procedure, and data handling. The results suggested that the effect of the chromatographic system is probably not a major variable in measuring the carotenoid concentration. The standardization of the carotenoid stock solution would not appear to be a significant problem in the more experienced laboratory, although there were greater variations for lycopene calibration and measurement. Preliminary conclusions suggested that the preparation of the carotenoid extract may account for about 13% of the overall variance of around 23%.

## Quality Assurance

Garfield (1991) defines *quality control* as planned activities to provide a quality product and *quality assurance* as planned activities to ensure that the quality control activities are being properly implemented. The latter is the broad management concept of maintaining the ability of a laboratory to furnish reliable information (Horwitz et al. 1980).

Quoting the U.S. Consumer Product Safety Commission, Garfield (1984) cites the following common

objectives of quality assurance programs:

- to maintain a continuing assessment of the accuracy and precision of data generated by analysts within the laboratory group,
- to provide a measure of the accuracy and precision of analytic methods and to identify weak methodology,
- to detect training needs within the analytic group,
- to provide a permanent record of instrument performance as a basis for validating data and projecting repair or replacement needs, and
- to upgrade overall quality of laboratory performance.

A quality assurance program should include, among other things, the following elements (Inhorn 1978):

- maintenance of skilled personnel, written and validated methods, and properly constructed, equipped, and maintained laboratory facilities;
- provision of representative samples and controls;
- use of high-quality glassware, solvents, and other testing materials;
- calibration, adjustment, and maintenance of equipment;
- use of control samples and standard samples, with proper records;
- direct observation of the performance of certain critical tests;
- review and critique of results;
- tests of internal and external proficiency testing;
- use of replicate samples;
- comparison of replicate results with those of other laboratories; and
- monitoring of results.

The production of high-quality analytic data must be a commitment of any laboratory. However, the specific objectives and application of a quality assurance program will vary from one laboratory to another and will depend on the laboratory's size, complexity, purpose, and budget, although all programs should incorporate some basic recognized practices and procedures. Small laboratories can operate with a minimal but suitable program if supervisory personnel are aware of what is necessary to achieve quality results, staffing levels are adequate, and necessary expertise exists (Garfield 1984). The AOAC Laboratory Quality Assurance Committee developed a quality assurance checklist to serve as a guide for small laboratories (AOAC 1992). The Food and Agriculture Organization of the United Nations published a manual on quality assurance in the food control chemical laboratory (FAO 1993).

Quality assurance activities have associated costs, but the benefits of improved laboratory credibility and staff moral and the savings in not having to reanalyze, correct, or even discard unreliable data or misjudged product samples will justify the cost of the program (Garfield 1991).

# CALCULATION OF RETENTION IN PROCESSED FOODS

In food composition tables the carotenoid concentration must be expressed in terms of the weight of the carotenoid per unit weight of the raw food or per unit weight of the cooked or processed food as the food is eaten. Because of the instability of carotenoids, it is also important to verify losses during cooking and processing so that optimum conditions can be recommended to retain as much as possible of these important compounds.

Results of published retention studies are difficult to assess because of the following (Rodriguez-Amaya 1997):

- processing and storage conditions are not described or are only partially described;
- foods are prepared, cooked, or processed differently, making comparisons of processing methods difficult;
- different conditions are used for the same method of processing;
- the procedure followed for the calculation of the retention or loss is not specified; and
- no correction or compensation is made for weight changes during cooking and processing and the greater extraction efficiency with cooked compared to raw samples during analysis.

Although losses of carotenoids have been calculated in published paper simply by the difference of the carotenoid concentration before (e.g., µg/g raw weight) and after cooking and processing (e.g., µg/g cooked weight), this calculation does not account for changes in the weight of the food during cooking (e.g., loss of water and soluble solids, gain of water or oil) and, therefore, does not represent true losses of the carotenoids.

Calculations that account or compensate for loss or gain of food weight during cooking have been done by one of the formulas in Equation 2. Murphy et al. (1975) recommended the first formula for calculating retentions of nutrients in cooked foods and found that it more accurately measured retentions of the different nutrients under different situations of weight changes. Calculation on a dry weight basis overestimated retentions in nearly all instances. It is not always feasible, however, to obtain data on weights of foods before and after processing, especially under industrial production conditions. In our experience, the third calculation gives practically the same results as the first formula (Rodriguez-Amaya et al., unpublished).

Several papers reported carotenoid retentions of over 100% in cooked foods calculated on a dry weight basis. These results cannot be considered as true increases; there is no way carotenoids can be biosynthesized during cooking. The heat treatment inactivates the enzymes responsible for carotenoid biosynthesis and, in fact, stimulates isomerization and oxi-

**Equation 2.**

$$\% \text{ retention} = \frac{\text{carotenoid content per g of cooked food} \bullet \text{g of food after cooking}}{\text{carotenoid content per g of raw food} \bullet \text{g of food before cooking}} \bullet 100$$

$$\% \text{ retention} = \frac{\text{carotenoid content per g of cooked food (dry basis)}}{\text{carotenoid content per g of raw food (dry basis)}} \bullet 100$$

$$\% \text{ retention} = \frac{\text{carotenoid content (after cooking) per g of original raw food}}{\text{carotenoid content per g of raw food}} \bullet 100$$

dative degradation of carotenoids. These alleged increases could simply be due to the greater ease with which carotenoids are extracted from cooked or processed samples compared with carotenoids in fresh foods, where they are physically protected or combined with other food components. Extraction efficiency of fresh samples must be enhanced to make it as equivalent as possible to that of cooked samples (such as soaking the sample in water or extracting solvent before extraction), and extraction must be exhaustive. Apparent increases may also be due to appreciable leaching of soluble solids, as in carrots, concentrating the carotenoids per unit weight of food. Calculating the retention on the insoluble solid basis has been proposed in this case. Moreover, enzymatic oxidation of carotenoids can substantially lower their concentrations in raw samples, especially when these samples are left standing for some time after being cut or grated.

In studies on retention it is important to specify the processing and storage conditions (time, temperature, etc.). Paired samples (i.e., equivalent raw and cooked samples) must be used to offset variations due to varietal differences, seasonal or climatic effects, degree of maturity, nonuniform distribution of carotenoid in the food or food lot, etc. Speek et al. (1988), for example, prepared samples of leafy vegetables by systematically picking leaves off the stalk from the top to the roots, alternately dividing them into two portions. One portion was analyzed raw and the other after processing. In our laboratory, fruits or fruits vegetables are quartered longitudinally; two opposite sections are taken for analysis of raw samples and the other two opposite sections are submitted to processing before analysis.

It is also recommended that results be analyzed statistically so that the true meaning of the results can be appreciated.

# REFERENCES


ACS Committee on Environmental Improvement (1980) Guidelines for data acquisition and data quality evaluation in environmental chemistry. Anal Chem 52:2242-2249

AOAC International (1992) Quality assurance checklist for small laboratories. Referee 16(11):13

Adewusi SRA, Bradbury JH (1993) Carotenoids in cassava: comparison of open-column and HPLC methods of analysis. J Sci Food Agric 62:375-383

Aina JO (1990) Physico-chemical changes in African mango (*Irvingia gabonensis*) during normal storage ripening. Food Chem 36:205-212

Arima HK, Rodriguez-Amaya DB (1988) Carotenoid composition and vitamin A value of commercial Brazilian squashes and pumpkins. J Micronutr Anal 4:177-191

Arima HK, Rodriguez-Amaya DB (1990) Carotenoid composition and vitamin A value of a squash and a pumpkin from Northeastern Brasil. Arch Latinoam Nutr 40:284-292

Bickoff EM, Atkins ME, Bailey GF, Stitt F (1949) Stereoisomeric analysis of β-carotene. J Assoc Off Anal Chem 32:766-774

Britton G (1991) Carotenoids. Methods Plant Biochem 7:473-518

Britton G (1995) UV/visible spectroscopy. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: spectroscopy, vol 1B. Birkhäuser Verlag, Basel, pp 13-63

Bureau JL, Bushway RJ (1986) HPLC determination of carotenoids in fruits and vegetables in the United States. J Food Sci 51:128-130

Burton GW (1989) Antioxidant action of carotenoids. J Nutr 119:109-111

Bushway RJ (1985) Separation of carotenoids in fruits and vegetables by high performance liquid chromatography. J Liquid Chromatogr 8:1527-1547

Bushway RJ, Wilson AM (1982) Determination of α- and β-carotene in fruits and vegetables by high-performance liquid chromatography. Can Inst Food Sci Technol J 15:165-169

Carvalho PRN, Collins CH, Rodriguez-Amaya DB (1992) Comparison of provitamin A determination by normal-phase gravity-flow column chromatography and reversed-phase high performance liquid chromatography. Chromatographia 33:133-137

Cavalcante ML, Rodriguez-Amaya DB (1992) Carotenoid composition of the tropical fruits *Eugenia uniflora* and *Malpighia glabra*. In Charalambous G (ed), Food science and human nutrition. Elsevier Science Publishers, Amsterdam, pp 643-650

Chandler LA, Schwartz SJ (1987) HPLC separation of *cis-trans* carotene isomers in fresh and processed fruits and vegetables. J Food Sci 52:669-672

Chandler LA, Schwartz SJ (1988) Isomerization and losses of *trans*-β-carotene in sweet potatoes as affected by processing treatments. J Agric Food Chem 36:129-133.

Chen BH, Chen YY (1992) Determination of carotenoids and chlorophylls in water convolvulus (*Ipomoea aquatica*) by liquid chromatography. Food Chem 45:129-134

Chen BH, Peng HY, Chen HE (1995) Changes of carotenoids, color, and vitamin A contents during processing of carrot juice. J Agric Food Chem 43:1912-1918

Conacher HBS (1990) Validation of methods used in crisis situations: task force report. J Assoc Off Anal Chem 73:332-334

Craft NE (1992) Carotenoid reversed-phase high performance liquid chromatography methods: reference compendium. Methods Enzymol 213:185-205

Craft NE, Soares JH Jr (1992) Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents. J Agric Food Chem 40:431-434

Craft NE, Sander LC, Pierson HF (1990) Separation and relative distribution of all-*trans*-β-carotene and its *cis* isomers in ß-carotene preparations. J Micronutr Anal 8:209-221

Craft NE, Wise SA, Soares JH Jr (1992) Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. J Chromatogr 589:171-176

Craft NE, Wise AS, Soares JH Jr (1993) Individual carotenoid content of SRM 1548 total diet and influence of storage temperature, lyophilization, and irradiation on dietary carotenoids. J Agric Food Chem 41:208-213

Cunniff P, ed (1997) Official methods of analysis of AOAC international, 16th ed, vol 1. AOAC International, Gaithersburg, MD

Davies, BH (1976) Carotenoids. In Goodwin TW (ed), Chemistry and biochemistry of plant pigments, 2nd ed, vol 2, Academic Press, London, pp 38-165

Di Mascio P, Kaiser S, Sies H (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 274:532-538

El-Tinay AH, Chichester CO (1970) Oxidation of ß-carotene. Site of initial attack. J Org Chem 35:2290-2293

Emenhiser C, Sander LC, Schwartz SJ (1995) Capability of a polymeric $C_{30}$ stationary phase to resolve *cis-trans* carotenoid isomers in reversed-phase liquid chromatography. J Chromatogr A 707:205-216

Emenhiser C, Englert G, Sander LC, et al (1996a) Isolation and structural elucidation of the predominant geometrical isomers of α-carotene. J Chromatogr A 719:333-343

Emenhiser C, Simunovic N, Sander LC, Schwartz SJ (1996b) Separation of geometrical carotenoid isomers in biological extracts using a polymeric $C_{30}$ column in reversed-phase liquid chromatography. J Agric Food Chem 44:3887-3893

Englert G (1995) NMR spectroscopy. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: spectroscopy, vol 1B. Birkhäuser Verlag, Basel, pp 147-260

Enzell CR, Back S (1995) Mass spectroscopy. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: spectroscopy, vol 1B. Birkhäuser Verlag, Basel, pp 261-320

Epler KS, Sander LC, Ziegler RG, et al (1992) Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids. J Chromatogr 595:89-101

Eugster CH (1995) Chemical derivatization: microscale tests for the presence of common functional groups in carotenoids. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: isolation and analysis, vol 1A. Birkhäuser Verlag, Basel, pp 71-80

Ezell BD, Wilcox MS (1962) Loss of carotene in fresh vegetables as related to wilting and temperature. J Agric Food Chem 10:124-126

FAO (1993) Manual of food quality control 14. Quality assurance in the food control chemical laboratory. Food and Agriculture Organization of the United Nations, Rome

Flügel M, Gross J (1982) Pigment and plastid changes in mesocarp and exocarp of ripening muskmelon *Cucumis melo* cv. *Galia*. Angew Botanik 56:393-406

Foote CS, Chang YC, Denny RW (1970) Chemistry of singlet oxygen X. Carotenoid quenching parallels biological protection. J Am Oil Chem Soc 92:5216-5218

Garfield FM (1984) Quality assurance principles for analytical laboratories. Association of Official Analytical Chemists, Arlington

Garfield FM (1991) Quality assurance principles for analytical laboratories. AOAC International, Arlington

Godoy HT, Rodriguez-Amaya DB (1987) Changes in individual carotenoids on processing and storage of mango (*Mangifera indica*) slices and puree. Int J Food Sci Technol 22:451-460

Godoy HT, Rodriguez-Amaya DB (1991) Comportamento dos carotenóides de purê de mamão (*Carica papaya*) sob processamento e estocagem. Cienc Tecnol Aliment 11:210-220

Godoy HT, Rodriguez-Amaya DB (1994) Occurrence of *cis*-isomers of provitamins A in Brazilian fruits. J Agric Food Chem 42:1306-1313

Godoy HT, Rodriguez-Amaya DB (1995a) Buriti (*Mauritia vinifera* Mart), uma fonte riquíssima de pró-vitamina A. Arq Biol Tec 38:109-120

Godoy HT, Rodriguez-Amaya DB (1995b) Carotenoid composition and vitamin A value of Brazilian loquat (*Eriobotrya japonica* Lindl.). Arch Latinoam Nutr 45:336-339

Godoy HT, Rodriguez-Amaya DB (1998) Occurrence of *cis*-isomers of provitamin A in Brazilian vegetables. J Agric Food Chem 46:3081-3086

Godoy HT, Rodriguez-Amaya DB, Connor AE, Britton G (1990) Confirmation of the structure of papaya ß-cryptoxanthin monoepoxide. Food Chem 36:281-286

Gross J (1982a) Changes of chlorophylls and carotenoids in developing strawberry fruits (*Fragaria anonassa*) cv. *Tenira*. Gartenbauwiss 47:142-144

Gross J (1982b) Pigment changes in the pericarp of the Chinese goosebery or kiwi fruit (*Actinidia chinensis*) cv. Bruno during ripening. Gartenbauwiss 47:162-167

Gross J (1982/83). Chlorophyll and carotenoid pigments in Ribes fruits. Sci Hort 18:131-136

Gross J (1984) Carotenoid pigments in three plum cultivars. Gartenbauwiss 49:18-21

Gross J (1985) Carotenoid pigments in the developing cherry (*Prunus avium*). cv. 'Dönissen's Gelbe'. Gartenbauwiss 50:88-90

Gross J (1987) Pigments in fruits. Academic Press, London

Gross J (1991) Pigments in vegetables. Chlorophylls and carotenoids. Avi:Van Nostrand Reinhold Company Inc, New York

Gross J, Ikan R, Eckhardt G (1983) Carotenoids of the fruit of *Overrhoa carambola*. Phytochemistry 22:1479-1481

Hart DJ, Scott KJ (1995) Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. Food Chem 54:101-111

Heinonen MI (1990) Carotenoids and provitamin A activity of carrot (*Daucus carota* L.) cultivars. J Agric Food Chem 38:609-612

Heinonen MI, Ollilainen V, Linkola EK, et al (1989) Carotenoids in Finnish foods: vegetables, fruits, and berries. J Agric Food Chem 37:655-659

Horwitz W (1982) Evaluation of analytical methods used for regulation. J Assoc Off Anal Chem 65:525-530

Horwitz W (1988) Sampling and preparation of sample for chemical examination. J Assoc Off Anal Chem 71:241-245

Horwitz W, Kamps LR, Boyer KW (1980) Quality assurance in the analysis of foods for trace constituents. J Assoc Off Anal Chem 63:1344-1354

Hulshof PJM, Xu C, van de Bovenkamp P, et al (1997) Application of a validated method for the determination of provitamin A carotenoids in Indonesian foods of different maturity and origin. J Agric Food Chem 45:1174-1179

Huyskens S, Timberg R, Gross J (1985) Pigment and plastid ultra-structural changes in kumquat (*Fortunella margarita*) "Nagami" during ripening. J Plant Physiol 118:61-72

Inhorn SL, ed (1978) Quality assurance practices for health laboratories. American Public Health Service, New York

John J, Subbarayan C, Cama HR (1970) Carotenoids in 3 stages of ripening of mango. J Food Sci 35:262-265

Keith LH, Crummet W, Deegan J Jr, et al (1983) Principles of environmental analysis. Anal Chem 55:2210-2218

Khachik F, Beecher GR, Whitaker NF (1986) Separation, identification, and quantification of the major caro-

tenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. J Agric Food Chem 34:603-616

Khachik F, Beecher GR, Vanderslice JT, Furrow G (1988) Liquid chromatographic artifacts and peak distortion: sample-solvent interactions in the separation of carotenoids. Anal Chem 60:807-811

Khachik F, Beecher GR, Lusby WR (1989) Separation, identification, and quantification of the major carotenoids in extracts of apricots, peaches, cantaloupe, and pink grapefruit by liquid chromatography. J Agric Food Chem 37:1465-1473

Khachik F, Beecher GR, Goli MB, Lusby WR (1992a) Separation and quantification of carotenoids in foods. Methods Enzymol 213:347-359

Khachik F, Goli MB, Beecher GR, et al (1992b) Effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. J Agric Food Chem 40:390-398

Kimura M, Rodriguez-Amaya DB, Godoy HT (1990) Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. Food Chem 35:187-195

Kimura M, Rodriguez-Amaya DB, Yokoyama SM (1991) Cultivar differences and geographic effects on the carotenoid composition and vitamin A value of papaya. Lebens Wissen Technol 24:415-418

Konings EJM, Roomans HHS (1997) Evaluation and validation of an LC method for the analysis of carotenoids in vegetables and fruit. Food Chem 59:599-603.

Kopas-Lane LM, Warthesen JJ (1995) Carotenoid photostability in raw spinach and carrots during cold storage. J Food Sci 60:773-776

Koskitalo LN, Ormrod DP (1972) Effects of sub-optimal ripening temperatures on the color quality and pigment composition of tomato fruit. J Food Sci 37:56-59

Kramer A, Twigg BA (1970) Fundamentals of quality control for the food industry, 3rd ed, vol 1, Avi Publishing Co, Westport, CT

Kratochvil B, Taylor JK (1981) Sampling for chemical analysis. Anal Chem 53:924A-926A, 928A, 930A, 932A, 934A, 936A, 938A

Krinsky NI (1989) Antioxidant functions of carotenoids. Free Radical Biol Med 7:617-635

Lesellier E, Marty C, Berset C, Tchapla A (1989) Optimization of the isocratic non-aqueous reverse phase (NARP) HPLC separation of *trans*/*cis*-α- and β-carotenes. J High Resolut Chromatogr 12:447-454

Lessin WJ, Catigani GL, Schwartz SJ (1997) Quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables. J Agric Food Chem 45:3728-3732

Liaaen-Jensen S (1971) Isolation, reactions. In Isler O. (ed), Carotenoids. Birkhäuser Verlag, Basel, pp 61-188

Lietz G, Henry CJK (1997) A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil. Food Chem 60:109-117

Lin SD, Chen AO (1995) Major carotenoids in juices of ponkan mandarin and liucheng orange. J Food Biochem 18:273-283

Lynch JM (1998) Use of AOAC International method performance statistics in the laboratory. J AOAC Int 81:679-684

Mantoura RFC, Wright SW, Jeffrey SW, et al (1997) Filtration and storage of pigments from microalgae. In Jeffrey SW, Mantoura RFC, Wright SW (eds), Phytoplankton pigments in oceanography: guidelines to modern methods. UNESCO Publishing, Paris, pp 283-305

Mercadante AZ, Rodriguez-Amaya DB (1990) Carotenoid composition and vitamin A value of some native Brazilian green leafy vegetables. Int J Food Sci Technol 25:213-219

Mercadante AZ, Rodriguez-Amaya DB (1991) Carotenoid composition of a leafy vegetable in relation to some agricultural variables. J Agric Food Chem 39:1094-1097

Mercadante AZ, Rodriguez-Amaya DB (1998) Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango. J Agric Food Chem 46:128-130

Mercadante AZ, Steck A, Rodriguez-Amaya DB, et al (1996) Isolation of methyl (9'Z)-apo-6'-lycopenoate from *Bixa orellana*. Phytochemistry 41:1201-1203

Mercadante AZ, Rodriguez-Amaya DB, Britton G (1997a) HPLC and mass spectrometric analysis of carotenoids from mango. J Agric Food Chem 45:120-123

Mercadante AZ, Steck A, Pfander H (1997b) Isolation and identification of new apocarotenoids from annatto (*Bixa orellana*) seeds. J Agric Food Chem 45:1050-1054

Mercadante AZ, Britton G, Rodriguez-Amaya DB (1998) Carotenoids from yellow passion fruit (*Passiflora edulis*). J Agric Food Chem 46:4102-4106

Mercadante AZ, Steck A, Pfander H (1999) Carotenoids from guava (*Psidium guajava* L.): isolation and structure elucidation. J Agric Food Chem 47:145-151

Mínguez-Mosquera MI, Garrido-Fernandez J (1989) Chlorophyll and carotenoid presence in olive fruit. J Agric Food Chem 37:1-7

Mínguez-Mosquera MI, Garrido-Fernández J, Gandul-Rojas B (1989) Pigment changes in olives during fermentation and brine storage. J Agric Food Chem 37:8-11

Murphy EW, Criner PE, Gray BC (1975) Comparisons of methods for calculating retentions of nutrients in cooked foods. J Agric Food Chem 23:1153-1157

Nelis HJCF, Leenheer AP de (1983) Isocratic nonaqueous reversed-phase liquid chromatography of carotenoids. Anal Chem 55:270-275

O'Neil CA, Schwartz SJ (1992) Chromatographic analysis of *cis-trans* carotenoid isomers. J Chromatogr 624:235-252

O'Neil CA, Schwartz SJ, Catignani GL (1991) Comparison of liquid chromatographic methods for determination of *cis-trans* isomers of β-carotene. J Assoc Off Anal Chem 74:36-42

Padula M, Rodriguez-Amaya DB (1986) Characterization of the carotenoids and assessment of the vitamin A value of Brazilian guavas (*Psidium guajava* L.). Food Chem 20:11-19

Padula M, Rodriguez-Amaya DB (1987) Changes in individual carotenoids and vitamin C on processing and storage of guava juice. Acta Alimentaria 16:209-216

Palozza P, Krinsky NI (1992) Antioxidant effects of carotenoids *in vivo* and *in vitro*:An overview. Methods Enzymol 213:403-420

Park YW (1987) Effect of freezing, thawing, drying, and cooking on carotene retention in carrots, broccoli, and

spinach. J Food Sci 52:1022-1025
Pettersson A, Jonsson L (1990) Separation of *cis-trans* isomers of alpha- and beta-carotene by adsorption HPLC and identification with diode array detection. J Micronutr Anal 8:23-41
Pfander H, Riesen R (1995) Chromatography: part IV high-performance liquid chromatography. In Britton G, Liaaen-Jensen S, Pfander H (eds), Carotenoids: isolation and analysis, vol 1A. Birkhäuser Verlag, Basel, pp 145-190
Pfander H, Riesen R, Niggli U (1994) HPLC and SFC of carotenoids scope and limitations. Pure Appl Chem 66:947-954.
Pocklington WD (1990) Harmonized protocols for the adoption of standardized analytical methods and for the presentation of their performance characteristics. Pure Appl Chem 62:149-162.
Pomeranz Y, Meloan CE (1994) Food analysis: theory and practice, 3rd ed. Chapman & Hall, New York
Porsch B (1993) Experimental consequences of differences between composition of the sample solvent and of the mobile phase in HPLC. J Liquid Chromatogr 16:3409-3421
Quackenbush FW, Smallidge RL (1986) Nonaqueous reverse phase liquid chromatographic system for separation and quantitation of provitamins A. J Assoc Off Anal Chem 69:767-772
Rahman FMM, Buckle KA (1980) Pigment changes in capsicum cultivars during maturation and ripening. J Food Technol 15:241-249
Ramos DMR, Rodriguez-Amaya DB (1987) Determination of the vitamin A value of common Brazilian leafy vegetables. J Micronutr Anal 3:147-155
Ramos DMR, Rodriguez-Amaya DB (1993) Avaliação das perdas de carotenóides e valor de vitamina A durante desidratação e liofilização industrial de espinafre. Arq Biol Tecnol 36:83-94
Raymundo LC, Griffiths AE, Simpson KL (1967) Effect of dimethyl sulfoxide (DMSO) on the biosynthesis of carotenoids in detached tomatoes. Phytochemistry 6:1527-1532
Riso P, Porrini M (1997) Determination of carotenoids in vegetable foods and plasma. Int J Vitam Nutr Res 67:47-54
Rodriguez-Amaya DB (1989) Critical review of provitamin A determination in plant foods. J Micronutr Anal 5:191-225
Rodriguez-Amaya DB (1990) Provitamin A determination—problems and possible solutions. Food Nutr Bull 12:246-250
Rodriguez-Amaya DB (1993). Nature and distribution of carotenoids in foods. In Charalambous G (ed), Shelf-life studies of foods and beverages. Chemical, biological, physical and nutritional aspects. Elsevier Science Publishers, Amsterdam, pp 547-589
Rodriguez-Amaya DB (1996) Assessment of the provitamin A contents of foods—the Brazilian experience. J Food Comp Anal 9:196-230
Rodriguez-Amaya DB (1997) Carotenoids and food preparation: the retention of provitamin A carotenoids in prepared, processed, and stored foods. OMNI, Washington DC.
Rodriguez-Amaya DB, Kimura M (1989) Carotenóides e valor de vitamina A em cajá (*Spondias lutea*). Cienc Tecnol Aliment 9:148-162
Rodriguez-Amaya DB, Amaya-Farfan J (1992) Estado actual de los metodos analiticos para determinar provitamina A. Arch Latinoam Nutr 42:180-191
Rodriguez-Amaya DB, Tavares CA (1992) Importance of *cis*-isomer separation in determining provitamin A in tomato and tomato products. Food Chem 45:297-302
Rodriguez-Amaya DB, Simpson KL, Chichester CO (1973) The biosynthesis of astaxanthin XVII. Intermediates in the conversion of β-carotene. Int J Biochem 4:213-222
Rodriguez-Amaya DB, Lee TC, Chichester CO (1975) Comparative study of the carotenoid composition of the seeds of ripening *Momordica charantia* and tomatoes. Plant Physiol 56:626-629.
Rodriguez-Amaya DB, Raymundo LC, Lee TC, et al (1976a) Carotenoid pigment changes in ripening *Momordica charantia* fruits. Ann Bot 40:615-624
Rodriguez-Amaya DB, Tanaka Y, Katayama T, et al (1976b) Hydroxylation of β-carotene on micro-cel C. J Agric Food Chem 24:819-822
Rodriguez-Amaya DB, Bobbio PA, Bobbio FO (1983) Carotenoid composition and vitamin A value of the Brazilian fruit *Cyphomandra betacea*. Food Chem 12:61-65
Rodriguez-Amaya DB, Kimura M, Godoy HT, Arima HK (1988) Assessment of provitamin A determination by open column chromatography/visible absorption spectrophotometry. J Chromatogr Sci 26:624-629
Rouchaud J, Moons C, Meyer JA (1984) Effects of pesticide treatments on the carotenoid pigments of lettuce. J Agric Food Chem 32:1241-1245
Rouseff RL, Sadler GD, Putnam TJ, Davis JE (1992) Determination of β-carotene and other hydrocarbon carotenoids in red grapefruit cultivars. J Agric Food Chem 40:47-51
Sander LC, Craft NE (1990) Device for subambient temperature control in liquid chromatography. Anal Chem 62:1545-1547
Sander LC, Sharpless KE, Craft NE, Wise SA (1994) Development of engineered stationary phases for the separation of carotenoid isomers. Anal Chem 66:1667-1674
Schiedt K, Liaaen-Jensen S (1995) Isolation and analysis. In Britton G, Liaaen-Jensen S, Pfander H. (eds), Carotenoids:isolation and analysis, vol 1A. Birkhäuser Verlag, Basel, pp 81-108
Schmitz HH, van Breemen RB, Schwartz SJ (1992) Fast-atom bombardment and continuous-flow fast-atom bombardment mass spectrometry in carotenoid analysis. Methods Enzymol 213:322-336
Schmitz HH, Emenhiser C, Schwartz SJ (1995) HPLC separation of geometric carotene isomers using a calcium hydroxide stationary phase. J Agric Food Chem 43:1212-1218
Scott KJ (1992) Observations on some of the problems associated with the analysis of carotenoids in foods by HPLC. Food Chem 45:357-364
Scott KJ, Hart DJ (1993) Further observations on problems associated with the analysis of carotenoids by HPLC—2: Column temperature. Food Chem 47:403-405
Scott KJ, Finglass PM, Seale R, et al (1996) Interlaboratory studies of HPLC procedures for the analysis of caro-

tenoids in foods. Food Chem 57:85-90
Simonetti, P, Porrini M, Testolin G (1991) Effect of environmental factors and storage on vitamin content of *Pisum sativum* and *Spinacea oleracea*. Italian J Food Sci 3:187-196
Speek AJS, Speek-Saichua S, Schreurs WHP (1988) Total carotenoid and β-carotene contents of Thai vegetables and the effect of processing. Food Chem 27:245-257
Sweeney JP, Marsh AC (1971) Effect of processing on provitamin A in vegetables. J Am Diet Assoc 59:238-243.
Takama F, Saito S (1974) Studies on the storage of vegetables and fruits II. Total carotene content of sweet pepper, carrot, leek and parsley during storage. J Agric Sci (Japan) 19:11
Tanaka Y, KatayamaT, Simpson KL, Chichester CO (1981) Stability of carotenoids on silica gel and other adsorbent. Bull Jpn Soc Fish 46:799
Tavares CA, Rodriguez-Amaya DB (1994) Carotenoid composition of Brazilian tomatoes and tomato products. Lebens Wiss Technol 27:219-224
Terão J (1989) Antioxidant activity of β-carotene-related carotenoids in solution. Lipids 24:659-661
Taylor RF (1983) Chromatography of carotenoids and retinoids. Adv Chromatogr 22:157-213
Thomas P, Janave MT (1975) Effects of gamma irradiation and storage temperature on carotenoids and ascorbic acid content of mangoes on ripening. J Sci Food Agric 26:1503-1512
Tonucci LH, Holden JM, Beecher GR, et al (1995) Carotenoid content of thermally processed tomato-based food products. J Agric Food Chem 43:579-586
Tsukida K (1992) Separation of isomers of *cis*-β-carotenes. Methods Enzymol 213:291-305
Tsukida K, Saiki K, Sugiura M (1981) Structural elucidation of the main *cis*-β-carotenes. J Nutr Sci Vitaminol 27:551-561
Tsukida K, Saiki K, Takii T, Koyama Y (1982) Separation and determination of *cis*/*trans*-β-carotenes by high performance liquid chromatography. J Chromatogr 245:359-364
Van Breemen RB (1996) Innovations in carotenoid analysis using LC/MS. Anal Chem 68:299A-304A
Van Breemen RB (1997) Liquid chromatography/mass spectrometry of carotenoids. Pure Appl Chem 69:2061-2066
Van Breemen RB, Schmitz HH, Schwartz SJ (1995) Fast atom bombardment tandem mass spectrometry of carotenoids. J Agric Food Chem 43:384-389
Van Calsteren MR, Bissonnette MC, Cormier F, et al (1997) Spectroscopic characterization of crocetin derivatives from *Crocus sativus* and *Gardenia jasminoides*. J Agric Food Chem 45:1055-1061.
Walpole RE, Myers R (1972) Probability and statistics for engineers and scientists. Macmillan, New York
Wilberg VC, Rodriguez-Amaya DB (1995) HPLC quantitation of major carotenoids of fresh and processed guava, mango and papaya. Lebensm Wiss Technol 28:474-480
Wills RBH, Rangga A (1996) Determination of carotenoids in Chinese vegetables. Food Chem 56:451-455
Wu Y, Perry AK, Klein BP (1992) Vitamin C and β-carotene in fresh and frozen green beans and broccoli in a simulated system. J Food Qual 15:87-96
Young A, Britton G (1990) Carotenoids and stress. In Alscher RG, Cumming JR (eds), Stress responses in plants: adaptation and acclimation mechanisms. Plant Biology, vol 12. Wiley-Liss Inc, New York
Zapata M, Garrido JL (1991) Influence of injection conditions in reversed-phase high-performance liquid chromatography of chlorophylls and carotenoids. Chromatographia 31:589-594
Zapata M, Ayala AM, Franco JM, Garrido JL (1987) Separation of chlorophylls and degradation products in marine phytoplankton by reversed-phase high-performance liquid chromatography. Chromatographia 23:26-30

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