Pure & Appl. Chem., Vol. 63, No. 1, pp. 71–80, 1991.
Printed in Great Britain.

Separation, identification, and quantification of carotenoids in fruits, vegetables and human plasma by high performance liquid chromatography

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Abstract- Separation, identification, and quantitation by high performance liquid chromatography of prominent carotenoids and carotenol fatty acid esters in various fruits and vegetables are reviewed. Examples of transformations of carotenoids as a result of food preparation are discussed. Recent developments in separation and identification of several carotenoids in human plasma are presented.

INTRODUCTION

In the past decade a number of foods and food constituents have been studied for their inhibitory effect on carcinogenesis (ref. 1). Several of these studies have demonstrated an inverse relationship between the consumption of certain fruits and vegetables and the risk of epithelial cancer (ref. 2 & 3). The experimental (ref. 4) and human epidemiological evidence (ref. 5) for the nutritional prevention of cancer have recently been reviewed. Nutritional prevention of cancer in human populations is based upon the mechanisms of action, toxicity, and efficacy of certain chemical compounds, also referred to as dietary agents or micronutrients, that are present in foods associated with reduced cancer risks. These dietary components include carotenoids, retinoids, tocopherols, ascorbic acid, selenium, and several other minor constituents known to occur in specific classes of foods, e.g., indoles, phenols, and aromatic isothiocyanates. Technological advances in chemical instrumentation during the past two decades have provided analysts with powerful new tools to quantify low levels and various forms of dietary nutrients. Characterization and accurate quantitative measurement of these nutrients, in foods associated with reduced cancer risks, are essential in interpreting the epidemiological studies and identifying a specific chemical component or combinations of micronutrients that may be responsible for lowering the risk of cancer.

The micronutrients considered in this text will be largely limited to carotenoids, but the analysis of vitamin A and two forms of vitamin E (γ- and α-tocopherol) in human plasma will be briefly discussed. Carotenoids are among the most abundant naturally occurring pigments that are found in plants and plant foods. The present number of naturally occurring carotenoids isolated from various sources is in excess of 560 (ref. 6). Current food composition tables (ref. 7 & 8) lack detailed analytical information in that they only provide data on vitamin A activity which is largely contributed by about four carotenoids in plant foods (ref. 9). In the past decade, with the implementation of high performance liquid chromatography (HPLC), more attention has been focused on the separation of all the carotenoid constituents of foods. In the following section, the separation of various classes of carotenoids by HPLC is described.

SEPARATION OF CAROTENOIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The separation of carotenoids by HPLC may be divided into:

1) separation of cartenoids containing different end-groups
2) separation of stereoisomers of carotenoids
   a) geometrical isomers of carotenoids
   b) configurational (optical) isomers of carotenoids

The choice of the HPLC column for separating stereoisomers of carotenoids is critical. Whereas the geometrical isomers of β-carotene are best separated on an HPLC lime column (ref. 10), the geometrical isomers of several other carotenoids that are abundant in fruits and vegetables can be better separated on a C_{18} reversed phase column (refs. 11-14). The configurational isomers of hydroxycarotenoids such as lutein and zeaxanthin have been separated by HPLC via derivatization with (S)-(+)-α-(1-naphthyl) ethyl isocyanate to their corresponding dicarbamates (ref. 15). In some cases, the configurational isomers of certain carotenoids have been separated without derivatization by HPLC employing nitrile bonded (ref. 16) and chiral columns (ref. 17 & 18). Unfortunately, in the separation of

carotenoids in extracts of fruits and vegetables, where numerous carotenoids may be present, the full resolution of all carotenoids, their geometrical isomers and, where applicable, their configurational isomers cannot be accomplished simultaneously in a single chromatographic run. Because of this complexity, it is more practical to establish HPLC conditions that separate carotenoids with various end-groups in an initial evaluation of extracts from biological sources. This may then be followed by isolating individual carotenoids and elucidating their structures. Each of the isolated carotenoids can be further examined under appropriate HPLC conditions to determine the presence or absence of certain geometrical and configurational isomers. In chromatographic separation of carotenoids it is essential to avoid artifacts that can result from interactions between solute molecules (carotenoids), injection solvent, and mobile phase. These interactions can result in production of HPLC peaks that could be interpreted erroneously as indicating the presence of impurities or cis carotenoids (ref. 19). In studies where a correlation between the distribution of certain carotenoids in fruits and vegetables and prevention of cancer is investigated, the separation of carotenoids with different constitution is usually a primary concern. Stereochemistry is not of major importance. However, in understanding the metabolic pathways of carotenoids in animals, knowledge of the stereochemistry and absolute configuration of the carotenoids involved is important (ref. 20).

We have extensively evaluated the distribution of carotenoids in common fruits and vegetables and have developed HPLC conditions that can separate and quantify the various carotenoids in these foods. By employing a C_{18} reversed phase column and gradient HPLC conditions, carotenoids from the extracts of fruits and vegetables with complex profiles have been separated (ref. 21).

SEPARATION AND IDENTIFICATION OF CAROTENOIDS IN FRUITS AND VEGETABLES BY HPLC

Common fruits and vegetables, with the exception of green ones, each have their own unique carotenoid distribution. Although it is difficult to categorize the fruits and vegetables according to their carotenoid HPLC profile, one can attempt to divide these foods into three major groups as shown in Tables 1, 2, and 3. The carotenoid identifications are listed in Table 4. For convenience, only the trivial names of the carotenoids have been used throughout this text. The semi-systematic names and chemical structures of these carotenoids have been tabulated by Pfander (ref. 6). The major carotenoid constituents of the fruits and vegetables listed in Tables 1, 2, and 3 have been isolated from the extracts of these foods by preparative thin-layer and high performance liquid chromatography. The HPLC separations were performed on a Microsorb (25-cm length x 4.6-mm i.d.) C_{18} (5-μm spherical particles) column (Rainin Instrument Co.), which was protected with a Brownlee guard cartridge (3-cm length x 4.6-mm i.d.) packed with spheri-5-C_{18} (5-μm particle size). An isocratic mixture of methanol (10%), acetonitrile (85%), and dichloromethane/hexane : 1/1 (5%) at time 0 was followed by a linear gradient beginning at time 10 min and completed at time 40 min. The final composition of the gradient mixture at time 40 min was methanol (10%), acetonitrile (45%), and dichloromethane/hexane : 1/1 (45%). The column flow rate was 0.7 ml/min. The individually purified carotenoids were identified from their UV/visible and mass spectra, and by comparison of their HPLC retention times and UV/visible absorption spectra (determined by photodiode array detector), with those of synthetic carotenoids. In certain cases, functional group reactions of carotenoids were employed to characterize a particular carotenoid end-group.

The first category of the fruits and vegetables, the green ones (Table 1), all have similar chromatographic profiles showing three major groups of pigments. In the order of chromatographic elution from a C_{18} reversed phase column these are: (a) xanthophylls (oxygenated carotenoids), (b) chlorophylls and their derivatives, and (c) hydrocarbon carotenoids. A typical chromatogram of an extract from raw green beans is shown in Fig. 1. The gradient chromatographic procedure allows the separation of all the major carotenoid and chlorophyll constituents of the green fruits and vegetables without the need for saponification and removal of chlorophylls and their derivatives (ref. 11).

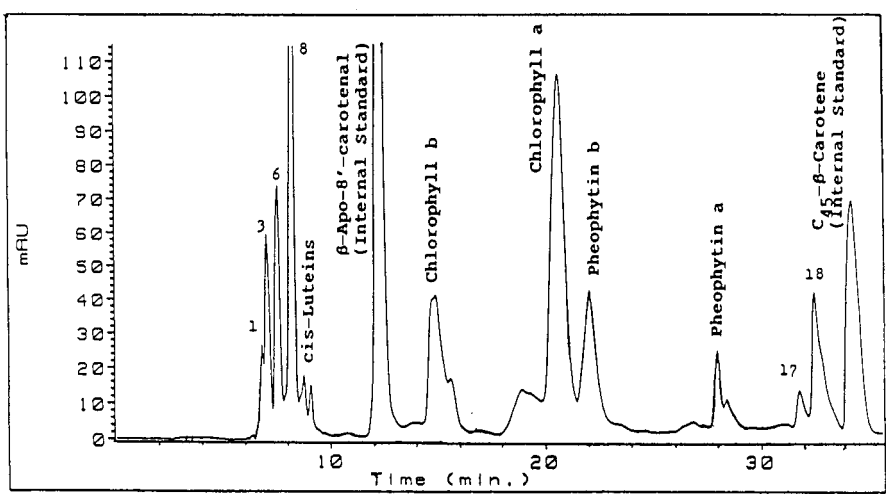

Fig. 1. Chromatogram of an extract from raw green beans; for peak identification see Table 4.

TABLE 1. Distribution of carotenoids in common green fruits and vegetables with similar HPLC profiles.

Common name	Scientific name	Carotenoids *	Reference
Beans			
common, green	Phaseolus vulgaris	1, 2, 3, 4, 5, 6, 7, 8, 17, 18	22, 23, 24
lima	Phaseolus limensis	1, 2, 3, 4, 5, 6, 7, 8, 17, 18	22
Broccoli	Brassica oleracea var. botrytis	1, 2, 3, 4, 5, 6, 7, 8, 18	11, 23
Brussels sprouts	Brassica oleracea var. gemmifera	1, 2, 3, 6, 8, 18	11
Cabbage	Brassica oleracea var. capitata	1, 2, 3, 6, 8, 18	11
Chinese cabbage			
pakchoy or bokchoy	Brassica chinensis	1, 2, 3, 5, 8, 18	24
wild	Brassica pekinensis	1, 2, 3, 5, 7, 8, 18	24
Kale	Brassica oleracea var. acephala	1, 2, 3, 6, 8, 18	11
Kiwi, or chinese gooseberry	Actinidia chinensis	1, 3, 6, 8, 18	22
Lettuce	Lactuca sativa	1, 3, 5, 6, 8, 18	22, 24
Muskmelon, honeydew	Cucumis melo	1, 3, 6, 8, 18	22
Spinach	Spinacia oleracea	1, 2, 3, 4, 5, 8, 18	11, 23
Peas, green	Pisum sativum	1, 3, 6, 8, 17, 18	22

TABLE 2. Distribution of carotenoids in common yellow/red fruits and vegetables, containing mostly hydrocarbon carotenoids.

Common name	Scientific name	Carotenoids *	Reference
Apricots	Prunus armeniaca	14, 15, 16, 18, 19, 20	14
Cantaloupe	Cucumis melo	16, 18, 19, 20	14
Carrots	Daucus carota var. sativa	16, 17, 18	12
Grapefruit, pink	Citrus paradisi var. Ruby Red	14, 16, 18, 19, 20	14
Pumpkin	Cucurbita pepo	8, 16, 17, 18, 19, 20	12, 24
Sweet potato	Ipomoea batatas	18	12, 24
Tomatoes	Lycopersicon esculentum	14, 16, 18, 19, 20	22, 24, 28 29, 30, 31

TABLE 3. Distribution of carotenoids in yellow/orange fruits and vegetables with complex HPLC profiles, mostly containing carotenol acyl esters.

Common name	Scientific name	Carotenoids *	Reference
Mango	Mangifera indica	3, 13, 16, 18, 19, 20, 27, 28, 29, 30, 31, 32, 33	22, 24
Papaya	Carica papaya	3, 11, 13, 16, 18, 19, 20, 21, 22, 25, 26, 29, 30, 31, 32, 33 37, 38, 39	22, 24
Peaches	Prunus persica	9, 13, 16, 18, 19, 20, 25, 26, 37, 38, 39, 40, 41, 42	14
Prunes	Prunus species	8, 13, 17, 18, 25, 26, 36, 37, 38, 39	22
Squash, acorn	Cucurbita pepo	3, 4, 5, 8, 18, 27, 29, 31, 32, 33, 36, 37, 38, 39	13
Squash, winter	Cucurbita maxima		
	var. Northrup King	7, 8,10, 13, 18, 34, 35, 36, 37, 38, 39	13
	var. Butternut	8, 17, 18, 36, 37, 38, 39	13
Oranges	Citrus sinensis	3, 4, 5, 16, 18, 19, 20, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33	22

* For identification of carotenoids see Table 4.

TABLE 4. Identification of carotenoids listed in Tables 1, 2, and 3.

1.	Neoxanthin	21.	β-Cryptoxanthin epoxide myristate
2.	Neochrome	22.	β-Cryptoxanthin epoxide palmitate
3.	Violaxanthin	23.	α-Cryptoxanthin myristate
4.	Luteoxanthin	24.	α-Cryptoxanthin palmitate
5.	Auroxanthin	25.	β-Cryptoxanthin myristate
6.	Lutein-5,6-epoxide	26.	β-Cryptoxanthin palmitate
7.	Flavoxanthin	27.	Violaxanthin monomyristate
8.	Lutein	28.	Violaxanthin monopalmitate
9.	Zeaxanthin	29.	Violaxanthin bislaurate
10.	2', 3'-Anhydrolutein	30.	Violaxanthin laurate/myristate
11.	β-Cryptoxanthin-5, 6-epoxide	31.	Violaxanthin bismyristate
12.	α-Cryptoxanthin	32.	Violaxanthin myristate/palmitate
13.	β-Cryptoxanthin	33.	Violaxanthin bispalmitate
14.	Lycopene	34.	Lutein monomyristate
15.	γ-Carotene	35.	Lutein monopalmitate
16.	ζ-Carotene	36.	Lutein bislaurate
17.	α-Carotene	37.	Lutein bismyristate
18.	β-Carotene	38.	Lutein myristate/palmitate
19.	Phytofluene	39.	Lutein bispalmitate
20.	Phytoene	40.	Zeaxanthin bismyristate
		41.	Zeaxanthin myristate/palmitate
		42.	Zeaxanthin bispalmitate

Two synthetic carotenoids, namely β-apo-8'-carotenal (ref. 11) and 'C_{45}-β-carotene' (ref. 12 & 25), have been employed as internal standards for quantitative analysis of the xanthophylls and the carotenes, respectively. The major chlorophyll constituents of green fruits and vegetables are chlorophylls **b** and **a**, which may be accompanied by their most common degradation products pheophytins **b** and **a**. The general chromatographic profiles of the other green fruits and vegetables are very similar to that of raw green beans illustrated above. These similarities are consistent with the extensive surveys carried out by Strain (ref. 26), who has shown that the leaves of higher plants usually contain the same carotenoids. The major differences among the green fruits and vegetables appear to be the concentrations at which the various components are present.

The second category (Table 2) contains the yellow/red fruits and vegetables, which have similar chromatographic profiles and contain mostly hydrocarbon carotenoids. A typical chromatogram of an extract from pumpkin is shown in Fig. 2. The major carotenoid constituents in this vegetable are: lutein, ζ-carotene, α-carotene, β-carotene, phytofluene, and phytoene. Samples of this particular variety of pumpkin were obtained from the Cook Islands, which is one of the several Pacific Island nations, where a large collaborative survey of lifestyle risk factors for cancer is presently being conducted. The major difference between the carotenoid profiles of pumpkin commonly consumed in the US (ref. 12) and that of the Cook Islands is the presence of ζ-carotene in unusually high concentrations in the latter (ref. 24). The levels of carotenoids in the extracts of fruits and vegetables in general are highly dependent on the origin of the sample (cultivar, growing season, location). The effect of environment on the synthesis of carotenoids in plants has been reported by Goodwin (ref. 27). The presence of ζ-carotene, phytofluene, and phytoene in the fruits and vegetables listed in Table 2 is not surprising as these compounds are precursors of α- and β-carotene and participate in the overall scheme for the biosynthesis of cyclic carotenes in plants (ref. 27). The chromatographic separation shown in Fig. 2 has been monitored at four different wavelengths simultaneously by means of a photodiode array detector. This is due to the nature of the UV/visible absorption spectra of lutein (λ_{max}= 446 nm), ζ-carotene (λ_{max}= 400 nm), α-carotene (λ_{max}= 446 nm), β-carotene (λ_{max}= 454 nm), phytofluene (λ_{max}= 350 nm), and phytoene (λ_{max}= 286 nm) in the HPLC solvents (ref. 14).

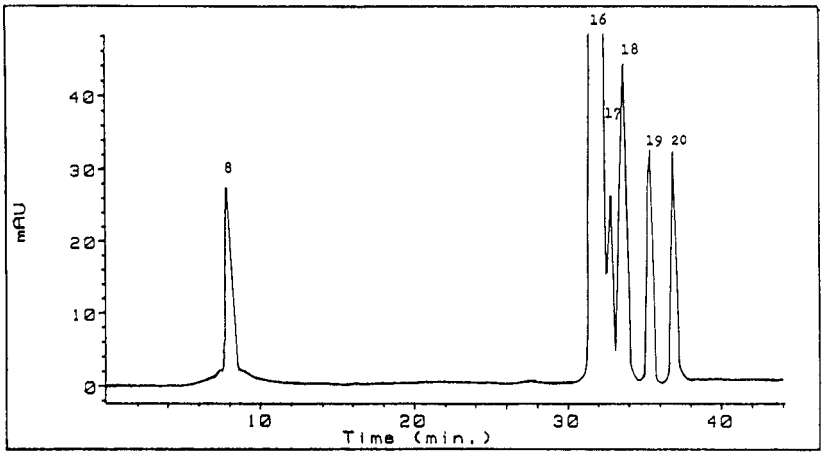

Fig. 2. Chromatogram of an extract from pumpkin; for peak identification see Table 4.

Although according to the chromatogram shown in Fig. 2, there seems to be significant HPLC peak overlap between ζ- and α-carotene at the monitoring wavelengths of 400 and 450 nm, this interference can be minimized by monitoring ζ-carotene at 380 nm and α- and β-carotene at 475 nm.

The third category of the fruits and vegetables are the yellow/orange foods (Table 3) which usually contain carotenol acyl esters. In general, the carotenoid HPLC profiles of the extracts from these fruits and vegetables are quite complex, because the naturally occurring hydroxycarotenoids (i.e. lutein, zeaxanthin, β-cryptoxanthin) and violaxanthin (an epoxycarotenoid) in these fruits and vegetables are esterified with straight chain fatty acids such as lauric, myristic, and palmitic acids. In certain extracts from fruits and vegetables that contain carotenoids with two hydroxyl groups (i.e. lutein, zeaxanthin, and violaxanthin), carotenol esters with a mixture of fatty acid side chains are also present. As a result, the chromatographic separation of these compounds becomes quite complicated. Because of this complexity, carotenoid extracts from natural sources, that are suspected to contain carotenol acyl esters, are customarily saponified to remove the fatty acids and liberate the parent carotenoids. We reported HPLC separation conditions for the acyl esters of a number of hydroxy- and epoxycarotenoids (ref. 32) and have successfully applied this technique to the separation and identification of carotenoids in fruits and vegetables (ref. 13 & 14). Although, in certain cases, the saponification of carotenoid extracts from biological sources may be inevitable, the existing methodology can successfully be implemented to separate carotenol acyl esters in the extracts from most of the fruits and vegetables. The saponification of carotenoid extracts can result in the destruction and/or structural transformation of some carotenoids, so such HPLC methods which separate the various classes of carotenoids without saponification can be highly advantageous and provide valuable information on the identity and the levels of these compounds in their natural state in foods.

Typical chromatograms of an extract from papaya before and after saponification are shown in Figs. 3A and 3B, respectively. This fruit, from Hawaii, is commonly consumed in the US. The major carotenoid constituents in the unsaponified extract (Fig. 3A) are: ζ-carotene, β-carotene, phytofluene, phytoene, β-cryptoxanthin acyl esters, β-cryptoxanthin-5,6-epoxide acyl esters, violaxanthin acyl esters, and lutein acyl esters. Low levels of free β-cryptoxanthin-5,6-epoxide and β-cryptoxanthin are also present in the unsaponified extract. Unfortunately, under these conditions, the HPLC peaks of phytofluene and phytoene are masked by the HPLC peaks of the acyl esters of violaxanthin and β-cryptoxanthin. Therefore, for quantitative analysis of carotenoids in papaya, saponification may provide a more satisfactory approach.

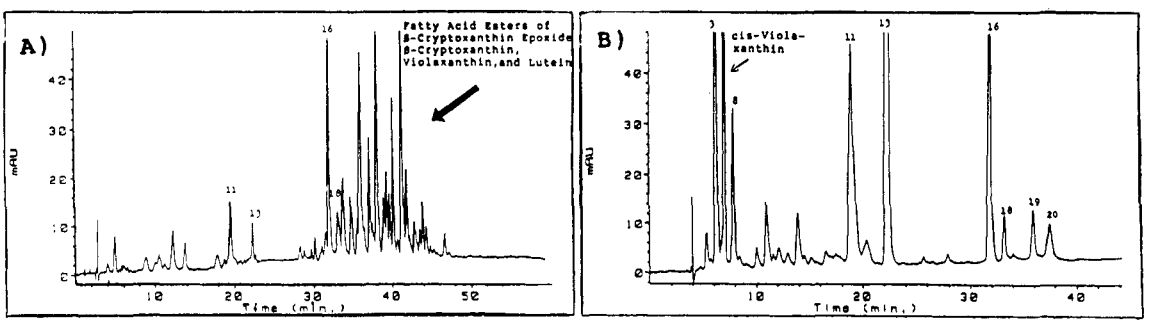

Fig. 3 Chromatograms of an extract from papaya before (A) and after (B) saponification; for peak identification see Table 4.

The HPLC profile of the saponified extract of papaya (Fig. 3B) shows the presence of ζ-carotene, phytofluene, and phytoene. This chromatogram also confirms the liberation of free violaxanthin, lutein, β-cryptoxanthin-5,6-epoxide, and β-cryptoxanthin from their corresponding acyl esters. Small amounts of luteoxanthin and β-cryptoxanthin-5,8-epoxide were also detected in the saponified extract of papaya; these may be artifacts of the extraction and work-up procedure, resulting from the rearrangement of violaxanthin and β-cryptoxanthin-5,6-epoxide, respectively. Samples of another variety of papaya, obtained from the Cook Islands in the South Pacific, showed the presence of the same carotenoids as the papaya commonly consumed in the US, but the carotenoid levels, particularly those of β-cryptoxanthin and ζ-carotene, were much higher than in fruit imported into the US mainland from Hawaii. The HPLC profile of extracts from other fruits and vegetables that are listed in Table 3 show similar separations for carotenol acyl esters.

CHEMICAL TRANSFORMATION OF CAROTENOIDS AS A RESULT OF VARIOUS FOOD PREPARATION AND PROCESSING

The effect of processing on cis-trans isomerization and on the carotene content of fruits and vegetables has been reported by many researchers (refs. 11-14 & 33-38). Most of the qualitative and quantitative data, generated in our laboratory, suggest that different classes of carotenoids differ in their stability towards heat treatment. For example, a recent study on the effects of cooking and processing on a number of yellow/orange vegetables has revealed that the destruction of the hydrocarbon carotenoids such as α- and β-carotene as a result of heat treatment is about 8-10% (ref. 12). In another study, the epoxycarotenoids such as neoxanthin, violaxanthin, and lutein epoxide that are

particularly abundant in green vegetables were shown to be much more labile towards heat treatment and underwent complete destruction or partial conversion into their corresponding 5,8-epoxides (ref. 11). Often, it is difficult to compare analytical data on the effects of cooking and processing on carotenoid levels in fruits and vegetables that have been reported in different studies. In most cases, there is insufficient information on the history of the samples (i.e., cultivar, growing season, location), the length of cooking, and the method of preparation (i.e., frying, steaming, boiling, baking, microwave cooking) of cooked foods. In determining changes in the carotenoid content in a cooked food, the food must be analyzed in raw and cooked forms. Measures for correction of the weight to allow for evaporation of water and volatiles during cooking must be included when analytical data are generated in such studies. We have studied extensively the effect of various means of cooking (microwaving, boiling, steaming) on the qualitative and quantitative distribution of carotenoids in several green vegetables, namely: broccoli, green beans, and spinach (ref. 23). The results for the major carotenoids in extracts from green beans prepared by various methods of cooking, including a long-term cooking, have been tabulated in Table 5. These carotenoid data are the means of at least three extractions and analyses of various samples of green beans for each method of preparation. In this quantitative study, two synthetic internal standards, namely β-apo-8'-carotenal (ref. 11) and 'C_{45}–β-carotene' (ref. 12 & 25), were employed to minimize analytical errors as a result of extraction and work-up procedures.

TABLE 5. Carotenoid content of green beans.

Cooking method	Carotenoids (mg/ 100 g raw)					
	neoxanthin	violaxanthin	lutein-5,6-epoxide	lutein	α-carotene	β-carotene
Raw	0.13	0.23	0.20	0.60	0.08	0.47
Microwave [a]	0.13	0.09	0.09	0.71	0.09	0.53
Boiling [b]	0.13	0.08	0.07	0.61	0.08	0.54
Long-term cooking study:						
Raw	0.20	0.23	0.23	0.69	0.10	0.51
Boiling for 1 h	-- [c]	--	--	0.72	0.09	0.58

[a], microwave samples (60 g) were prepared by adding 25 g of deionized water and heating the vegetable for 4 minutes in a microwave oven at full power; [b], boiled samples (60 g) were prepared by adding 1000 ml of water and boiling the vegetable for 9 minutes; [c], insufficient level in aliquot to permit quantification. Limits of detection for carotenoids are approximately 1 μg/ 100 g of food extracted.

From these results it is apparent that under the various cooking conditions employed (see footnotes in Table 5), neoxanthin, lutein, α- and β-carotene are quite heat resistant; the levels of these carotenoids in cooked green beans in comparison with raw are not statistically different. In contrast, regardless of method of cooking, lutein epoxide and violaxanthin are quite labile towards heat treatment and suffer losses of about 55% and 65%, respectively. The one hour boiling experiment with samples of green beans revealed that the levels of lutein, α- and β-carotene remained unchanged, while the epoxycarotenoids such as neoxanthin, violaxanthin, and lutein epoxide were completely destroyed. No significant cis/trans isomerization of lutein was shown by HPLC to have taken place in the extracts from green beans cooked under various conditions. Results similar to those described above were obtained with broccoli and spinach cooked under various conditions (microwaved and steamed). The complete details of these cooking studies will be published elsewhere.

Another chemical transformation of carotenoids that may be triggered by food processing is a dehydration reaction. In an earlier report, we observed the presence of 3-hydroxy-2',3'-didehydro-β,ε-carotene in a variety of processed baby food squash (ref. 13). The presence of this compound was related to an acid-catalyzed dehydration of lutein in the acidic squash (pH ≈ 4) at the processing stage. We have now shown that although this compound can be readily prepared by acidic dehydration of lutein in organic solvents, it can also be formed rather easily from lutein bisacyl esters. When a solution of lutein bispalmitate in hexane was treated with catalytic amounts of acid (i.e. hydrochloric or sulfuric acid) or phosphorus oxychloride at room temperature, two products were formed, which were separated by preparative thin layer and high performance liquid chromatography. After hydrolysis, these dehydration products were identified from their UV/visible absorption and mass spectra as 3-hydroxy-2',3'-didehydro-β,ε-carotene (70%) and 3'-hydroxy-3,4-didehydro-β-carotene, also known as anhydrolutein (30%). In the dehydration reaction of lutein, organic solvents that contain traces of methanol or ethanol should be avoided because, in the presence of these alcohols and acidic media, lutein can undergo alkylation of the allylic hydroxyl group (ref. 39). The ease with which the dehydration products of lutein bisesters are formed in acidic media suggests that the

dehydration product in processed squash may originate from lutein esters as well as from free lutein. The dehydration products of lutein were not detected in the cooked green vegetables described in Table 5, indicating that, in green vegetables that contain weak organic acids such as oxalic acid, lutein does not undergo dehydration under these conditions. However, when a sample of raw spinach was cooked in an autoclave at 132 ° C for 1 h., the two dehydration products of lutein were shown by HPLC to be present. The levels of carotenoids such as lutein and β-carotene in the extract of the autoclaved sample of spinach were substantialy lower than in the raw samples because of significant degradation of these carotenoids at high temperature. From the limited data available, it remains uncertain whether 3-hydroxy-2',3'-didehydro-β,ε-carotene (2', 3'-anhydrolutein) is an artifact of sample preparation and processing of vegetables or is a naturally occurring carotenoid. The source and the natural occurrence of 3-hydroxy-2',3'-didehydro-β,ε-carotene is of particular interest, since for the first time we have recently isolated and identified this compound in extracts from human plasma.

RECENT DEVELOPMENTS IN SEPARATION AND STRUCTURE ELUCIDATION OF CAROTENOIDS IN HUMAN PLASMA

The experimental and human epidemiological studies that have associated the high consumption of carotenoid-containing foods with a lower incidence of cancer have led to numerous studies of carotenoid metabolism in higher animals including humans. In these studies, the absorption and metabolism of carotenoids ingested by subjects either as food or as synthetic supplements were investigated by evaluating the plasma carotenoid profile of these subjects after various time intervals. Reversed phase HPLC has been found to be superior to normal adsorption phase for the separation of plasma carotenoids. The first successful separation of carotenoids from an extract of human serum was reported by Nelis and De Leenheer (ref. 40), who developed nonaqueous reversed phase chromatographic conditions on a Zorbax ODS column, with a mixture of acetonitrile (70%), dichloromethane (20%), and methanol (10%) as eluent. Under these conditions six carotenoids were separated and identified in an extract from human serum; these were: lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene, and β-carotene. Although many other researchers have developed nonaqueous reversed phase HPLC conditions employing a variety of organic solvents and various HPLC columns (refs. 41-43), the HPLC conditions of Nelis and De Leenheer provide better separation of carotenoids, and presently it can be considered state of the art for the separation of these compounds from extracts of human plasma. This has been demonstrated by Bieri et al. (ref. 44), who successfully employed this chromatographic procedure to separate carotenoids from extracts of human plasma on a C_{18} reversed phase HPLC column. In addition to the six carotenoids reported by Nelis and De Leenheer, Bieri and co-workers reported the presence of two unidentified carotenoids, one of which has been referred to as pre-cryptoxanthin, in human plasma.

From our extensive studies on the distribution of carotenoids in common fruits and vegetables, it appeared that as many as 42 carotenoids may be available from the diet and absorbed, metabolized, or utilized by the human body. The fact that only 6 to 8 carotenoids were detected in human plasma suggested that either the methodology for the detection of other carotenoids in the extracts from human plasma has not yet been fully developed or only selected carotenoids are actually absorbed and metabolized by humans. To clarify these points, we have investigated extensively the HPLC separation of carotenoids extracted from human plasma, employing conditions that were shown to allow the effective separation of various classes of carotenoids in fruits and vegetables listed in Tables 1, 2, and 3. The chromatographic profile of an extract from human plasma under these conditions is shown in Fig. 4. This chromatogram was monitored by a photodiode array detector at five different wavelengths (470, 450, 400, 350, 290 nm) to ensure the detection of all components in the plasma extract. According to this chromatogram, 14 carotenoids as well as vitamin A and two forms of vitamin E (γ- and α-tocopherol) were shown to be present in human plasma. These various components were each isolated by preparative thin layer and high performance liquid chromatography and were identified by comparison of their HPLC retention times and their UV/visible absorption and mass spectra with those of synthetic compounds. The later peaks in the chromatogram of an extract from human plasma are the hydrocarbon carotenoids, which were identified as lycopene, ζ-carotene, α-carotene, β-carotene (and cis-isomers), phytofluene, and phytoene (see Fig. 4 for HPLC peak identification). The presence of these hydrocarbons in human plasma is not surprising because they are abundant in fruits and vegetables listed in Tables 2 and 3. The more polar components with shorter retention times than the hydrocarbons in the chromatogram shown in Fig. 4 have been identified as α-cryptoxanthin, β-cryptoxanthin, γ-tocopherol, and α-tocopherol. Although the HPLC peaks of β-cryptoxanthin and γ-tocopherol coincide (Fig. 4), the absorption spectra of these compounds in the HPLC solvents with maxima at 450 and 292 nm, respectively, allow accurate determination of these two components in the extracts from human plasma. There are two possible chemical structures for α-cryptoxanthin (ref. 6) depending on whether the 3-hydroxyl group is located on the ε- or β end-group. We have prepared and separated these compounds by HPLC under the conditions that were employed for the separation of plasma carotenoids. Comparison of the HPLC retention times established that the hydroxyl group in α-cryptoxanthin in human plasma is substituted in the β- rather than the ε- end-group. This conclusion was further supported by the finding that the α-cryptoxanthin isolated from human plasma did not react with methanol in the presence of trace amounts of hydrochloric acid, indicating the absence of an allylic hydroxyl group in this compound. It is now evident that one of the two unknown carotenoids that was referred to as pre-cryptoxanthin by Bieri and co-workers (ref. 44) is α-cryptoxanthin. The presence of α-cryptoxanthin in human plasma is rather unusual as this naturally occurring carotenoid is rare in the plant kingdom; the only dietary source among the common fruits and vegetables is oranges and citrus fruits. It is interesting to note that all the α-cryptoxanthin in

oranges and citrus fruits is esterified with straight chain fatty acids. From the more polar carotenoids in the HPLC profile shown in Fig. 4, we have separated and identified 3-hydroxy-2',3'-didehydro-β,ε-carotene (2',3'-anhydrolutein) in the extracts from human plasma. We have now positively identified this compound to be the other unknown carotenoid 'pre-cryptoxanthin', that was first detected in human plasma by Bieri and co-workers (ref. 44). Dietary sources of 2',3'-anhydrolutein are limited as this compound has only been detected in one variety of squash, as described earlier. Therefore it seems logical to assume that the presence of this compound in the extracts from human plasma may be related to an enzymic dehydration of lutein or de-esterification of lutein acyl esters in strong acidic media in the human digestive system. In a recent publication, Fukasawa (ref. 45) has shown the presence of 3'-hydroxy-3,4-didehydro-β-carotene (3,4-anhydrolutein) in an extract from human serum analyzed by HPLC. Although, under our HPLC conditions, a synthetic mixture of 2',3'-anhydrolutein (λ_{max}= 446 nm) and 3,4-anhydrolutein (λ_{max}= 464 nm) were well separated and exhibited quite different absorption maxima in the HPLC solvents, we were unable to detect 3,4-anhydrolutein in the extracts from human plasma.

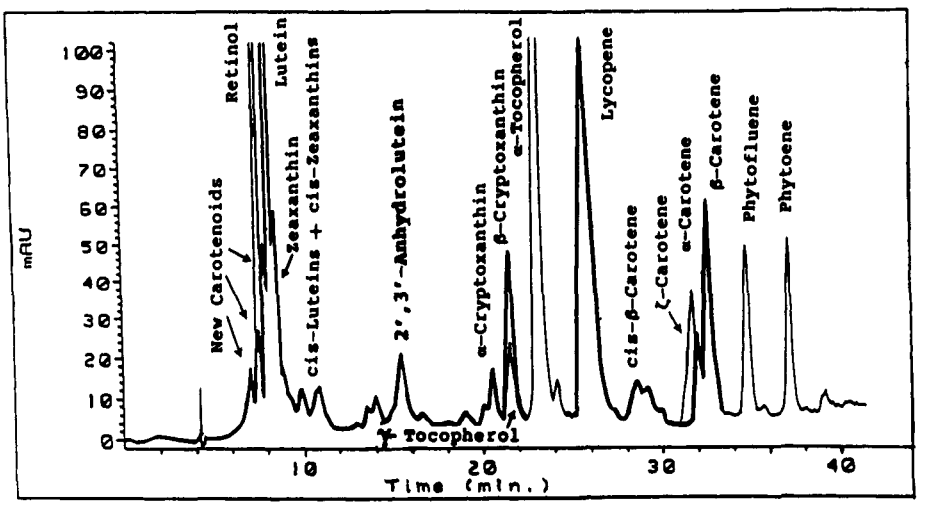

Fig. 4 Chromatographic profile of an extract of human plasma.

The most polar fraction of the extracts from human plasma consisted of several components that have short retention times (6-12 min) on a C_{18} reversed phase HPLC column as shown in the chromatogram in Fig. 4. These components have been identified as vitamin A alcohol (retinol), lutein, and zeaxanthin, together with several new carotenoids, which were only partially separated because of their high polarity and low affinity for the C_{18} reversed phase column. Although, under these HPLC conditions, retinol and lutein could be accurately estimated at 325 nm and 450 nm, respectively, the separation of the various components of this polar fraction of human plasma needed to be modified to allow the identification and accurate determination of the new carotenoids. Therefore the most polar fraction of extracts from human plasma, which only contained these low retention-time components was concentrated by preparative thin layer chromatography (TLC) on C_{18} reversed phase plates and examined on a nitrile bonded column.

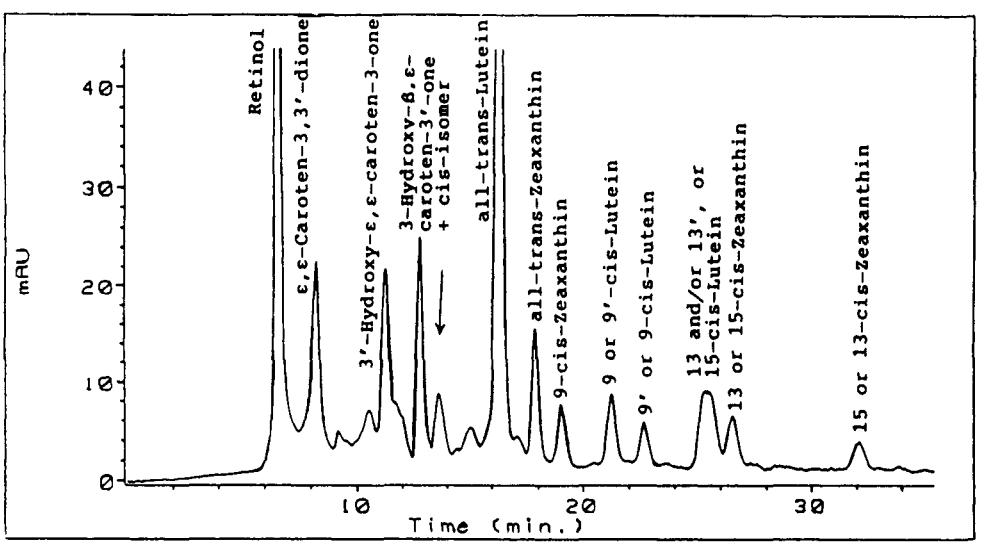

Fig. 5 Chromatogram of the most polar xanthophyll fraction of an extract from human plasma.

The HPLC separations were performed on a nitrile bonded silica (25-cm length x 4.6-mm i.d.; 5-μm particle size) column (Regis Chemical Co.), which was protected with a guard column (1-cm length x 4.6-mm i.d.) packed with the same adsorbent as the column. An isocratic mixture of hexane (74.6%) , dichloromethane (25%), methanol (0.3%), and ethyl diisopropylamine (0.1%) was employed with a flow rate of 1 ml/min. In addition to retinol, lutein, and zeaxanthin, the chromatographic profile (Fig. 5) of the most polar fraction of human plasma under these conditions, showed the presence of carotenoids, which were identified as ε, ε-carotene-3,3'-dione, 3'-hydroxy-ε,ε-caroten-3-one, and 3-hydroxy-β,ε-caroten-3'-one. The chemical structures of these carotenoids are shown in Fig. 6. Also present were several geometrical isomers of lutein and zeaxanthin, which were tentatively identified from their UV/visible absorption spectra.

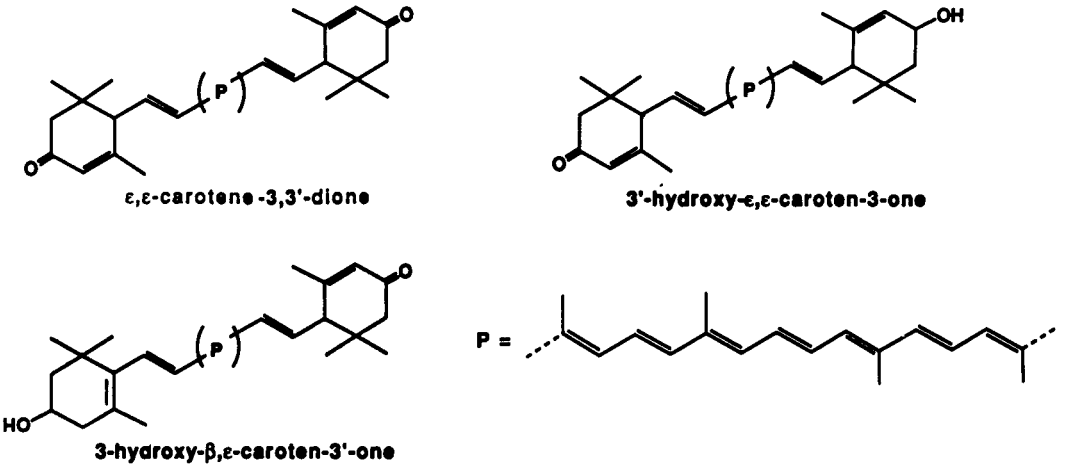

Fig. 6 Chemical structures of polar ketocarotenoids isolated from an extract of human plasma

The presence of these carotenoids in human plasma is of particular interest, because they have been isolated from hen's egg yolk by several researchers (refs. 46-48). Schiedt et al. (ref. 47) and Matsuno et al. (ref. 48) reported that these ketocarotenoids are oxidation products of lutein and zeaxanthin in hen's egg yolk and suggested possible metabolic pathways for their formation. Although the presence of ketocarotenoids in human plasma may suggest that these compounds are metabolic products of lutein, or perhaps even zeaxanthin, such assumptions may be premature at the present time, in the absence of human studies with labelled carotenoids. From our extensive studies of the distribution of carotenoids in common fruits and vegetables, it appears that the sole dietary source of these ketocarotenoids is limited to egg yolk and egg products. At the present the absolute configurations of the ketocarotenoids isolated from extracts of human plasma are not known; knowledge of their stereochemistry could provide an insight into the metabolic pathways of carotenoids in humans. If the ketocarotenoids isolated from human plasma are indeed metabolites of dietary carotenoids, their presence may support the idea of a protective role for carotenoids against oxidation. For a comprehensive review of antioxidant functions of carotenoids the reader is referred to the articles by Krinsky (ref. 49 & 50). One of the hypotheses to explain the possible role of carotenoids as cancer preventing agents is based on the antioxidant capability of these compounds to quench singlet oxygen and other oxidizing species, inhibit lipid peroxidation and prevent the neoplastic cells from further promotion and replication (ref. 51). If a free radical mechanism is involved in the initiation and promotion of carcinogensis, it is possible that carotenoids such as lutein may participate in the quenching of peroxides and the prevention of cellular oxidative damage. Under such conditions, lutein could undergo allylic oxidation to 3-hydroxy-β,ε-caroten-3'-one. Although the mechanism by which carotenoids may assist in prevention of cancer is not known, the isolation of these oxidation products of carotenoids from human plasma opens a new era in research on carotenoid metabolism in humans.

Acknowledgement

We thank F. Hoffmann-La Roche & Co. (Basel, Switzerland) for their gift of carotenoid reference samples. We thank Dr. J. C. Smith and Mrs. Anne Dulin (USDA, Vitamins & Minerals Lab.) for their helpful advice and assistance in the extraction of a large volume of human plasma. Partial support by the National Cancer Institute through reimbursable Agreement Y01-CN-30609 is acknowledged.

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