ELSEVIER

Journal of Chromatography B, 781 (2002) 393–418

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Carotenoids: separation methods applicable to biological samples

Qing Su[a,*], Kevin G. Rowley[b], Nicholas D.H. Balazs[a]

[a]*Biochemistry Unit, Southern Cross Pathology Australia, Monash Medical Centre, Locked Bag 29, Clayton, Melbourne, VIC 3168, Australia*

[b]*Department of Medicine, University of Melbourne, St Vincent's Hospital, Princes Street, Fitzroy, VIC 3065, Australia*

Abstract

Epidemiologic and clinical studies have shown that a high intake of vegetables and fruit, with consequently high intakes and circulating concentrations of carotenoids, is associated with reduced risk of cardiovascular and other chronic diseases. The antioxidant properties of carotenoids are thought to contribute to these effects. The analysis of carotenoids in plasma, foods and tissues has thus become of interest in studies examining the role of diet in chronic disease prevention and management. High-performance liquid chromatography with ultra-violet or photodiode array detection is most often employed in routine use. We review these and other current methods for carotenoid analysis and information on sample stability relevant to epidemiological studies. The carotenoids remain an important and intriguing subject of study, with relevance to prevention of several important ''lifestyle-related'' diseases. Research into their physiological functions and their use as dietary markers requires sensitive, accurate and precise measurement. Further advances in these methodological areas will contribute to basic, clinical and public health research into the significance of carotenoid compounds in disease prevention.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Carotenoids

Contents

1. Introduction 394
   1.1. Epidemiological and clinical studies of carotenoids 394
   1.2. Chemical structure, function and distribution of carotenoids 396
      1.2.1. Structure and function 396
      1.2.2. Carotenoids in human blood and tissues 396
         1.2.2.1. Lutein and zeaxanthin 397
         1.2.2.2. Lycopene 397
         1.2.2.3. β-Cryptoxanthin 397
         1.2.2.4. β- And α-carotene 397
2. Methods of analysis 398
   2.1. Conventional chromatography 398

*Corresponding author. Tel.: +61-3-9594-4516; fax: +61-3-9594-4517.
E-mail address: q.su@southernhealth.org.au (Q. Su).

1570-0232/02/$ – see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S1570-0232(02)00502-0

2.2. Capillary electrochromatography (CEC) ... 398
2.3. High-performance liquid chromatography (HPLC) ... 398
2.3.1. Analytical methods using HPLC ... 398
2.3.1.1. UV–visible absorbance ... 398
2.3.1.2. Electrochemical array detection (ED) ... 399
2.3.1.3. Thermal lens spectrometry (TLS) ... 399
2.3.1.4. Mass spectroscopy (MS) ... 399
2.3.1.5. Nuclear magnetic resonance (NMR) ... 399
2.3.1.6. Resonance Raman excitation spectroscopy ... 400
2.3.2. HPLC methods for measurement of carotenoids in plasma or serum ... 400
2.3.2.1. Extraction of retinol, tocopherols and carotenoids from plasma and serum ... 400
2.3.3. HPLC methods for measurement of carotenoids in food ... 402
2.3.3.1. Extraction methods ... 405
2.3.3.2. Columns and mobile phase ... 406
2.3.4. HPLC methods for detection of carotenoids in tissues ... 406
2.3.4.1. Extraction of carotenoids from tissues ... 406
3. Stability of carotenoids during sample handling ... 406
3.1. Problems with carotenoid assay ... 406
3.2. Standardization of carotenoid analysis ... 408
3.3. Photosensitivity and stability of carotenoids in plasma and after extraction ... 409
3.4. Stability of carotenoids in whole blood ... 409
3.5. Stability of carotenoids in frozen serum or plasma ... 410
3.6. Summary of sample handling requirements ... 411
3.7. Effects of food processing on carotenoid content and bioavailability ... 411
4. Biological relevance of analytical results ... 412
4.1. Comparison of plasma antioxidant status across populations ... 412
5. Nomenclature ... 413
References ... 413

1. Introduction

1.1. Epidemiological and clinical studies of carotenoids

Epidemiologic studies have shown that a high vegetable intake is associated with reduced risk of chronic degenerative disease, such as cancer [1,2], cardiovascular disease [3–7], and age-related eye disease [8–10]. Reactive oxygen species and other free radicals are thought to be important in the aetiology of such diseases. Vegetables are a major source of carotenoids and, because of their anti-oxidant properties, these compounds are thought to contribute to the beneficial effects of vegetable consumption [11–14]. This hypothesis is consistent with studies examining chronic disease risk in relation to dietary carotenoid intake [15–19], although not all studies have found such associations [20,21]. Consistent with these observations, inverse associations across populations between plasma concentrations of carotenoids and cardiovascular disease or cancer mortality have been reported [7,12,13].

Population-based cohort studies have identified inverse associations of plasma carotenoids with risk of chronic disease [22–24]. Case-control studies have demonstrated similar inverse associations of plasma carotenoid levels with chronic disease risk. Table 1 summarizes a number of such studies, including two longitudinal studies of cardiovascular events, one of which found an inverse association of risk with certain carotenoids [25] and one of which reported no association [26]. A population-based nested case-control study from the European Anti-oxidant Myocardial Infarction and Breast Cancer (EURAMIC) study showed that increased adipose tissue lycopene levels were associated with lower incidence of myocardial infarction [27]. Other studies found low plasma levels of β-carotene to be associated with risk of cardiovascular events [23,25,28] or cancer [31–33]. Brennan and co-workers [34] have examined the role of dietary patterns

Table 1
Case-control studies of circulating carotenoids in relation to chronic disease risk

Study and reference	Location	n, case/control	Study finding
Street et al. (1994) [25]	USA	123/123	Low carotenoids predictive of subsequent myocardial infarction among smokers
Evans et al. (1998) [26]	USA	240/480	No significant association of "total carotenoids" with subsequent fatal or non-fatal CHD in high-risk men
Hsueh et al. (1998) [27]	Taiwan	74/193	Lower α- and β-carotene in arsenic-associated ischaemic heart disease
Kontush et al. (1999) [28]	Germany	34/40	α- And β-carotene lower in the presence of CHD
Kardinaal et al. (1993) [29]	10 European countries	683/727	Adipose tissue β-carotene inversely associated with risk of myocardial infarction in smokers
Kohlmeier et al. (1997) [30]	10 European countries	662/717	Adipose tissue lycopene inversely associated with myocardial infarction
Malvy et al. (1993) [31]	France	418/632	β-Carotene inversely associated with cancer risk in children
Torun et al. (1995) [32]	Turkey	208/156	β-Carotene lower in newly diagnosed cancer cases
Nagata et al. (1999) [33]	Japan	156/156	α-Carotene and lycopene inversely associated with risk of cervical dysplasia

and specific dietary nutrients in the etiology of lung cancer among non-smokers, using a multicenter case-control approach. Protective effects against lung cancer were observed for high consumption of tomatoes, lettuce, carrots, margarine and cheese. Only weak protective effects were observed for high consumption of all carotenoids, β-carotene and retinol. Protective effects for high levels of fruit consumption were restricted to squamous cell carcinoma and small cell carcinoma, and were not apparent for adenocarcinoma. Low plasma carotenoid levels have been reported in the presence of angina pectoris [35,36], diabetes [37] and intimal-medial thickening of the carotid artery [38], but not age-related maculopathy [39].

Thus a number of observational, cohort and case-control studies provide consistent data suggesting that consumption of foods rich in antioxidant vitamins and carotenoids may reduce the risk for cardiovascular disease and certain cancers. However, several large-scale randomized controlled trials of vitamin E and/or β-carotene supplementation for the prevention of cardiovascular events and cancer have largely proven supplementation to be ineffective [40–45]. The β-carotene arm of at least one of these studies resulted in an increased risk of lung cancer among smokers. In contrast, several studies using antioxidant-rich diets have shown large benefits in reducing the risk of cardiac events [46,47]. These studies imply that α-tocopherol or β-carotene, in isolation, are less important than the food sources from which they are derived, and that it may be their interactions with other phytochemicals such as the co-ingested carotenoids that are more relevant. Hence studies of the role of nutrition in chronic disease need to consider a wider range of antioxidant molecules. Several recent review papers [48–51] concluded that further studies are necessary to define the populations that can benefit from carotenoids and to define the proper doses, lengths of treatment, and whether mixtures, rather than single carotenoids, are more advantageous. The intervention studies cited above have been carried out in generally well-nourished populations, and it is possible that greater gains may be made in populations with more marginal nutritional status. Despite the lack of a clear

demonstrated benefit, there has been a rapid increase in the consumption of these micronutrient supplements.

1.2. Chemical structure, function and distribution of carotenoids

1.2.1. Structure and function

Carotenoids are naturally occurring colored compounds that are abundant as pigments in plants. Between 500 and 600 specific carotenoids have been identified. They have been identified in photosynthetic and non-photosynthetic organisms: in plants, algae, fungi, bacteria, and in at least one species of animal [52]. However, a far smaller number of carotenoids are found in human blood and tissues, the major ones being α-carotene, β-carotene, lutein, zeaxanthin, lycopene and cryptoxanthin (Fig. 1; for structures of other carotenoids and their isomers, see Refs. [52–54]). Carotenoids have the capacity to trap not only lipid peroxyl radicals, but also singlet oxygen species [55]. The essential role of carotenoids as a major dietary source of vitamin A has been known for many years. Although all carotenoids contain extensive conjugated double bonds (see Fig. 1), individual carotenoids differ in their antioxidant potential in humans [56]. Carotenoids also have remarkable effects on the immune response and intercellular communication [57–59]. Some have no measurable antioxidant potential in vitro. The true antioxidant capacity of the most prevalent carotenoids in vivo is still in question.

1.2.2. Carotenoids in human blood and tissues

The hydrocarbon carotenoids α- and β-carotene and lycopene, the monohydroxy xanthophyll, β-cryptoxanthin, and the dihydroxy xanthophylls, lutein and zeaxanthin, are among the main carotenoids present in human plasma and tissue [60]. During normal dietary intake, the hydrocarbon carotenoids (mainly β-carotene and lycopene) and the oxy-carotenoids (mainly lutein) each account for about half of the total plasma carotenoids, with a total serum carotenoid concentration of about 1–2 μmol/L. This apparent difference in carotenoid profile between food products and biologic matrices (such as plasma and tissues) is suggestive of selective uptake and/or differences in metabolism between carotenoids [61].

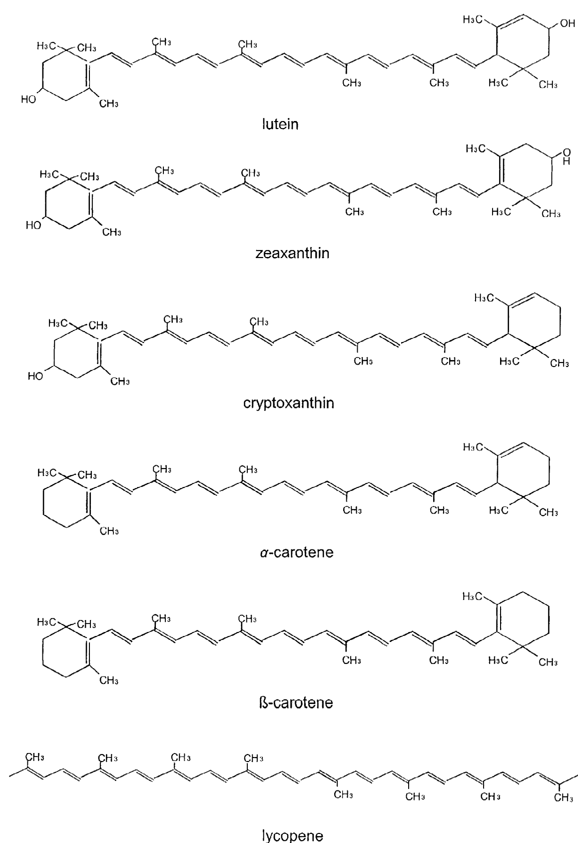

Fig. 1. Chemical structures of major carotenoids appearing in the human circulation.

The carotenoids of fruit, vegetables and animal products are usually fat soluble and are associated with lipid fractions. They may also be esterified or complexed with protein [62]. During proteolytic digestion, carotenoids are released from associated proteins and aggregate with other lipids. In humans, it has been reported that between 5 and 50% of carotenoids are absorbed [63]. Absorption efficiency of carotenoids is known to be affected by the presence or absence of other components in the diet such as dietary fat and protein [64,65] and by bile salts [66]. Carotenoids in foods are usually less well absorbed from the intestine than is preformed vitamin A [67]. As the amount of carotenoids in the diet

increases, the absorption efficiency decreases [68]. After absorption, provitamin A carotenoids are cleaved in the mucosal cells to form retinal, which is then reduced to retinol. Some unconverted carotenoids are directly absorbed and pass into the blood where their composition reflects the diet. They may be deposited in the liver or elsewhere, such as fat depots and various organs [69].

1.2.2.1. Lutein and zeaxanthin. These xanthophylls exist in the retina, plasma and other tissues. In the retina, they function to protect photoreceptor cells from light-generated oxygen radicals, and may thus play a key role in preventing advanced macular degeneration [70–74]. Lutein possesses chemopreventive activity, induces gap junction communication between cells and inhibits lipid peroxidation in vitro more effectively than do carotenes and lycopene [75]. Lutein and zeaxanthin are also prevalent in ovaries and adipocyte tissue. Lutein occurs in high concentrations in green leafy vegetables and also in mango, papaya, oranges, kiwi fruit, peaches, squash, peas, lima beans, green beans, broccoli, brussel sprouts, cabbage, kale, lettuce, prunes, pumpkin, sweet potatoes and honeydew melon. Commercial sources are obtained from the extraction of marigold petals. Lutein does not possess provitamin A activity. Lutein and zeaxanthin also have protective effects against lipid peroxidation [76,77].

1.2.2.2. Lycopene. The all-*trans* isomer of this carotenoid is typically quantified in serum, although signals for the 9-, 13- and 15-*cis* isomers are detectable and account for as much as 50% of total lycopenes [78]. In experiments performed in vitro, lycopene quenched singlet oxygen more efficiently than α-carotene, β-carotene, zeaxanthin, lutein and cryptoxanthin [79]. Serum levels of lycopene have been inversely related to the risk of cancer of the bladder, pancreas and digestive tract [80,81]. In the study of Goodman and coworkers [82] involving 147 confirmed cervical cancer patients and 191 noncancerous subjects, lycopene was found to be significantly lower in the cancer patients. This carotenoid has been identified in various tissues: thyroid, kidney, adrenal, spleen, liver, heart, testis, adipose tissue and pancreas [83,84]. The red color of fruits and vegetables such as tomatoes, pink grapefruit, the skin of red grapes, watermelon and red guavas is due to lycopene. Other dietary sources include papaya and apricots. Lycopene is not converted to retinol in vivo. It has attracted attention due to its biological and physicochemical properties, especially related to its effects as a natural antioxidant. Tomatoes and related tomato products are the major source of lycopene compounds, and are also considered an important source of other carotenoids in the human diet. Lycopene in fresh tomato fruits occurs essentially in the all-*trans* configuration. Lycopene bioavailability in processed tomato products is higher than in unprocessed fresh tomatoes [53]. Food processing would improve lycopene bioavailability by breaking down cell walls, which weakens the bonding forces between lycopene and the fruit matrix.

1.2.2.3. β-Cryptoxanthin. β-Cryptoxanthin is capable of quenching singlet oxygen [79]. It occurs in oranges, mango, papaya, cantaloupe, peaches, prunes, squash, and is used to color butter [85,86]. β-Cryptoxanthin exhibits provitamin A activity. Wingerath et al. [87] studied the uptake of β-cryptoxanthin after ingestion of tangerine juice and found increasing amounts of β-cryptoxanthin in chylomicrons and serum. Concentrations in liver are four times greater than in serum [88].

1.2.2.4. β- And α-carotene. Six times as much β-carotene as α-carotene is found in the body. A study by Dugas et al. [89] showed that β-carotene appears to function as an antioxidant in protecting LDL from cell-mediated oxidation. The three- to six-fold enrichment of LDL with β-carotene achieved by dietary supplementation was more effective in inhibiting oxidation than were the 11- to 12-fold enrichment achieved in vitro. A study by Kontush and co-workers [28] found that α-carotene and γ-tocopherol were significantly lower in plasma of CHD patients compared to controls. Carrots are the only rich source of α-carotene. β-Carotene and α-carotene have been identified in tissues of the thyroid, kidney, spleen, liver, heart, pancreas, adipose tissue, ovary, adrenal gland and mucosal cells [27,78,83,90,91].

2. Methods of analysis

2.1. Conventional chromatography

Open-column and thin-layer chromatography methods were originally used for analysis of carotenoids [92]. Historically, much of the ''carotene'' data in tables of food composition has been obtained by measuring absorption at a specified wavelength and quantified against a β-carotene standard; or, more usually, by open-column chromatography to separate carotenoid pigments, which are then quantified spectrophotometrically. Open-column and thin-layer chromatography methods need large amounts of sample and there are difficulties with total recovery of the carotenoids from TLC plates [93,94]. Compared to more modern methods, resolution and assay speed are poor. Research into the structure of carotenoids has been greatly advanced by the rapid development of spectroscopic techniques, nuclear magnetic resonance (NMR) [95] and mass spectrometry (MS), in particular [96–100]. The development of high-performance liquid chromatography (HPLC) has greatly improved the isolation and quantification of carotenoids [101–104].

2.2. Capillary electrochromatography (CEC)

Interest in capillary electrochromatography continues to grow at a rapid pace as refinements in the technique are reported. The motivation for this interest is the promise of high separation efficiencies comparable to those obtained with gas chromatography or capillary electrophoresis. Sander et al. [105] first reported the use of polymeric C_{30} stationary phases in CEC for the separation of carotenoid isomers in baby food, and *Dunaliella* algae. The polymeric C_{30} stationary phase was prepared on either Rainin or ProntoSIL 30 nm pore size, 3 μm silica particles [105,106]. Isocratic CEC separations were carried out using a Hewlett-Packard 3D capillary electrophoresis instrument with vial pressurization modification. Absorbance detection at 450 nm was employed with acetone and 1 m*M* borate buffer as mobile phase. This method was able to separate lycopene isomers, β-carotene isomers, α-carotene isomers, lutein isomers, zeaxanthin isomers and β-cryptoxanthin isomers in food samples (including: creamed corn, tomatoes, beef, orange juice, papaya juice, creamed spinach and powdered infant formula) over a run time of 35 min. Although peak area reproducibility was not assessed, retention time reproducibility was typically 5% relative standard deviation. Separation efficiency for lycopene isomers was lower compared to other carotenoid isomers, probably because of reduced electro-osmotic flow with the mobile phase composition (acetone–buffer, 99:1, v/v) [105]. The combination of high efficiency and high selectivity, enabling better separation of carotenoid mixtures, was demonstrated. Roed et al. [107] developed a CEC method using a C_{30} column for retinyl ester analysis. The separation and quantification of retinyl palmitate, heptadecanoate, stearate, oleoate and linoleoate in liver extracts of Arctic seals was completed in less than 30 min using 30 cm columns.

2.3. High-performance liquid chromatography (HPLC)

HPLC offers significant advantages in terms of simplicity, speed, cost (depending on detection method), sensitivity, specificity, precision and sample preservation. HPLC methods use isocratic or gradient mobile phases in either reversed-phase or normal-phase mode [93,108–113]. Since carotenoids are easily oxidized, it is useful to add antioxidants such as butylated hydroxytoluene (BHT) to the extraction solvent and mobile phase and to keep the column temperature low and constant [114,115]. Infrared spectroscopy gives information on the nature of the bonds and atoms in the analysed compound, while nuclear magnetic resonance (NMR) of proton and carbon permits the assigning of these atoms to a specific structure [116,117]. HPLC coupled to mass spectrometry (MS) has been used in several studies to identify carotenoids from biological samples [54,97,111,118,119].

2.3.1. Analytical methods using HPLC

2.3.1.1. UV–visible absorbance. Standard ultraviolet (UV) and visible single wavelength absorbance detectors have been used for many years as components of HPLC systems for quantitation of carotenoids. UV absorbance detection allows sufficient

sensitivity for many routine purposes. Coupling a photodiode array (PDA) detector to the HPLC allows for a continuous collection of spectrophotometric data during analysis [120], which greatly aids determination of peak purity and identification of unknown compounds in some cases. However, the complexity of the carotenoid profile in many biological samples requires more sophisticated methods for definitive peak identification.

2.3.1.2. Electrochemical array detection (ED). Electrochemical array detection has been successfully applied to significantly increase sensitivity for both hydrocarbon (β-carotene and α-carotene) and oxygenated (lutein and zeaxanthin) carotenoids [121–123]. A method for measuring the lipophilic antioxidants α-tocopherol, γ-tocopherol, ubiquinol-10, ubiquinone-10, β-cryptoxanthin, and β-carotene in 5 or 10 μl neonatal plasma samples was developed by Finckh et al. [121]. This highly sensitive method enabled routine use of capillary microsamples and avoided long HPLC runs, while enabling measurement of the most important lipophilic antioxidants. Recently, Ferruzzi and colleagues [124,125] described two C_{30} HPLC–ED methods optimized for the separation of *trans*-lycopene and several of its *cis* isomers. Separation of 13 lycopene isomers, including prolycopene (a novel tetra-*cis*-lycopene found in tangerine tomatoes), was accomplished with both isocratic and gradient methods using different proportions of methanol, methyl *tert*.-butyl ether, water and 1 *M* ammonium acetate buffer. The method was successfully applied in the analysis of small quantities of plasma, buccal mucosal cells, prostate and cervical tissues. Limits of detection for *trans*-lycopene by ED were found to be 50 fmol, representing a 10–100-fold increase over conventional UV–Vis absorbance methods. The methods take into account several distinctive chromatographic and physicochemical properties of lycopene compared to other common dietary carotenoids. Separation of these analytes was achieved in less than 30 min. Reproducibility was not reported.

2.3.1.3. Thermal lens spectrometry (TLS). TLS, one of the laser photothermal detection methods, has proven to be an ultra-sensitive detection method, successfully applied in biomedical research [126]. TLS was applied to the detection of carotenoids in plasma [127,128] and fish oil [129]. This combined HPLC–TLS method was validated by comparison with HPLC–UV–Vis analysis of plasma under identical chromatographic conditions. Detection limits for plasma samples were 70, 85, 100 and 120 pg/ml, respectively, for cryptoxanthin, α-carotene, β-carotene and lycopene [127]. This represents a 100-fold improvement in sensitivity compared to HPLC analysis with UV detection.

2.3.1.4. Mass spectroscopy (MS). HPLC–MS systems have a number of advantages over UV detection: sample quantity required for analysis is very small, with limits of detection around 500 fmol for individual carotenoids [130]. LC–MS provides information on carotenoid molecular mass, and fragmentation patterns allow determination of the carotenoid structure. Much effort has been devoted to developing HPLC–MS methods with mainly atmospheric pressure ionization interfaces (APCI) or electrospray ionization interfaces (ESI) [130–132]. Dachtler et al. [133] have reported that, using HPLC–MS with atmospheric pressure chemical ionization, the lutein stereoisomers can be distinguished from zeaxanthin stereoisomers in the upper picogram range within one chromatographic run. Recently, the matrix-assisted laser desorption ionization technique was used and its detection limits are in the femtomolar–attomolar range [132,134]. LC–MS has been used in several studies to identify carotenoids from biological samples [54,111,136].

2.3.1.5. Nuclear magnetic resonance (NMR). The identification and determination of unknown carotenoid stereoisomers from biological samples is possible by using coupled HPLC–MS and HPLC–NMR. Dachtler et al. [133] have reported using HPLC–MS with atmospheric pressure chemical ionization, whereas HPLC–NMR coupling allows the unequivocal identification of each stereoisomer with a concentration in the upper nanogram range. Strohschein et al. [95] have shown that with the 3-μm C_{30} stationary phase a better separation efficiency for the different β-carotene *cis*/*trans* isomers can be achieved, resulting in sharper peaks and

better resolution, which greatly facilitated the structure determination of the carotenoid isomers. Measurement of NMR spectra requires a very pure compound in higher amounts than for UV–visible and MS detection. The isolation of carotenoids usually employs column chromatography (CC) or thin-layer chromatography (TLC), while the final purification step is carried out by HPLC.

2.3.1.6. Resonance Raman excitation spectroscopy. Resonance Raman excitation spectroscopy has permitted the study of carotenoids in biological systems. Ruban and coworkers [137] first undertook a systematic search for the characteristic features of isolated carotenoids that are found in the photosynthetic membrane. They have obtained for the first time the resonance Raman spectra of violaxanthin and zeaxanthin in vivo. Their work established a new approach to the study of complex carotenoid-containing systems and offers a broad range of applications, from identification and assessment of xanthophyll configuration in reconstituted/isolated complexes to in vivo investigation in order to establish their role in photoprotective mechanisms.

2.3.2. HPLC methods for measurement of carotenoids in plasma or serum

Table 2 summarizes a number of published HPLC methods for quantitation of carotenoids in plasma or serum. The list is not exhaustive and includes only selected methods that were well characterized with respect to precision, recovery and detection limits. Most published methods use reversed-phase HPLC to separate major carotenoids, but do not resolve their geometric isomers [92,146,147]. Some workers have focussed on the separation of just β-carotene isomers [148] or isomers or both α- and β-carotene [90,143]. Hess and coworkers [138] and Sowell et al. [141] separated retinol, tocopherols, α-carotene, β-carotene, lycopene, β-cryptoxanthin and some of their isomers in human serum, and measured them with both UV absorbance and fluorescence detection. However, they were unable to resolve lutein and zeaxanthin.

Lycopene in serum is reported to exist as several different isomers. Use of C_{30} NIST silicon phase columns, selected solvent systems and electrochemical array detection resolve or partially resolve these isomers [124]. However, most routine HPLC methods do not separate lycopene isomers (Table 2).

For routine assessment of vitamin A activity and for characterization of retinoids and carotenoids in biological samples, HPLC is clearly the best analytical technique. For clinical purposes and large epidemiological studies using human plasma, reversed-phase HPLC with UV detection using C_{18} columns is an optimal approach. It is quick, simple, relatively cheap, shows good recovery (90–105%), low limits of detection (<10 μg/L) and has good reproducibility (inter-assay C.V. <10%). For foods and complex biological samples, HPLC–ED, HPLC–MS and HPLC–NMR using C_{30} columns may be the better techniques. They would be able to separate, identify and quantitate unknown carotenoids and their complex isomers in biological samples.

2.3.2.1. Extraction of retinol, tocopherols and carotenoids from plasma and serum. The steps required for carotenoid studies are extraction, separation, identification, and quantitation. Carotenoids are usually soluble in lipids or in non-polar solvents, except when they exist as complexes with proteins and sugars. The review paper by Wyss [150] describes in detail the extraction methods for biological fluids and tissues. In general, extraction must be carried out very quickly, avoiding exposure to light, oxygen and high temperatures in order to minimize degradation [151,54]. Most extraction methods for carotenoids in plasma and serum use hexane as solvent [85,103,113,152–156]. Generally, the process includes precipitation of protein materials with a polar solvent (usually ethanol containing an internal standard) and extraction of retinol, tocopherols and carotenoids into a non-polar solvent (usually hexane). Since carotenoids are light-sensitive compounds, all experimental procedures should be carried out in a darkened room under red lighting. We have found that optimal recovery is obtained when the extracted residue was first reconstituted in 30 μL of chloroform, vortexed for 40 s, then diluted in 70 μL of acetonitrile–methanol (1:1; mobile phase) solution and vortexed again for 40 s. Under these conditions, the recovery for the individual carotenoids ranged from 90 to 102%.

Table 2
Selected HPLC methods for analysis of carotenoids in plasma and serum

Ref.	Method	Mobile phase	Column	Detector	Analysis	C.V. (%)	LOD	Recovery (%)	External QC e.g. [149]
[138]	Isocratic	CH_3CN $CHCl_3$ CH_3OH	C_{18} (Nova Pak)	UV	α-Carotene β-carotene β-cryptoxanthin lycopene	11.8 10.4 11.6 12.7	– – – –	– – – –	– – – –
[139]	Isocratic	CH_3CN THF CH_3OH	ODS (Ultrasphere)	UV	α-Carotene β-carotene β-cryptoxanthin lycopene	10.0 9.3 5.8 6.6	10 μg/L 10 μg/L 10 μg/L 5 μg/L	100 101 95 97	– – – –
[92]	Isocratic	CH_3CN THF CH_3OH	ODS (Zorbax)	UV	α-Carotene β-carotene lutein cryptoxanthin lycopene	7.4 7.6 10.4 7.9 6.4	15 μg/L 12 μg/L 4.0 μg/L 8.0 μg/L 23 μg/L	– 77 84 – –	– – – – –
[140]	Isocratic	CH_3CN THF CH_3OH	C_{18} (Nucleosil)	UV	α-Carotene β-carotene lutein *trans*-lycopene	11.3 10.5 4.5 10.9	– – – –	96 101 99 99	– – – –
[141]	Isocratic	CH_3CN CH_3OH	ODS (Ultramex)	UV	α-Carotene β-carotene lutein/zeaxanthin β-cryptoxanthin lycopene	8.2 6.4 10.8 7.7 6.7	6.0 μg/L 6.5 μg/L 4.0 μg/L 5.5 μg/L 6.4 μg/L	– – – – –	Yes
[142]	Isocratic	CH_3OH CH_3CN THF	ODS1 (Nucleosil)	UV	Lutein, cryptoxanthin lycopene α-carotene β-carotene	6.9 7.3 9.0 7.6 7.6	0.74 μg/L 0.74 μg/L 1.48 μg/L 1.48 μg/L 1.48 μg/L	94 96 89 87	Yes
[143]	Gradient	CH_3OH CH_3CN H_2O	ODS1 (Spherisorb)	UV	β-Carotene lutein/zeaxanthin β-cryptoxanthin lycopene	3.4 12.3 6.5 8.0	7.0 μg/L 6.2 μg/L 7.0 μg/L 15 μg/L	100 – – –	Yes
[144]	Gradient	CH_3CN CH_3OH CH_2Cl_2	ODS (Supelcosil)	UV	α-Carotene β-carotene	8.5 5.2	3.5 μg/L 9.4 μg/L	96 95	– –
[103,229]	Gradient	CH_3CN CH_3OH $CHCl_3$ NH_4AC	S5-ODS2 (Spherisorb)	UV	α-Carotene β-carotene lutein/zeaxanthin β-cryptoxanthin *trans*-lycopene *cis*-lycopene	8.1 5.6 7.4 5.4 7.8 9.2	1.5 μg/L 1.5 μg/L 2.3 μg/L 2.0 μg/L 3.0 μg/L 3.3 μg/L	99 99 98 98 99 –	Yes
[145]	Gradient	C_6H_{14} CH_2Cl_2 Dioxane	C_{18} (Aluspher A1)	UV	Lycopene	6	2.6 μg/L	93	–

Table 2. Continued

Ref.	Method	Mobile phase	Column	Detector	Analysis	C.V. (%)	LOD	Recovery (%)	External QC e.g. [149]
[121]	Isocratic	LiCl	C_2/C_{18}	ED	Ubiquinol-10	3–14[a]	10.2 ng/L	105	–
		CH_3OH	Super Pac		Ubiquinone-10		12 ng/L	97	–
		C_2H_5OH	Pep-S RP		α-tocopherol		4–12 ng/L	103	–
					γ-tocopherol			105	–
					β-cryptoxanthin			105	–
					β-carotene			102	–
[124]	Gradient	CH_3OH	C_{30}	ED	13 lycopene	7.7	3 ng/L	90	–
		MTBE	(NIST)		isomers				
		NH_4AC							
[127]	Isocratic	CH_3OH	C_{18}	TLS	β-Carotene	5[a]	100 ng/L	–	–
		THF	(Vydac		α-carotene	5	85 ng/L	–	–
			218TP54)		lycopene	12	120 ng/L	–	–
					cryptoxanthin	5	70 ng/L	–	–
[129]	Isocratic	CH_3OH	C_{18}	TLS	β-Carotene	4.1	580 ng/L	101	–
		THF	(Vydac						
			218TP54)						
[133]	Isocratic	C_2H_4O	C_{30}	APCI-MS	Lutein, zeaxanthin	–	13 ng/L to	–	–
		H_2O	Silica	NMR	α-carotene	8.8	3 ng/L	–	–
					β-carotene	6.5		–	–
					lycopene	11.2		–	–
[130]	Gradient	C_2H_4O	C_{30}	ESI-MS	Lycopene isomers	–	–	–	–
		H_2O	Silica		β-carotene,	–	15 ng/L	–	–
					canthaxanthin	–	30 ng/L	–	–
[97]	Isocratic	CH_3CN	ODS	APCI-MS	Lutein isomers	1.8	12.5 ng/L	–	–
		CH_3OH	Silicon C_{18}		zeaxanthin	1.8	1.2 ng/L	–	–
		$CHCl_3$	Spherisorb C_8		β-carotene	3	15.1 ng/L	–	–
		Heptane	Nucleosil C_{18}		β-cryptoxanthin	2.4	8.7 ng/L	–	–
[119]	Isocratic	C_2H_4O	C_{18}	APCI-MS	α-Carotene	8.8	3 ng/mL	89	–
		CH_3OH	(ODS-5)		β-carotene	6.5	3 ng/mL	102	–
					lycopene	11.2	3 ng/mL	92	–

[a] Intra-assay C.V. (%).

2.3.3. HPLC methods for measurement of carotenoids in food

Carotenoids from fruits and vegetables or from mixed foods may be routinely assayed by HPLC with PDA detection. Other methods for food analysis are often subject to overestimation of vitamin A, because biologically inactive carotenoids are difficult to completely remove from the provitamin A carotenoid by the open-column chromatography employed in colorimetric or spectrophotometric methods [157]. Furthermore, such methods cannot clearly identify individual carotenoids. More recently, interest has been extended to the possible importance of naturally occurring carotenoids other than those normally quantified in blood. Scott [115] reported two main problem areas: (i) the reaction between carotenoids and metal surfaces with particular reference to the stainless steel frits in HPLC systems; and (ii) the reaction between carotenoids, injection solvents and the mobile phase. These may result in peak distortion and the production of artifacts. Carotenoid measurement in natural products involves extraction and chromatography with organic solvents, with the potential for loss during sampling, extraction and saponification. There are many HPLC methods reported in the literature (Table 3) which differ in their

Table 3
Selected HPLC methods for analysis of carotenoids in foods

Food	Analyte	Extraction	Column	Mobile phase	Detection	Ref.
Raw tomatoes	α-, β-Carotene, lycopene	C_2H_4O petroleum	ODS C_{18} Partisil PX5-5 (5 μm)	CH_3CN–$CHCl_3$	UV	[158]
Corn grain	Lutein, β-carotene, β-cryptoxanthin, zeaxanthin	C_2H_5OH, KOH, C_6H_{14} C_6H_6O (10:8)	C_{18} Ultrasphere-Si (5 μm)	C_3H_7OH–C_6H_{14}	UV	[159]
Palm-pressed fiber	α-, β-, γ-Carotene, lycopene, phytoene, tocopherols, tocotrienols	C_6H_{14}, $CHCl_3$ CO_2	ODS C_{18} Zorbax (5 μm)	CH_3CN–CH_2Cl_2	UV	[160]
Green vegetables	α-, β-, γ-Carotene, lutein, zeaxanthin β-cryptoxanthin, phytoene, phytofluene	THF	C_{18} Spherisorb-S (5 μm)	CH_3CN–CH_3OH –CH_2Cl_2	UV	[161]
Spice red pepper	Violaxanthin, lutein, β-cryptoxanthin, β-carotene, capsanthin, autheroxanthin, tocopherols, ascorbic acid	CH_2Cl_2–CH_3OH (6:1)	C_{18} Chromsil (6 μm)	CH_3CN–CH_3OH –C_3H_7OH	UV	[162]
Olive oil	Tocopherol, β-carotene	C_2H_5OH, KOH, C_6H_{14}; EtOAc	ODS-C_{18} Tracer Extrasil (5 μm)	CH_3OH–H_2O –C_4H_9OH	PDA	[163]
Tomato juice, paste, puree, sauce	α-, β-Carotene, lutein, lycopene, β-cryptoxanthin, phytoene, phytofluene	THF	C_{18} Spherical (5 μm)	CH_3CN–CH_3OH –C_3H_7OH	PDA	[164]
Tomato paste, juice	α-, β-Carotene, lycopene, β-cryptoxanthin	CH_2Cl_2	C_{18} Spherical (5 μm)	CH_3CN–CH_2CI_2 –C_6H_{14}	PDA	[165]
Tomato paste waste	Lycopene, β-carotene	SC-CO_2, KOH C_6H_{14}–C_2H_4O–C_2H_5OH–C_6H_6O	C_{18} Symmetry (5 μm)	CH_3OH–THF –H_2O	PDA	[166]
Green beans, vegetables, fruits, tomatoes	Lycopene, β-cryptoxanthin, α-, β-carotene, lutein, zeaxanthin isomers	THF	C_{18} Spherisorb (5 μm)	CH_3CN–CH_2CI_2 –C_6H_{14}	PDA	[85]
Rose mosqueta hips	Rubixanthin, zeaxanthin, β-cryptoxanthin, lycopene, gazaniaxanthin, β-carotene	$(CH_3CH_2)_2O$	ODS2-C_{18} Spherisorb (5 μm)	CH_3CN–CH_3OH –CH_2CI_2	PDA	[167]
Red paprika	Violaxanthin, α-, β-carotene, β-cryptoxanthin, lutein, capsanthin, autheroxanthin, antheraxanthin and several *cis* isomers	CH_3OH, $(CH_3CH_2)_2O$ KOH	C_{18} Chromsyl (6 μm)	CH_3CN–CH_3OH –H_2O	PDA	[168]
Wild green vegetables, figs, olive oil	β-Cryptoxanthin, lycopene, α-, β-carotene, lutein	THF, $CHCl_3$ –CH_3OH	ODS2-C_{18} Spherisorb (5 μm)	CH_3CN–CH_3OH –$CHCl_3$	PDA	[169]

Table 3. Continued

Food	Analyte	Extraction	Column	Mobile phase	Detection	Ref.
Processed green beans	Violaxanthin, lutein, neochrome, α-, β-carotene, chlorophyll and several isomers	C_2H_4O	C_{18} Lihrospher (5 μm)	$(CH_3CH_2)_2O$–CH_3OH –H_2O	PDA	[170]
Tomato products, vegetables, broccoli, carrot, kale	Lutein, α-, β-carotene, β-cryptoxanthin zeaxanthin	THF CH_3OH	Silica C_{18} Nucleosil C_{18} Techsphere ODS Spherisorb C_8 (3 μm, 5 μm)	CH_3CN–CH_2CI_2–C_6H_{14} CH_3OH	UV–MS	[97]
Milk	Lycopene, β-cryptoxanthin, α-, β-carotene, lutein, zeaxanthin isomers	PBS solution C_6H_{14}	C_{18} Spherical 5 μm	CH_3CN–CH_2Cl_2–C_6H_{14}–CH_3OH	UV–MS NMR	[111]
Gardenia fruits, saffron	Carotenoids-glycosyl esters	$(CH_3CH_2)_2O$	C_{18} Nitrile (5 μm)	CH_3OH–H_2O	UV–MS NMR	[173]
Annatto seeds	Lycopenoate isomers, methyl-8′-apo-β-carotene	Ethyl acetate t-BME	C_{18} Nucleosil (5 μm)	C_2H_4O–C_6H_{14} –H_2O ethyl acetate	LC–MS	[172]
Mango	β-Carotene, zeaxanthin, luteoxanthin isomers β-cryptoxanthin, violaxanthin, neoxanthin	C_2H_4O $(CH_3CH_2)_2O$	Spherisorb C_{18}	C_2H_4O–C_6H_{14}	UV-MS	[136]
Tomato processed by-product	Lycopene, α-tocopherol	CO_2, $CHCl_3$	C_{18} (2) Luna (3 μm)	CH_3OH– C_3H_7OH– ammonium acetate	ECD	[173]
Orange juice	α-, β-Cryptoxanthin, lutein, zeaxanthin isomers, auroxanthin, phytofluene, α-, β-, γ-carotene, etc.	$K_4Fe(CN)_6$ C_2H_4O	Silica C_{30} Spheres (3 μm)	t-BME– CH_3OH– H_2O	PDA	[174]
Guava	β-Carotene isomers, lycopene, β-cryptoxanthin, rubixanthin, lutein, neochrome, phytofluene	C_6H_{14} $(CH_3CH_2)_2O$	C_{18} Vydac C_{30} YMC	CH_3OH–t-BME	PDA MS NMR	[54]
Spinach	Lutein, zeaxanthin and β-carotene isomers	THF	Silica C_{30} Pronto Sil (3 μm)	C_2H_4O–H_2O	UV–MS HPLC–NMR	[133]
Tomato juice, infant food	Tocopherol isomers, canthaxanthin, echineone, lycopene, α-, β-carotene, zeaxanthin, lutein,	CH_2Cl_2	Silica C_{30} Pronto Sil (3 μm)	C_2H_4O–H_2O	UV–MS	[130]
Vegetable juice	Astaxanthin, zeaxanthin, canthaxanthin, echineone, lycopene, β-carotene	t-BME	Silica C_{30} (3 μm)	CH_3OH– t-BME	UV–MS	[98]
Orange, mandarin	Neochrome, α-, β-carotene, α-, β-cryptoxanthin, lutein, mutatoxanthin, antheroxanthin, unknown autheroxanthin	$ZnSO_4$ $K_4Fe(CN)_6$– C_2H_4O $(CH_3CH_2)_2O$	Silica C_{30} (5 μm)	t-BME–CH_3OH– H_2O	PDA	[175]

Table 3. Continued

Food	Analyte	Extraction	Column	Mobile phase	Detection	Ref.
Fresh and processed vegetables	α-Carotene isomers, β-carotene isomers, β-cryptoxanthin isomers	CH_3OH C_6H_{14}–C_2H_4O	C_{30} (NIST) (5 μm)	t-BME–CH_3OH	PDA	[176]
Orange juice	Valenciaxanthin, neochrome, α-, β-cryptoxanthin, lutein, mutatoxanthin A and B, antheraxanthin, trolichrom, neoxanthin, auroxanthin, leutoxanthin, phytofluene	$ZnSO_4$ $K_4Fe(CN)_6$ C_2H_4O petroleum ether $(CH_3CH_2)_2O$	C_{30} Silica (5 μm)	t-BME– CH_3OH–H_2O	PDA	[177]
Flowers	Neoxanthin, β-cryptoxanthin, lutein, α-, β-carotene, violaxanthin, violeoxanthin and several *cis* isomers	C_6H_{14}–C_2H_5OH –C_2H_4O– C_6H_6O (10:6:6:7), KOH	Silica C_{30} (5 μm)	CH_3OH–CH_2Cl_2 –C_3H_7OH	PDA	[178]

analytical conditions, making it difficult to choose the most appropriate technique. Careful selection of the mobile phase, column and extraction solvent is likely to improve resolution [170].

2.3.3.1. Extraction methods. Up to now, no single solvent has been found optimal for the extraction of all carotenoids from food samples. Carbon disulfide is the best solvent, but volatility, flammability, toxicity, and degradation limit its use [52]. Other solvents such as hexane, heptane, and isooctane are not as efficient for extraction, but possess other favorable characteristics. In addition, it must be considered that polar solvents (such as acetone, methanol, ethanol) are good for extraction of xanthophylls but not for carotenes. As a general rule, the extraction process consists of the removal of hydrophobic carotenoids from a hydrophilic medium. Thus, complete extraction was reported from samples with low moisture content, using slightly polar plus non-polar solvents [178]. For the isolation of carotenes, it is useful to extract the sample first with a highly polar solvent (e.g. methanol) that removes water and the xanthophylls partially, both of which are discarded, after which the carotenes may be extracted with a suitable, less-polar solvent.

Many methods are reported in the literature for the extraction of food samples (Table 3) using solvents such as acetone, petroleum ether, diethyl ether, tetrahydrofuran (THF), methanol, hexane and dichloromethane [85,111,171,179,180]. Most methods for the extraction of vegetables use THF [86,161,166,179,181]. We have found (Su et al., unpublished) that chloroform–methanol (2:1) extracted lycopene and β-carotene from tomatoes with greater efficiency than did THF. Compared to extraction with THF, chloroform–methanol extraction gave a four-fold greater value for lycopene content of tomatoes and a two-fold greater value for β-carotene. For extraction of lutein and β-carotene from green leafy vegetables, THF gave higher results than did chloroform–methanol (2:1).

In foods that contain a significant amount of water, it is desirable to use an organic solvent that is miscible with water in an attempt to facilitate the denaturing of carotenoid–protein complexes and to prevent the formation of emulsions [165]. Acetone and THF can be employed as extracting solvents. However, lycopene has a higher solubility in dichloromethane or chloroform and poor solubility in other organic solvents. Therefore, lycopene-rich foods, such as tomatoes, need to be extracted with a solvent containing chloroform or dichloromethane. The food matrix in which the carotenoids are held also affects extraction efficiency. There are large differences reported in the measurement of carotenoids in fruits and vegetables, such as lycopene in raw tomato (116–600 μg/100 g); β-carotene in pumpkin (490–20 000 μg/100 g), kale (2840–14 600 μg/100 g) and carrot (1830–14 700 μg/100 g); and cryptoxanthin in orange juice (14–489 μg/100 g) and fresh oranges (12–389 μg/100 g) [158,183–185]. These results indicate that polar and non-polar carotenoids have

different solubilities in different solvents and the choice of solvent is critical for food analysis. Specific solvents for optimal extraction of carotenoids from fruits and vegetables may be required for quantification of each carotenoid. The addition of sodium, magnesium or calcium carbonate (0.1 g/g sample) and antioxidants (BHT) is recommended to neutralize tissues containing acids and to avoid oxidation, respectively.

2.3.3.2. Columns and mobile phase. With reversed-phase HPLC, C_8 and C_{18} bonded phase columns have proved to be well suited for the solution of a myriad of separation problems. For the separation of naturally occurring carotenoids, as well as for carotenoid isomers, C_{30} bonded silica exhibits a higher selectivity than the conventionally used C_8 and C_{18} materials [133,156]. Since the initial introduction of the C_{30} stationary phase by Sander, their synthesis has been further improved and this C_{30} stationary phase is very useful for the analysis of plant extracts, biological tissues, and mixtures of geometric isomers [100,117,186]. C_{30} bonded phases allow baseline separation of carotenoid stereoisomers, even that of similar stereoisomers such as (9-*Z*)- and (9′-*Z*)-lutein [133]. Fig. 1 and Refs. [52–54,59] show the structure of the identified carotenoid stereoisomers of β-carotene, zeaxanthin, and lutein separated using HPLC–MS and a C_{30} column. Dachtler and colleagues [133] reported that C_{30} stationary phases, coupled to HPLC–NMR, combine two advantages. First, the increased selectivity of C_{30} versus C_{18} phases results in sharper peak shapes with higher sample concentrations, and, secondly, C_{30} phases have a higher sample loading capacity than C_{18} phases.

In our analyses of fruits and vegetables [169] we have used two different columns: Spherisorb ODS-2, 5 μm and Discovery C_{18}, 5 μm. Using the Spherisorb ODS-2 column, we could separate lycopene and carotene isomers. However, the Discovery C_{18} column was able to resolve lutein isomers better than did the Spherisorb ODS-2 column.

2.3.4. HPLC methods for detection of carotenoids in tissues

In 1997, Khachick et al. [101] reported the isolation and identification of several oxidation products of lutein and zeaxanthin in human and monkey retinas. Furthermore, Bernstein et al. [74] have demonstrated that the tissues of the uveal tract—the retinal pigment epithelium/choroids (RPE/choroids), the ciliary body, and the iris—are enriched in lutein, zeaxanthin, and their metabolites. Nierenberg and Nann [91] reported on tissue samples prepared using enzymatic digestion followed by mechanical homogenization; saponification was avoided and tocopherol nicotinate was used as an internal standard. This method is of interest for the quantitative assessment of carotenoids in solid tissue samples because it avoids the usual saponification step required in other reported techniques. Table 4 summarizes extraction and detection methods for the analysis of carotenoids in tissues.

2.3.4.1. Extraction of carotenoids from tissues. Delgado-Vargas et al. [52] in their recent review paper suggested that "if the tissue is previously dried, then water-immiscible solvents are used such as petroleum or ether; with fresh materials acetone or ethanol are used as solvents, which have two functions, extraction and dehydration. Solvents used in extraction must be pure (without oxygen, acids, halogens) to avoid degradation". Various homogenization and extraction methods have been reported in the literature (Table 4). For a further discussion of methods applicable to tissue samples, see the Wyss review [150].

3. Stability of carotenoids during sample handling

3.1. Problems with carotenoid assay

The carotenoids are extremely reactive and consequently unstable due to their long chain of conjugated double bonds (Fig. 1). Several precautions should be taken during isolation and chromatography, such as protection against light and oxygen, use of low temperature and antioxidants, with analysis in the shortest possible time. Scott [115] reported that using an acetonitrile–dichloromethane (ACN–DCM, 80:20, v/v) mobile phase with a Zorbax ODS column, although initially giving a lutein and zeaxanthin combined peak without complete separation, as the chromatography continued a considerable

Table 4
Selected HPLC methods for the analysis of carotenoids in tissues

Sample	Analyte	Procedure	Detector	Ref.
Human lung, skin, breast, colon	α-, β-Carotene, lutein, lycopene, β-cryptoxanthin, retinol, α-tocopherol	Homogenization of sample in phosphate-buffered saline, with or without detergent (no enzymatic digestion or saponification) saponification in 5% methanolic KOH	HPLC–UV	[91]
Human buccal mucosal cells	α-, β-Carotene, lutein, lycopene, β-cryptoxanthin, γ-, α-tocopherol, retinyl palmitate, retinol	Homogenization of sample in 1% protease solution, incubated 30 min, 1% sodium dodecyl sulfate in ethanol, hexane extracted	HPLC–UV	[206]
Rat colon mucosa	α-, β-Carotene, lycopene	Colon was cut open along its length with cold 0.9% NaOH, the distal colon placed in 4% neutral formalin solution	HPLC–ED	[123]
Monkey retinas	Lutein, zeaxanthin isomers and oxidation product	Homogenization of sample in THF containing 0.1% BHT, THF phase evaporated and dissolved in 10% sodium chloride and mixture of C_6H_{14}–CH_2Cl_2–C_2H_5OH (75:25:0.25)	HPLC–UV	[101]
Rat liver, spleen, brain, heart, intestine, lung, muscle, skin, eye, testis, adrenal, thyroid gland	Lycopene	Homogenization of sample in acetone containing 0.25% BHT, acetone phase evaporated and dissolved in ethanol–water extracted twice with hexane	HPLC–UV	[145]
Human prostate, cervical, buccal mucosal cells	γ-, α-, β-carotene, lycopene	Homogenization of sample in 1% protease solution, incubated 30 min, 1% sodium dodecyl sulfate in ethanol, hexane–acetone (1:1) extracted	HPLC–ED	[124]
Bovine retinas, chicken retinas	Lutein, zeaxanthin and isomers, γ-, α-, β-carotene	Homogenization of tissue sample in ethanol –water (1:1), extracted twice with hexane	HPLC–APCI-MS NMR	[135]
Bovine retinas, chicken retinas	lutein, zeaxanthin and isomers	Homogenization of tissue sample in ethanol –water (1:1), extracted twice with hexane	HPLC–UV	[135]
Human and rat liver, intestine, spleen, brain, heart, skeletal muscle, kidney, lung	Retinoids, tocopherols, lycopene, β-carotene	Homogenization of tissue sample in 2-propanol–CH_2Cl_2 (2:1) and kept under argon overnight	HPLC–UV	[187]
Free living gulls liver, breast muscle, heart, lung, brain, kidney, pancreas, abdominal fat	Lutein, zeaxanthin, canthaxanthin, echinenone, β-carotene, cryptoxanthin	Homogenization of sample in 5% NaCl with ethanol–water (1:1), extracted twice with hexane	HPLC–UV	[188]
Human macular anterior sclera, peripheral retina, ciliary body, iris, lens	Lutein, zeaxanthin and isomers, xanthophyll, lycopene, canthaxanthin, α-, β-carotene	Homogenization of sample in hexane containing 0.5% BHT, extracted twice with hexane	HPLC–UV	[73]
Human eyes, RPE/choroid, peripheral retina, ciliary body, iris, lens	Lutein, zeaxanthin and their metabolites, α-, β-cryptoxanthin, tocopherols, lycopene, γ-, α-, β-carotene	Homogenization of sample in methanol containing 0.1% BHT, extracted twice with 0.5% BHT in CH_2Cl_2–hexane (30:70)	HPLC–UV	[74]

reduction of the lutein response accompanied by peak distortion was observed. Separation and peak shape were only temporarily restored by washing the column with 100% methanol and re-equilibrating with mobile phase. They suggested that this deterioration could be resolved by including 5% methanol in the solvent mixture (ACN–DCM–MeOH, 75:20:5). Also, the concentration of a stock *trans*-lycopene standard in chloroform solution (stored at −20 °C) progressively decreased over a 20-day period by 48%, as measured by absorbance at 472 nm. Inclusion of 0.1% BHT reduced the loss to 11%. Froescheis et al. [145] reported that stock solutions of lycopene (3 mg of carotenoid together with 250 mg BHT in 20 ml DCM) stored in an amber flask at −20 °C were stable for at least 3 weeks with only minor isomerization observed. The contribution of *trans*-lycopene to the sum of all isomers declined by 2–3% within 4 weeks and the percentage of (*Z*) isomers increased by roughly the same amount. To check the stability of lycopene during the maximum analysis time of a run sequence, lycopene standards in a completely filled autosampler tray at room temperature were measured. The contribution of individual isomers to the total lycopene concentration changed with time. The decline of *trans*-lycopene in favor of (13*Z*)-lycopene was roughly 4–5% within 12 h. These results concur with the findings of Schierle et al. [189], who reported isomerization of the all-*E* to (13*Z*) isomers during analytical sample preparation. Details of lycopene bioactivity, bioavailability, degradation and stability have been reported by Shi and Le Maguer [53] using various analytical methods.

Ozone is a strong antimicrobial agent and commonly used in place of chlorine to increase the microbiological safety of foods [190]. The effect of ozone and oxygen on the degradation of carotenoids in an aqueous model system has been reported [191]: ''All-*trans*-β-carotene, 9-*cis*-β-carotene, β-cryptoxanthin and lycopene were adsorbed onto a C_{18} solid phase and exposed to a continuous flow of water saturated with oxygen or ozone at 30 °C. Approximately 90% of all-*trans*-β-carotene, 9-*cis*-β-carotene and β-cryptoxanthin were lost after exposure to ozone for 7 h. A similar loss of lycopene occurred in only 1 h. The degradation of all the carotenoids followed zero-order reaction kinetics with the following relative rates: lycopene>β-cryptoxanthin> all-*trans*-β-carotene>9-*cis*-β-carotene. The major degradation products of β-carotene were tentatively identified by LC–MS. Predominant isomers of β-carotene were 13-*cis*, 9-*cis* and a di-*cis* isomer.''

3.2. Standardization of carotenoid analysis

Scott et al. [192] reported on collaborative studies involving 17 European laboratories to assess the accuracy of HPLC procedures for the measurement of lutein, zeaxanthin, lycopene, α-carotene and β-carotene in a vegetable mix. The studies investigated possible problem areas, including chromatographic systems, standardization of carotenoid stock solutions, extraction procedures and data handing. The results suggested that the effect of the chromatographic system is probably not a major contributor to inter-laboratory variation, although some systems achieved a more discrete separation of carotenoid isomers than did others. In the more experienced laboratories, variation in standardization of the carotenoid solution was not thought to be a significant problem. However, there were greater variations for lycopene calibration and measurement.

In our laboratory, we have found β-carotene, α-carotene, lutein, zeaxanthin, and β-cryptoxanthin in hexane with 0.1% BHT stock solutions were stable for up to 10 days at −20 °C. Lycopene in hexane with 0.1% BHT stock solutions were stable for up to 3 days at −20 °C. We have previously published the characteristics of our HPLC–UV detection method for the routine analysis of lipid-soluble antioxidants in plasma [103]. The availability of a stable freeze-dried human serum matrix material, Standard Reference Material 968 [149,193,194], from the NIST in the USA should go a long way to improving the agreement of results of antioxidant vitamin and carotenoid assays. This material has certified concentration values for *trans*-retinol, δ-tocopherol, γ-tocopherol, α-tocopherol and total β-carotene. Reference concentration values which do not meet the stringent accuracy criteria for certified values are also available for *trans*-lutein, total lutein, total zeaxanthin, total β-cryptoxanthin, *trans*-α-carotene, total α-carotene, *trans*-lycopene and total lycopene.

3.3. Photosensitivity and stability of carotenoids in plasma and after extraction

We have previously reported that carotenoids, as measured by reversed-phase gradient HPLC, are stable in plasma exposed to laboratory lighting conditions for at least 24 h [103]. In plasma samples held at room temperature under fluorescent lighting for up to 72 h, we found no significant variation in measured concentrations of β-carotene, α-carotene, cryptoxanthin or *cis*-lycopene. However, the variation in the lutein+zeaxanthin concentration approached statistical significance ($P=0.054$) and the *trans*-lycopene concentration fell significantly over this period, with a 10% decrease after 48 h. This study examined the stability of small volumes (300 μL) in standard 2 mL polypropylene storage vials. Hence, the samples had a relatively large surface-area-to-volume ratio, and were exposed to greater oxidative stress than samples of a larger volume. The stability of carotenoids is probably enhanced by their interactions with proteins and lipids in plasma [59].

In contrast to the situation in plasma, carotenoids become unstable once extracted into organic solvents, particularly on exposure to light and heat. We have reported that, at −20 °C, there were statistically significant variations in the levels of β-carotene, α-carotene and lutein/zeaxanthin over time in samples extracted from plasma into hexane containing 0.01% BHT [103]. For β- and α-carotene, these changes were small in magnitude and random, and are likely to represent fluctuations due to methodological variability. For lutein/zeaxanthin, the results suggested a downward trend in measured levels after 24 h. Similar observations were made for extracts held at 4 °C. At room temperature, the variability in measurement, as indicated by a greater standard deviation, was greater than in samples kept cold. Thus, storage of extracts at room temperature is not recommended.

The study by Cavina and coworkers [195] determined the stability of individual carotenoids in extracted plasma left at room temperature for 0, 4, 12, and 24 h. They too observed no significant changes in the measured concentrations of retinol, tocopherol, or individual carotenoids. For lycopene, an apparent downward trend was observed between 0 and 4 h, however this was not statistically significant. They did not report the stability of extracted analytes for periods longer than 24 h.

3.4. Stability of carotenoids in whole blood

The stability of carotenoids in whole blood under various conditions is also of interest, as this is a common circumstance for samples collected as part of clinical and epidemiological studies. We have extended our previous observations of stability to cover a range of storage and handling conditions commonly encountered.

The stability of analytes during storage of whole blood was tested under three typical laboratory conditions: 4 °C in darkness, room temperature (19–22 °C) and elevated temperature (35 °C) exposed to light [196]. The last condition was chosen to represent field conditions in tropical areas. There were no significant changes in the measured concentrations of retinol, tocopherols and individual carotenoids in blood held at 4 °C in the dark, except for lycopene which was 8% lower after 24 h. At room temperature during exposure to light, there were no significant changes in the measured concentrations of analytes, but the reduction in lycopene again approached statistical significance. Retinol, γ-tocopherol and α-carotene appear to be stable when held at 35 °C and exposed to light for up to 72 h. However, α-tocopherol, β-cryptoxanthin, lutein/zeaxanthin, β-carotene and lycopene were all unstable at 35 °C in whole blood under these conditions. At 72 h, these analytes were 10, 11, 16, 15 and 22% lower than baseline, respectively. Hence (not surprisingly), storage at 35 °C is not recommended. However, collection in the field with storage at 4 °C and subsequent transfer within 24 h is a feasible operation that will not compromise results.

In the collection and processing of whole blood, Gross and coworker [197] showed that carotenoids were generally stable in samples that were (a) exposed to air and light, (b) exposed to air but protected from light, or (c) protected from air and light. Blood specimens were protected from air by flushing plasma aliquots with nitrogen within a few minutes of their removal from the collection tubes.

Protection from fluorescent light was by the use amber storage vials in a darkened room. The blood was centrifuged at 1500 *g* for 20 min in a cooled (4 °C) centrifuge, and aliquots of EDTA–plasma were placed in clear or amber glass 2-mL crimp-top vials. All samples were stored frozen at −70 °C for 8 weeks. Comparisons between carotenoids and α-tocopherol concentrations of samples collected and processed under the three sample processing procedures did not reveal any significant differences in concentration. Key and coworkers [198] also reported that storage of whole blood at 4 °C for 24 h before freezing does not result in significant changes in retinol, tocopherols and carotenoid concentrations.

In order to identify differences between plasma and serum in the measured concentration of lipid-soluble antioxidants, triplicate plasma and serum samples were prepared from each of four subjects and the antioxidant concentrations in plasma versus serum were compared using paired *t*-test. There were no significant differences in the measured concentrations of retinol, tocopherols, β-cryptoxanthin, β-carotene, α-carotene or lycopene between serum and plasma (Su et al., unpublished data).

3.5. Stability of carotenoids in frozen serum or plasma

By far the largest study relating to the loss of β-carotene during storage is that by Smith and Waller [199], who collected serum from 2073 persons. Specimens were frozen at −20 °C and analysed for β-carotene after storage ranging from 1 month to 2.75 years. For the first 6 months, there was no detectable loss. By 9 months, about 30% had been lost; this was followed by a subsequent slow decline in the serum β-carotene concentration of approximately 50 μg/L per year. Similarly, Comstock et al. [200] concluded that, in assaying β-carotene, and possibly other carotenoids as well, storing serum at −20 °C for more than 6 months is unwise. They periodically assayed aliquots of several large plasma pools [201] and reported that α-tocopherol, γ-tocopherol, β-cryptoxanthin, lutein/zeaxanthin, β-carotene, α-carotene and lycopene were all stable at −77 °C for 4 years. In other studies, plasma carotenoid levels were reported to be stable at −20 °C for 5 months [202]; Thomas et al. [203] observed losses of β-carotene of 10 mg/L per year in serum at −25 °C. Retinol, α-tocopherol, and β-carotene were less stable at −25 °C in frozen serum than they were in lyophilized serum. Ocke et al. [204,205] studied the effect of frozen storage on (pro)vitamin concentrations in 55 samples of EDTA–plasma and whole blood. The samples were analyzed before storage and after 3, 6, 12, 24, 36 and 48 months at −20 °C. Dramatic decreases occurred for the plasma concentration of vitamin E between 6 and 12 months, and for vitamin A, total carotenoids and β-carotene after 1 year. We have also found evidence of degradation of these compounds over 20 months stored at −20 or −30 °C: in a sample of pooled serum the concentrations of α-tocopherol, γ-tocopherol, β-cryptoxanthin, lutein/zeaxanthin, α-carotene, β-carotene and lycopene decreased by 13, 15, 31, 16, 30, 33 and 24%, respectively (Su et al., unpublished result).

Peng et al. [206] found that lutein, zeaxanthin, cryptoxanthin, lycopene, α-carotene, β-carotene, γ-tocopherol and α-tocopherol in plasma and buccal mucosal cells stored at −80 °C were stable for up to 8 months. At −70 °C, plasma carotenoid levels were reported to be stable for up to 28 months [202]. It has been reported that retinol, α-tocopherol and β-carotene concentrations in serum stored at −70 °C were not affected by two freeze–thaw cycles over a 1-week period [207]. Six to 10 freeze–thaw cycles over a 5–8-year period were reported to have no effect on retinol stored at −20 °C [208].

Retinol, α-tocopherol, *trans*-lycopene, and *trans*-β-carotene in reconstituted lyophilized serum stored at −20 °C were stable for at least 3 days with minimal (less than five) freeze–thaw cycles [203]. Long-term storage of lyophilized serum pools at room temperature, −25, −80 and 4 °C was studied at the National Institute of Standards and Technology (NIST) in 1985. These materials were analyzed periodically to determinate the stability of retinol, α-tocopherol, and β-carotene, which are part of the NIST-managed Micronutrients Measurement Quality Assurance Program [209], and to provide information regarding the potential stability and handing conditions for NIST Standard Reference Materials and other related samples [149,193,194]. They found that β-carotene levels were stable for up to 3 years,

and retinol and α-tocopherol appeared stable in lyophilized serum for at least 5 years at −80 or −25 °C. Retinal appeared stable in samples stored at 4 °C for at least 7 years, but α-tocopherol and, especially, β-carotene showed evidence of degradation after 3 years [203]. The concentration of retinol, α-tocopherol, and β-carotene in lyophilized serum stored at −80 °C has been monitored over 10 years [203] and there was no evidence of degradation of any of these compounds over the 10-year period.

3.6. Summary of sample handling requirements

The results of these studies indicate that, during storage of whole blood at room temperature (19–22 °C) for up to 72 h, lipid-soluble antioxidants are generally stable (with the possible exception of lycopene) and therefore the results of analyses can be considered suitable for inclusion in retrospective studies. However, storage of whole blood at higher temperature (35 °C) is not recommended due to degradation of these analytes. We found no significant difference between human serum and plasma in the measured concentration of retinol, tocopherols, or carotenoids. Thus, the analyses of carotenoid levels from human serum or plasma are likely to be comparable. In plasma exposed to laboratory lighting conditions, carotenoids were generally stable, with lycopene again the exception. However, once extracted, samples should be kept in the dark, cold and analysed within 24 h for optimal accuracy. For long-term storage, −20 to −30 °C does not appear to be suitable: storage at −70 °C or lower is necessary to preserve carotenoids in plasma or serum for extended periods.

3.7. Effects of food processing on carotenoid content and bioavailability

Lycopene in fresh tomato fruits occurs essentially in the *trans* configuration. The main causes of tomato lycopene degradation during processing are isomerization and oxidation. Heat induces isomerization of the *trans* to *cis* forms and *cis* isomers increase with temperature and processing time [53]. The bioavailability of *cis*-isomers in food is higher than that of all-*trans* isomers. Lycopene bioavailability in processed tomato products is higher than in unprocessed fresh tomatoes [210]. The composition and structure of the food also has an impact on the bioavailability of lycopene and may affect the release of lycopene from the tomato tissue matrix. Food processing may improve lycopene bioavailability by breaking down cell walls, which weakens the bonding forces between lycopene and the tissue matrix, thus making lycopene more accessible and enhancing the *cis* isomerization [53]. Lycopene is very stable in frozen and heat-sterilized foods, but not in dehydrated tomatoes [53].

We have found no significant difference in concentrations of lutein or β-carotene between vegetable soup and the fresh vegetables from which it was prepared (Su et al., unpublished data). Thus, lutein and β-carotene in vegetables appear to be stable during cooking. The study of the effects of various means of cooking on the levels of carotenoids in raw and cooked (microwaved, boiled, steamed, stewed) green vegetables and tomatoes by Khachik and coworkers [110] also showed that lutein, α-carotene, β-carotene, lycopene, phytofluene and phytoene were stable during the various heat treatments. However, carotenoid epoxides were sensitive to heat treatment [110,211].

Most studies have focused on the changes in the levels of all-*trans*-α- and β-carotene and subsequent losses in vitamin A activity due to thermal degradation and isomerization of these compounds [182]. Khachik and coworkers [85,110] examined the effect of various methods of cooking and processing on a number of yellow/orange vegetables. They reported that the destruction of the hydrocarbon carotenoids such as α- and β-carotene as a result of heat treatment is about 8–10%. In green beans subjected to various cooking conditions (microwave for 4 min; boiled for 9 min, boiled for 1 h), lutein, α- and β-carotene were quite heat resistant. After 1 h boiling of green beans, the levels of lutein, and α- and β-carotene remained unchanged [85], consistent with our finding for green leafy vegetables. Given the many variables that food preparation presents (temperature, duration, presence of oils, mechanical stirring) it is difficult to predict the extent of degradation of the various carotenoids and, if required, these data must be determined experimentally for each situation. In addition, the growing con-

ditions of the fruit and vegetables can materially affect their carotenoid content.

4. Biological relevance of analytical results

4.1. Comparison of plasma antioxidant status across populations

At the very least, carotenoids are markers of a dietary intake which confers protection against cardiovascular disease and probably certain cancers. As such, they are useful plasma markers of dietary quality and disease risk for use in epidemiological and clinical studies. As part of risk factor screening and health program evaluation, we have determined circulating carotenoid and antioxidant vitamin concentrations in several Australian population groups. Average levels for Australian Aboriginal people are shown in Table 5, together with comparable data from several other populations.

Aboriginal and Torres Strait Islander people in Australia die at a greater rate and at younger ages than the general Australian population. About 76% of deaths among indigenous males and 67% of deaths among indigenous females occur before age 65. Among non-indigenous people, by contrast, only 27% males and 16% females die before age 65 [212]. Indigenous Australians have higher rates of some CVD risk factors, including diabetes [213], and the leading cause of death in Australian Aboriginal people is CVD [214]. We have found circulating carotenoid concentrations among Aboriginal people to be very low compared to Anglo-Celtic Australians and other populations (Table 5) [220,226], as are the reported fruit and vegetable intakes [215,216]. Hence, dietary factors probably make a substantial contribution to the excess risk of premature mortality in these populations.

Greek migrants also have high rates of certain CVD risk factors. However, the CVD mortality of Greek migrants is substantially lower than that for Australian-born people [217], and much lower than that of indigenous Australians. Greek migrants living in Melbourne have largely maintained the traditional Mediterranean diet from the 1950s and 60s, the period during which the majority migrated to Australia [218,219]. This dietary regimen includes high

Table 5
Average concentrations of lipid-soluble antioxidants in Australian and other populations. Data are medians except for NHANES III, which are geometric means

	Australia Aboriginal people[a]	USA NHANES III[b]	Finland[c]	Japan[d]
Men				
β-Carotene	4	18	20	19
α-Carotene	2	5	3	6
Lycopene	11	23	6	16
β-Cryptoxanthin	2	8	7	20
Lutein + zeaxanthin	5	24	10	35
α-Tocopherol	980	–	1602	990
Retinol	50	–	56	83
Women				
β-Carotene	7	25	–	34
α-Carotene	2	6	–	9
Lycopene	9	21	–	27
β-Cryptoxanthin	3	9	–	33
Lutein + zeaxanthin	6	23	–	39
α-Tocopherol	1030	–	–	1040
Retinol	40	–	–	67

[a] Pre-intervention, 15 y+, Rowley et al. [215].
[b] Ford et al. [35].
[c] Male smokers, Albanes et al. [227].
[d] 7 y+, Ito et al. [228].

intakes of fresh vegetables and fruit, particularly green leafy vegetables, which is reflected in their relatively high plasma lutein, lycopene and cryptoxanthin concentrations [220,226]. Greek migrants also exhibited higher concentrations of several unidentified compounds eluting between lutein and cryptoxanthin in our assay [103]. These unidentified carotenoids may be oxidative metabolites of lutein, zeaxanthin and/or lycopene [86,101], or may be oxidized metabolites of other components in fruits and vegetables. In our analyses of several wild green leafy vegetables commonly consumed by Greek persons in Melbourne, we could not detect compounds with the same spectral properties as those unidentified compounds appearing in plasma, although a number of other unidentified carotenoid compounds were observed [169]. Specific roles for these compounds, and even for those carotenoids usually quantified, remain to be determined. They are potentially important mediators of the protective effects against cardiovascular disease of the traditional Mediterranean diet.

Evidence is emerging of a role for carotenoids in modulating physiological functions important in chronic disease risk. The current concept of vascular disease is that it arises from the interactions of oxidative stress, inflammation, dyslipidaemia and endothelial dysfunction. We have identified inverse relationships of certain carotenoids with markers of vascular damage and inflammation, including urinary albumin excretion [221], and positive relationships with paraoxonase activity, an enzyme thought to partly mediate the protective function of HDL cholesterol [222]. Other workers have reported inverse associations of carotenoids with markers of lipid and DNA oxidation [223]. Carotenoids may also have hypocholesterolaemic effects [224] and modulate cellular interactions. For example, lycopene has been shown to inhibit binding of monocytes to endothelial cells (a primary step in the atherosclerotic process [225]). Carotenoids may also modulate immune responses and intercellular communication [57–59].

The carotenoids thus remain an important and intriguing subject of study, with relevance to the prevention of several important ‘‘lifestyle-related’’ diseases. Research into their physiological functions and their use as dietary markers requires sensitive, accurate, precise and efficient measurement. Further advances in these methodological areas will contribute to basic, clinical and public health research projects dealing with prevention and management of chronic disease through nutritional intervention.

5. Nomenclature

ACN	acetonitrile
AmAC	ammonium acetate
BHT	butylated hydroxytoluene
t-BME	*tert*.-butyl methyl ether
CFM	chloroform
DCM	dichloromethane
MeOH	methanol
EtOH	ethanol
PrOH	propanol
BuOH	Butanol
AcO	acetone
HEX	hexane
TOL	Toluene
DEE	diethyl ether
CHD	coronary heart disease
CI 95%	95% confidence intervals
C.V.	coefficient of variation
CVD	cardiovascular disease
ED	coulometric electrochemical array detector
EtOAC	ethyl acetate
HPLC	high-performance liquid chromatography
MS	mass spectrometer
NIST	National Institute of Standards and Technology, USA
NMR	nuclear magnetic resonance
PTE	petroleum ether
PDA	photodiode array detector
PBS	phosphate-buffered saline
TEA	triethylamine
THF	tetrahydrofuran
UV	ultraviolet light

References

[1] W.C. Willett, D. Trichopoulos, Cancer Causes Control 7 (1996) 178.
[2] S. Franceschi, E. Bidoli, C. La Veccia, R. Talamini, B. D’Avanzo, E. Negri, Int. J. Cancer 59 (1994) 181.

[3] E.B. Rimm, A. Ascherio, E. Giovannucci, D. Spiegelman, M.J. Stampfer, W.C. Willett, J. Am. Med. Assoc. 275 (1996) 447.
[4] A.R. Ness, J.W. Powles, Int. J. Epidemiol. 26 (1997) 1.
[5] J.M. Gaziano, J.E. Manson, L.G. Branch, R.M. Russel, AEP 5 (1995) 255.
[6] L. Zhang, W.J. Blot, W.C. You, Y.S. Chang, X.Q. Liu, R.W. Kneller, L. Zhao, W.D. Liu, J.K. Li, M.L. Jin, Int. J. Cancer 56 (1994) 650.
[7] M.C. Bellizzi, M.F. Franklin, G.G. Duthie, W.P. James, Eur. J. Clin. Nutr. 48 (1994) 822.
[8] G.E. Bunce, J. Nutr. Biochem. 5 (1994) 66.
[9] J. Mares-Periman, W. Brady, R. Klein, G.J. Haus, M. Palta, L.L. Ritter, S.M. Shoff, Am. J. Epidemiol. 141 (1995) 322.
[10] J.M. Seddon, U.A. Ajani, R.D. Sperduto, R. Hiller, N. Blair, T.C. Burton, M.D. Farber, E.S. Gragoudas, J. Haller, D.T. Miller, L.A. Yannuzzi, W. Willett, J. Am. Med. Assoc. 272 (1994) 1413.
[11] H. Sies, W. Stahl, Am. J. Clin. Nutr. 62 (1995) 1315.
[12] K.F. Gey, P. Puska, U.K. Moser, Am. J. Clin. Nutr. 53 (1991) 326S.
[13] M. Kristenson, B. Zieden, Z. Kucinskiene, L.S. Elinder, B. Bergdahl, B. Elwing, A. Abaravicius, L. Razinkoviene, H. Calkauskas, A. Olson, Br. Med. J. 314 (1997) 629.
[14] M.R. Law, J.K. Morris, Eur. J. Clin. Nutr. 52 (1998) 549.
[15] P. Knekt, A. Reunanen, R. Jävinen, R. Seppänen, M. Heliövaara, A. Aromaa, Am. J. Epidemiol. 140 (1994) 1180.
[16] E. Giovannucci, A. Ascherio, E.B. Rimm, M.J. Stampfer, G.A. Colditz, W.C. Willett, J. Natl. Cancer Inst. 87 (1995) 1767.
[17] K. Klipstein-Grobusch, J.M. Geleijnse, J.H. den Breeijen, H. Boeing, A. Hofman, D.E. Grebbee, J.C. Witteman, Am. J. Clin. Nutr. 69 (1999) 261.
[18] E.B. Rimm, M.J. Stampfer, A. Ascherio, New Engl. J. Med. 328 (1993) 1450.
[19] D.K. Pandey, R. Shekelle, B.J. Selwyn, C. Tangney, J. Stamler, Am. J. Epidemiol. 142 (1995) 1269.
[20] L.H. Kushi, A.R. Folsom, R.J. Prineas, P.J. Mink, Y. Wu, R.M. Bostick, New Engl. J. Med. 334 (1996) 1156.
[21] D.A. Cooper, A.L. Eldridge, J.C. Peters, Nutr. Rev. 57 (1999) 201.
[22] H.B. Stähelin, K.F. Gey, M. Eichholzer, E. Lüdin, F. Bermasconi, J. Thurneysen, G. Brubacher, Am. J. Epidemiol. 133 (1991) 766.
[23] K.F. Gey, H.B. Stähelin, M. Eichhozer, Clin. Invest. 71 (1993) 3.
[24] M. Eicholzer, H.B. Stähelin, K.F. Gey, E. Lüdin, F. Bernasconi, Int. J. Cancer 66 (1996) 145.
[25] D.A. Street, G. Comstock, R.M. Saüked, W. Schuep, M.J. Klag, Circulation 90 (1994) 1154.
[26] MRFIT Research Group, R.W. Evans, B.J. Shaten, B.W. Day, L.H. Kuller, Am. J. Epidemiol. 147 (1998) 180.
[27] L. Kohlmeier, J.D. Kark, E. Gomez-Gracia, B.C. Martin, S.E. Steck, A.F.M. Kardinaal, J. Ringstad, M. Thamm, V. Massaev, R. Riemersma, J.M. Martin-Moreno, J.K. Huttunen, F.J. Kok, Am. J. Epidemiol. 146 (1997) 618.
[28] A. Kontush, T. Spranger, A. Reich, K. Baum, U. Beisiegel, Atherosclerosis 144 (1999) 117.
[29] A.F.M. Kardinaal, F.J. Kok, J. Ringstad, J. Gomez-Aracena, V.P. Mazaev, L. Kohimeier, B.C. Martin, A. Aro, J.D. Kark, M. Delgado-Rodriguez, R.A. Riemersma, P. van't Veer, J.K. Huttunen, J.M. Martin-Moreno, Lancet 342 (1993) 1379.
[30] Y.M. Hsueh, W.L. Wu, Y.L. Huang, H.Y. Chiou, C.H. Tseng, C.J. Chen, Atherosclerosis 141 (1998) 249.
[31] The Cancer in Children and Antioxidant Micronutrients French Study Group, D.J.M. Malvy, B. Burtschy, J. Arnaud, D. Sommelet, G. Leverger, L. Dostalova, J. Drucker, O. Amëdëë-Manesme, Int. J. Epidemiol. 22 (1993) 761.
[32] A.M. Torun, S. Yardim, A. Gönenc, H. Sargin, A. Menevse, B. Simsek, J. Clin. Pharm. Ther. 20 (1995) 259.
[33] C. Nagata, H. Shimuzu, H. Yoshikawa, K. Noda, S. Nozawa, A. Yajima, S. Sekiya, H. Sugimori, Y. Hirai, K. Kanazawa, M. Sugase, T. Kawana, Br. J. Cancer 81 (1999) 1234.
[34] P. Brennan, C. Fortes, J. Butler, A. Agudo, S. Benhamou, S. Darby, M. Gerken, K.H. Jöckel, M. Kreuzer, S. Mallone, F. Nyberg, H. Pohlabeln, G. Ferro, P. Bo, Cancer Causes Control 11 (2000) 49.
[35] E.S. Ford, W.H. Giles, Ann. Epidemiol. 10 (2000) 106.
[36] R.A. Riemersma, D.A. Wood, C.C.A. Macintyre, R.A. Elton, K.F. Gey, M.F. Oliver, Lancet 337 (1991) 8732.
[37] A.Z. Chuang, P.N. Subramaniam, B.Y. LeGardeur, A. Lopez-S, Indian Heart J. 50 (1998) 285.
[38] T. Rissanen, S. Voutillainen, K. Nyyssönen, R. Salonen, J.T. Salonen, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 2677.
[39] L.C. Sander, K.E. Sharpless, N.E. Craft, S.A. Wise, Anal. Chem. 66 (1994) 1667.
[40] C.H. Hennekens, J.E. Buring, J.E. Manson, M. Stampfer, B. Rosner, N.R. Cook, C. Belanger, F. LaMotte, J.M. Gaziano, P.M. Ridker, W. Willett, R. Peto, New Engl. J. Med. 334 (1996) 1145.
[41] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens, B. Valanis, J.H. Williams, S. Barnhart, S. Hammar, New Engl. J. Med. 334 (1996) 1150.
[42] J.M. Rapola, J. Virtamo, J.K. Haukka, O.P. Heinonen, D. Albanes, P.R. Taylor, J.K. Huttunen, J. Am. Med. Assoc. 275 (1996) 693.
[43] J.P. Steghens, A.L. van Kappel, E.R. Iboli, C. Collombel, J. Chromatogr. B 694 (1997) 71.
[44] J. Virtamo, J.M. Rapola, S. Ripatti, O.P. Heinonen, P.R. Taylor, D. Albanes, J.K. Huttunen, Arch. Int. Med. 158 (1998) 668.
[45] The Heart Outcomes Prevention Evaluation Study Investigators, New Engl. J. Med. 342 (2000) 154.
[46] R.B. Singh, S.S. Rastogi, R. Verma, B. Laxmi, R. Singh, S. Ghosh, M.A. Niaz, Br. Med. J. 304 (1992) 1015.
[47] M. de Lorgeril, P. Salen, J.L. Martin, N. Mamelle, I. Monjaud, P. Touboul, J. Delaye, J. Am. Coll. Cardiol. 28 (1996) 1103.
[48] J.M. Gaziano, Proc. Assoc. Am. Physicians 111 (1999) 2.
[49] S.A. Paiva, R.M. Russell, J. Am. Coll. Nutr. 18 (1999) 426.
[50] S.B. Kritchevsky, J. Nutr. 129 (1999) 5.
[51] V.P. Palace, N. Khaper, Q. Qin, P.K. Singal, Free Radic. Biol. Med. 26 (1999) 746.
[52] F. Delgado-Vargas, A.R. Jiménez, O. Paredes-López, Crit. Rev. Food Sci. Nutr. 40 (2000) 173.

[53] J. Shi, M. Le Maguer, Crit. Rev. Food Sci. Nutr. 40 (2000) 1.
[54] A.Z. Mercadante, A. Steck, H. Pfander, J. Agric. Food Chem. 47 (1999) 145.
[55] W. Stahl, W.H. Sies, Diabetes 46 (Suppl.) (1997) S14.
[56] M.G. Simic, Methods Enzymol. 213 (1992) 444.
[57] W.K. Hong, M.B. Sporn, Science 278 (1997) 1073.
[58] A. Clairmont, D. Tessmann, H. Sies, FEBS Lett. 397 (1996) 22.
[59] G. Britton, FASEB J. 9 (1995) 1551.
[60] N.I. Krinsky, Free Radic. Biol. Med. 7 (1989) 617.
[61] H. van den Berg, Nutr. Rev. 57 (1999) 1.
[62] K.L. Simpson, T. Katayama, C.O. Chichester, in: J.C. Bauernfeind (Ed.), Carotenoids as Colorants and Vitamin A Precursors, Academic Press, New York, 1981, p. 463.
[63] R. Blomhoff, M.H. Green, J.B. Green, T. Berg, K.R. Norum, Physiol. Rev. 71 (1991) 951.
[64] D. Hollander, J. Lab. Clin. Med. 97 (1981) 449.
[65] A. Shiau, S.A. Mobarhan, M. Stacewicz-Sapuntzakis, J. Am. Coll. Nutr. 9 (1990) 533.
[66] J.A. Olson, Vitamin A, in: E.E. Ziegler, L.J. Filer, Jr., eds., ILSI Press, Washington, D.C., 1996, p. 109.
[67] J.A. Olson, in: M.L. Brown (Ed.), Vitamin A, International Life Sciences Institute, Nutrition Foundation, Washington, DC, 1990, p. 96.
[68] G. Tang, X. Gu, S.S. Hu, Q. Xu, J. Qin, G.G. Dolnikowski, C.R. Fjeld, X. Gao, R.M. Russell, S. Yin, Am. J. Clin. Nutr. 70 (1999) 1069.
[69] K.L. Sinpson, C.O. Chichester, Annu. Rev. Nutr. 1 (1981) 351.
[70] B. Olmediller, F. Granado, I. Blanco, C. Herrero, M. Vaquero, I. Millan, J. Nutr. Health Aging 6 (2002) 66.
[71] J.M. Seddon, U.A. Ajani, R.D. Sperduto, R. Hiller, N. Blair, T.C. Burton, M.D. Farber, E.S. Gragoudas, J. Haller, D.T. Miller, J. Am. Med. Assoc. 272 (1994) 1413.
[72] J. Mares-Periman, W. Brady, R. Klein, R. Klein, G.J. Haus, M. Palta, L.L. Ritter, S.M. Shoff, Am. J. Epidemiol. 141 (1995) 322.
[73] A.Y. Yemelyanov, N.B. Katz, P. Bernstein, Exp. Eye Res. 72 (2001) 381.
[74] P.S. Bernstein, F. Khachik, L. Carvalho, G.J. Muir, D.Y. Zhao, N.B. Katz, Exp. Eye Res. 72 (2001) 215.
[75] L.X. Zhang, R.V. Cooney, J.S. Bertram, Am. J. Clin. Nutr. 62 (Suppl.) (1991) 1327s.
[76] W. Stahl, A. Junghans, B. de Boer, E.S. Driomina, K. Briviba, H. Sies, FEBS Lett. 427 (1998) 305.
[77] C. Iribarren, A.R. Folsom, D.R. Jacobs, M.D. Gross, J.D. Belcher, J.H. Eckfeldt, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 1171.
[78] H. Sies, W. Stahl, A.R. Sundquist, Ann. NY Acad. Sci. 669 (1992) 7.
[79] P.M. Di Mascio, S. Kaiser, H. Sies, Arch. Biochem. Biophys. 274 (1989) 532.
[80] J. VanEenwyk, G.W. Comstock, J.S. Morris, Int. J. Cancer 48 (1991) 34.
[81] H. Gerster, A. Ascherio, E.B. Rimm, M.J. Stamfer, G.A. Colditz, W.C. Willett, J. Natl. Cancer Inst. 87 (1995) 1767.
[82] M.T. Goodman, N. Kiviat, K. McDuffie, J.H. Hankin, B. Hernandez, L.R. Wilkens, A. Franke, J. Kuypers, L.N. Kolonel, J. Nakamura, G. Ing, B. Branch, C.C. Bertram, L. Kamemoto, S. Sharma, J. Killeen, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 537.
[83] L.A. Kaplan, J.M. Lau, E.A. Stein, Clin. Physiol. Biochem. 8 (1990) 1.
[84] L.C. Su, M. Bui, A. Kardinaal, J. Gomez-Aracena, J. Martin-Moreno, B. Martin, M. Thamm, N. Simonsen, P. van't Veer, F. Kok, S. Strain, L. Kohlmeier, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 1043.
[85] F. Khachik, G.R. Bernstein, M.B. Goli, Pure Appl. Chem. 63 (1991) 71.
[86] F. Khachik, G.R. Beecher, J.C. Smith Jr., Cell. Biochem. Suppl. 22 (1995) 236.
[87] T. Wingerath, W. Stahl, H. Sies, Arch. Biochem. Biophys. 324 (1995) 385.
[88] L. Kohlmeier, S.B. Hastings, Am. J. Clin. Nutr. 62 (1995) 1370.
[89] T.R. Dugas, D.W. Morel, E.H. Harrison, Free Radic. Biol. Med. 26 (1999) 138.
[90] Y.S. Peng, Y.M. Peng, Cancer Epidemiol. Biomarkers Prev. 1 (1992) 375.
[91] D.W. Nierenberg, S.L. Nann, Am. J. Clin. Nutr. 56 (1992) 417.
[92] D. Steinberg, New Engl. J. Med. 320 (1989) 915.
[93] A.Z. Mercadabate, Arch. Latinoam. Nutr. 49 (1999) 52S.
[94] A.Z. Mercadabate, D.B. Rodriguez-Amaya, J. Agric. Food Chem. 39 (1991) 1094.
[95] S. Strohschein, M. Pursch, K. Albert, J. Pharm. Biomed. Anal. 21 (1999) 669.
[96] Y. Wang, X. Xu, M. van Lieshout, C.E. West, J. Lugtenburg, M.A. Verhoeven, A.F. Creemers, X. Muhilal, R.B. van Breemen, Anal. Chem. 72 (2000) 4999.
[97] C.W. Huck, M. Popp, H. Scherz, G.K. Bonn, J. Chromatogr. Sci. 38 (2000) 441.
[98] T. Lacker, S. Strohschein, K. Albert, J. Chromatogr. A 854 (1999) 37.
[99] T.W. Goodwin, G. Britton, in: T.W. Goodwin (Ed.), Plant Pigments, Academic Press, London, 1988, p. 62.
[100] M. Dachtler, H. Händel, D. Lindquist, R.M. Hawk, K.N. Craig, R.A. Komoroski, K. Albert, Magn. Reson. Chem. 38 (2000) 951.
[101] F. Khachik, P.S. Bernatein, D.L. Garland, Invest. Ophthalmol. Vis. Sci. 38 (1997) 1802.
[102] B. Olmedilla, F. Granado, I. Blanco, E. Rojas-Hidalgo, Food Chem. 45 (1992) 205.
[103] Q. Su, K.G. Rowley, K. O'Dea, J. Chromatogr. B 729 (1999) 191.
[104] P. Rosi, M. Porrini, Int. J. Vitam. Nutr. Res. 67 (1997) 47.
[105] L.C. Sander, M. Pursch, B. Marker, S.A. Wise, Anal. Chem. 71 (1999) 3477.
[106] L.C. Sander, K.E. Sharpless, N.E. Craft, S.A. Wise, Anal. Chem. 66 (1994) 1667.
[107] L. Roed, E. Lundance, T. Grelbrokk, Electrophoresis 20 (1999) 2373.
[108] H.J.C.F. Nells, A.P.D. Leenheer, Anal. Chem. 55 (1983) 270.

[109] O. Sommerburg, L.Y. Zang, F.J.G.M. van Kuijk, J. Chromatogr. B 695 (1997) 209.
[110] F. Khachik, G.R. Beecher, M.B. Goli, W.R. Lusby, J.C. Smith Jr., Anal. Chem. 64 (1992) 2111.
[111] F. Khachik, C.J. Spangler, J.C. Smith Jr., Anal. Chem. 69 (1997) 1973.
[112] N.E. Craft, E.D. Brown, J.C. Smith Jr., Clin. Chem. 34 (1988) 44.
[113] K.W. Miller, C.S. Yang, Anal. Biochem. 145 (1985) 21.
[114] D.J. Hart, K.J. Scott, Food Chem. 54 (1995) 101.
[115] J.J. Scott, Food Chem. 45 (1992) 357.
[116] S. Strohschein, M. Pursch, K. Albert, J. Pharm. Biomed. Anal. 21 (1999) 669.
[117] T. Glaser, M. Dachtler, K. Albert, GIT Fachz. Lab. 43 (1999) 904.
[118] D. Talwar, T.K.K. Ha, J. Cooney, Clin. Chim. Acta 270 (1998) 85.
[119] T. Hagiwara, T. Yatsuko, K. Funayama, S. Suzuki, J. Chromatogr. B 708 (1998) 67.
[120] T.K.W. Ng, K. Hassan, J.B. Lim, M.S. Lye, R. Ishak, Am. J. Clin. Nutr. 53 (1991) 1015S.
[121] B. Finckh, A. Kontush, J. Commentz, C. Hubner, M. Burdelski, A. Kohlschuter, Anal. Biochem. 232 (1995) 210.
[122] B. Finckh, A. Kontush, J. Commentz, C. Hubner, M. Burdelski, A. Kohlschutter, A.Z. Mercadante, Arch. Latinoam. Nutr. 49 (1999) 52S.
[123] T. Narisawa, Y. Fukaura, M. Hasebe, M. Ito, R. Aizawa, M. Murakoshi, S. Uemura, F. Khachik, H. Nishino, Cancer Lett. 107 (1996) 137.
[124] M.G. Ferruzzi, M.L. Nguyen, L.C. Sander, C.L. Rock, S.J. Schwartz, J. Chromatogr. B 760 (2001) 289.
[125] M.G. Ferruzzi, L.C. Sander, C.L. Rock, S.J. Schwartz, Anal. Biochem. 256 (1998) 74.
[126] R.M. Villanueva Camanas, J.M. Sanchis Mallols, E.F. Simo Alfonso, G. Ramis Ramos, Anal. Lett. 25 (1992) 1425.
[127] M. Franko, P. van de Bovenkamp, D. Bicanic, J. Chromatogr. B 718 (1998) 47.
[128] M. Franko, C.D. Tran, Rev. Sci. Instr. 67 (1996) 1.
[129] S. Luterotti, M. Franko, D. Bicanic, J. Pharm. Biomed. Anal. 21 (1999) 901.
[130] C. Rentel, S. Stroschein, K. Albert, E. Bayer, Anal. Chem. 70 (1998) 4394.
[131] R.B. van Breemen, Anal. Chem. News Feat. 1 (1996) 299.
[132] R.B. van Breemen, C.R. Huang, Y. Tan, L.C. Sander, A.B. Schilling, J. Mass Spectrom. 31 (1996) 975.
[133] M. Dachtler, T. Glaser, K. Kohler, K. Albert, Anal. Chem. 73 (2001) 667.
[134] R. Kaufmann, T. Wingerath, D. Kirsch, W. Stahl, H. Sies, Anal. Chem. 238 (1996) 117.
[135] M. Dachtler, K. Kohler, K. Albert, J. Chromatogr. B 720 (1998) 211.
[136] A.Z. Mercadante, S. Andrea, H. Pfander, J. Agric. Food Chem. 45 (1997) 120.
[137] A.V. Ruban, A.A. Pascal, B. Robert, P. Hoton, J. Biol. Chem. 276 (2001) 24862.
[138] D.I. Thurnham, E. Smith, P.S. Flora, Clin. Chem. 34 (1988) 377.
[139] D. Hess, H.E. Keller, B. Oberlin, R. Bonfanti, W. Schoep, Int. J. Vitam. Nutr. Res. 61 (1991) 232.
[140] M.H. Bui, J. Chromatogr. B 654 (1994) 129.
[141] A.L. Sowell, D.L. Huff, D.L. Yeager, S.P. Caudill, E.W. Gunter, Clin. Chem. 40 (1994) 411.
[142] D. Talwar, T.K. Ha, J. Cooney, C. Brownlee, D.S. O'Reilly, Clin. Chim. Acta 270 (1998) 85.
[143] A.Z. Zaman, P. Fielden, P.G. Frost, Clin. Chem. 39 (1993) 2229.
[144] M.A. Ababusain, J. Wright, J.W.T. Dickerson, M.A. El-Hazmi, H.Y. Enein, Biomed. Chromatogr. 12 (1998) 89.
[145] O. Froescheis, S. Moalli, H. Liechti, J. Bausch, J. Chromatogr. B 739 (2000) 291.
[146] C.S. Yang, M.J. Lee, Growth Cancer 4 (1987) 19.
[147] M. Stacewicz-Sapuntzakis, P.E. Bowen, J.W. Kikendall, J. Micronutr. Anal. 3 (1987) 27.
[148] J.G. Bieri, E.D. Brown, J.C. Smith Jr., J. Liq. Chromatogr. 8 (1985) 437.
[149] National Institute of Standards and Technology, US Department of Commerce Certificate of Analysis Standard Reference Material.
[150] R. Wyss, J. Chromatogr. B 671 (1995) 381.
[151] G. Britton, Method Plant Biochem. 7 (1991) 473.
[152] J.P. Steghens, A.L. van Kappel, E.R. iboli, C. Collombel, J. Chromatogr. B 694 (1997) 71.
[153] K.E. Sharpless, J.B. Thomas, L.C. Sander, S.A. Wise, J. Chromatogr. B 678 (1996) 187.
[154] H.J.C.F. Nells, A.P.D. De Leenheer, Anal. Chem. 55 (1983) 270.
[155] C. Emenhiser, L.C. Sander, S.J. Schwartz, J. Chromatogr. A 707 (1995) 205.
[156] K.S. Epler, R.G. Ziegler, N.E. Craft, J. Chromatogr. 619 (1993) 37.
[157] R.R. Eitenmiller, W.O. Landen, Vitamin analysis for the health and food sciences, CRC Press, London, 1999, p. 3.
[158] M. Zakaria, K. Simpson, P.R. Brown, A. Krstulovic, J. Chromatogr. 176 (1979) 109.
[159] E.J. Weber, JAOCS 64 (1987) 1129.
[160] Y.M. Choo, S.C. Yap, C.K. Ooi, A.N. Ma, S.H. Goh, A.S.H. Ong, JAOCS 73 (1996) 599.
[161] F. Granado, B. Olmedilla, I. Blanco, E. Rojas-Hidalgo, J. Agric. Food Chem. 40 (1992) 2135.
[162] F. Márkus, H.G. Daood, J. Kapitany, P.A. Biacs, J. Agric. Food Chem. 47 (1999) 100.
[163] A.E. Gimeno, E. Calero, A.I. Castellote, R.M. Lamuela-Raventos, M.C. De La Torre, M.C. Lopez-Sabater, J. Chromatogr. A 881 (2000) 255.
[164] L.H. Tonucci, J.M. Holden, G.R. Beecher, F. Khachik, C. David, G. Mulokozi, J. Agric. Food Chem. 43 (1995) 589.
[165] F. Khachik, M.B. Goil, G.R. Beecher, J. Holden, W.R. Luby, M.D. Tenorio, M.R. Barrera, J. Agric. Food Chem. 40 (1992) 390.
[166] T. Baysal, S. Ersus, D.A.J. Starmans, J. Agric. Food Chem. 48 (2000) 5507.
[167] X. Hornero-Mendez, M.I.M. Mosquera, J. Agric. Food Chem. 48 (2000) 825.
[168] J. Deli, P. Molnar, Z. Matus, G. Toth, J. Agric. Food Chem. 49 (2001) 1517.

[169] Q. Su, K.G. Rowley, C. Itsiopoulos, K. O'Dea, Eur. J. Clin. Nutr. (in press).
[170] M. Edelenbos, L.P. Christensen, K. Grevsen, J. Agric. Food Chem. 49 (2001) 4768.
[171] S. Pfister, P. Meyer, A. Steck, H. Pfander, J. Agric. Food Chem. 44 (1996) 2612.
[172] A.Z. Mercadante, A. Steck, H. Pfander, J. Agric. Food Chem. 45 (1997) 1050.
[173] N.L. Rozzi, R.K. Singh, R.A. Vierling, B.A. Watkins, J. Agric. Food Chem. 52 (2002) 111.
[174] P.P. Mouly, E.M. Gaydou, J. Corsetti, J. Chromatogr. A 844 (1999) 149.
[175] K.L. Goodner, R.L. Rouseff, H.J. Hofsommer, J. Agric. Food Chem. 49 (2001) 1146.
[176] W.J. Lesion, G.L. Catigani, S.J. Schwartz, J. Agric. Food Chem. 45 (1997) 3728.
[177] R. Rouseff, L. Raley, J. Agric. Food Chem. 44 (1996) 2176.
[178] C.Y. Tai, B.H. Chen, J. Agric. Food Chem. 48 (2000) 5962.
[179] K.L. Williams, L.C. Sander, S.A. Wise, J. Pharm. Biomed. Anal. 15 (1997) 1789.
[180] E. De Retter, A.E. Purcell, in: J.C. Bauerfein (Ed.), Carotenoids as Colorants and Vitamin A Precursors, Academic Press, New York, 1981, p. 815.
[181] P. Riso, M. Porrini, Int. J. Vitam. Nutr. Res. 67 (1997) 47.
[182] H. Nyambaka, J. Ryley, J. Food Chem. 55 (1995) 63.
[183] R.J. Bushway, Food. Chem. 55 (1996) 63.
[184] A.R. Mangels, J.M. Holden, G.R. Beecher, M.R. Forman, E. Lanza, J. Am. Diet. Assoc. 93 (1993) 284.
[185] I. Stewart, J. Am. Diet. Assoc. 60 (1977) 132.
[186] W. Vilegas, J.H.Y. Vilegas, M. Dachtler, T. Glaser, K. Albert, Phytochem. Anal. 11 (2000) 317.
[187] A.B. Barua, J.A. Olson, J. Chromatogr. B 707 (1998) 69.
[188] P.F. Surai, N.J. Royle, N.H.C. Sparks, Comp. Biochem. Physiol. A 126 (2000) 387.
[189] J. Schierle, W. Bretzel, I. Buhler, N. Faccin, D. Hess, K. Steiner, W. Schuep, Food Chem. 59 (1997) 459.
[190] J.G. Kim, A.E. Yousef, S. Dave, J. Food Prot. 62 (1999) 1071.
[191] L.K. Henry, N.L. Puspitasari-Nienaber, M. Jarén-Galán, R.B. van Breemen, G.L. Catigani, S.J. Schwartz, J. Agric. Food Chem. 48 (2000) 5008.
[192] K.J. Scott, P.M. Finglas, R. Seale, D.J. Hart, I. De Froidmont-Görtz, Food Chem. 57 (1996) 85.
[193] National Institute of Standards and Technology, US Department of Commerce Certificate of Analysis Standard Reference Material, 968b.
[194] National Institute of Standards and Technology, US Department of Commerce Certificate of Analysis Standard Reference Material, 968c.
[195] G. Cavina, B. Gallinella, R. Porra, P. Pecora, C. Suraci, J. Pharm. Biomed. Anal. 6 (1988) 259.
[196] Q. Su, K.G. Rowley, N.D.H. Balazs, Proc. Nutr. Soc. Australia 22 (2000) 189.
[197] M.D. Gross, C.B. Prouty, D.R. Jacobes Jr, Clin. Chem. 41 (1995) 943.
[198] T. Key, S. Oakes, G. Davey, J. Moore, L.M. Edmond, U.J. McLoone, D.L. Thurnham, Cancer Epidemiol. Biomarkers Prev. 5 (1996) 811.
[199] A.H. Smith, K.D. Waller, Am. J. Epidemiol. 133 (1991) 7.
[200] G.W. Comstock, A.J. Alberg, K.J. Helzlsouer, Clin. Chem. 39 (1993) 1075.
[201] G.W. Comstock, E.P. Norkus, S.C. Hoffman, M.W. Xu, K.J. Helzlsouer, Cancer Epidemiol. Biomarkers Prev. 4 (1995) 505.
[202] N.E. Craft, E.D. Brown, J.C. Smith, Clin. Chem. 34 (1988) 44.
[203] J.B. Thomas, D.L. Duewer, M.C. Kline, K.E. Sharpless, Clin. Chim. Acta 276 (1998) 75.
[204] M.C. Ocke, J. Schrijver, G.L. Obermann-De Boer, B.P.M. Bloemerg, G.R.M.M. Haenen, D. Kromhout, J. Clin. Epidemiol. 48 (1995) 1077.
[205] M.C. Ocke, J. Schrijver, G.L. Obermann-de Boer, B.P.M. Bloemberg, G.R.M.M.J. Miller, J. Sampson, L.P. Candeias, P.M. Bramley, C.A. Rice-Evans, FEBS Lett. 384 (1996) 240.
[206] Y.S. Peng, Y.M. Peng, D.L. McGee et al., Am. J. Clin. Nutr. 59 (1994) 636.
[207] I. Ahola, G. Alfthan, C.G. Gref, Clin. Chem. 34 (1988) 1923.
[208] W.J. Driskell, A.D. lackey, J.S. Hewett, M.M. Bashor, Clin. Chem. 31 (1985) 871.
[209] D.L. Duewer, J.B. Thomas, M.C. Kline, W.A. MacCrehan, R. Schaffer, K.E. Sharpless, W.E. May, J.A. Crowell, Anal. Chem. 69 (1997) 1406.
[210] C. Gärtner, W. Stahl, H. Sies, Am. J. Clin. Nutr. 66 (1997) 116.
[211] M. Padula, D.B. Rodriguez-Amaya, Acta Aliment. 16 (1987) 209.
[212] W. McLennan, R. Madden, Australian Bureau of Statistics, Canberra, 1999.
[213] K. O'Dea, M. Patel, D. Kubisch, J. Hopper, K. Traianedes, Diabetes Care 16 (1993) 1004.
[214] N. Thomson, Med. J. Aust. 54 (1991) 235.
[215] K.G. Rowley, Q. Su, M. Cincotta, M. Skinner, K. Skinner, B. Pindan, G.A. White, K. O'Dea, Am. J. Clin. Nutr. 74 (2001) 442.
[216] A.J. Lee, K. O'Dea, J.D. Mathews, Aust. J. Public Health 18 (1994) 109.
[217] C. Young, in: J. Donovan (Ed.), Immigrants in Australia: A Health Profile, AIHW Ethnic Series, Vol. No. 1, AGPS, Canberra, 1992, p. 34.
[218] A. Kouris-Blazos, M.L. Wahlqvist, A. Trichopoulou, E. Polychronopoulos, D. Trichopoulos, Age Ageing 25 (1996) 177.
[219] D. Kromhout, A. Keys, C. Aravanis, R. Buzina, F. Fidanza, S. Giampaoli, A. Jansen, A. Menotti, S. Nedeljkovic, M. Pekkarinen, Am. J. Clin. Nutr. 49 (1989) 889.
[220] Q. Su, M. Kaimakamis, M. Cameron, K. Fowler, K.G. Rowley, K. O'Dea, in: Proceedings of the Nutrition Society of Australia, Adelaide, 1998, p. 189, Vol. 22.
[221] K.G. Rowley, Q. Su, K. O'Dea, A.J. Jenkins, J.D. Best, Proc. Aust. Physiol. Pharmacol. Soc. 32 (Suppl. 2) (2001) 45.

[222] J. Cohen, A. Jenkins, C. Karschimkus, K. O'Dea, J.D. Best, K.G. Rowley, in: Proceedings of Redox Processes in Chemistry, Biology and Medicine, Society for Free Radical Research (Australasia)/Society for Free Radical Research, Japan, 2001, p. 26.
[223] A.D. Haegele, C. Gillette, C. O'Neill, P. Wolfe, J. Heimendinger, S. Sedlacek, H.J. Thompson, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 421.
[224] B. Fuhrman, A. Elis, M. Aviram, Biochem. Biophys. Res. Commun. 233 (1997) 658.
[225] K.R. Martin, D. Wu, M. Meydani, Atherosclerosis 150 (2000) 265.
[226] C. Itsiopoulos, R.M. Stoney, M.A. Cameron, M.A. Kaimakamis, K.Z. Walker, K.G. Rowley, P. Ireland, G. Giles, K. O'Dea, in: Proceedings of the 2nd South-West Pacific Nutrition and Dietetic Conference, Auckland, 1999, p. 125.
[227] D. Albanes, J. Vitamo, P.R. Taylor, M. Rautalahti, P. Pietineet, Am. J. Clin. Nutr. 66 (1997) 366.
[228] Y. Ito, J. Ochiai, R. Sasaki, K. Ishikawa, K. Tsutsumi, Clin. Chim. Acta 194 (1990) 131.
[229] Q. Su, Thesis, Dietary lipid-soluble antioxidants and risk of cardiovascular disease, Monash University, Australia, 2000, p. 46.