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Review

Chromatographic analysis of *cis/trans* carotenoid isomers☆

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ABSTRACT

Cis/trans configurations of carotenoids are known to effect the biochemistry of carotenoids in certain situations. Methodology for separating carotenoid *cis/trans* isomers is of importance to nutritionists and food scientists because *cis* isomers of provitamin A carotenoids have lower provitamin A activities than the all-*trans* form. Traditional food processing and preservation methods, especially thermal treatments, induce the formation of *cis* isomeric forms. However, many challenges are apparent for identifying and analyzing *cis/trans* isomers present in foods and other biological tissues. The development of current chromatographic methods for the separation of carotenoid *cis/trans* isomers is reviewed. For the separation of β-carotene isomers, most procedures employ either $Ca(OH)_2$ or Vydac C_{18} columns. In general, polymeric C_{18} columns allow for the detection of *cis* carotenes, while monomeric C_{18} columns provide for some separation of certain xanthophylls. The main *cis* isomers detected in foods are the 13-*cis* and 9-*cis* forms, although other forms have also been found (mainly 15-*cis* and various di-*cis* isomers). More studies involving the metabolism and physiological consequences of *cis/trans* isomers in the diet are needed. However, due to limitations in current techniques, further method development in the area of separation, detection and quantitation of *cis/trans* carotenoid isomers will be required.

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1. INTRODUCTION

Carotenoids have many biochemical roles, including the important functions of light harvesting and photoprotection in photosynthesis [1,2] as well as the provitamin A activity of certain carotenoids [3]. There is growing evidence that carotenoids may have a protective effect against the development of certain cancers [4], perhaps due to their antioxidant and free radical quenching activity [5]. In addition, some evidence suggests that carotenoids may have an immune enhancement effect that helps to limit cancer growth [6].

Currently, 563 naturally occurring carotenoids have been isolated and identified [7]. Individual carotenoids are capable of forming different *cis/trans* geometrical isomers. Geometrical configuration is known to effect the biochemistry of carotenoids in certain situations. For example, carotenoids exist in the 15-*cis* form in the reaction centers and the all-*trans* configuration in the light harvesting centers of photosynthetic organisms [1]. Animal studies indicate differences in provitamin activity among the *cis/trans* isomers of provitamin A carotenoids [8]. Isomer forms of carotenoids may have physiological or metabolic roles that are still being investigated. Also, methodology for the separation of *cis/trans* carotenoid isomers is necessary for accurate determination of provitamin A activity in foods. Analyses of processing effects on carotenoid content also require separation of the various *cis/trans* isomers. While many review articles on the analysis of carotenoids have been written [9–11], few have discussed recent studies concerning the various isomers of certain carotenoids. A comprehensive text on *cis/trans* isomers of carotenoids and polyenes was written in 1962 by Zechmeister [12]. More recent reports are scattered throughout the literature.

The purpose of this paper is to present a thorough review of the chromatographic methods used to separate carotenoid *cis/trans* isomers and to discuss the results of the work for which these methods were developed. Also, included are discussions on the identity of the various isomers being detected through the application of new chromatographic methods. Furthermore, information is presented on the chemistry, biochemistry and/or nutritional implications of these isomers. An overview of the extraction and separation procedures for carotenoids in general is included along with some of the historical developments which contributed to our current understanding of carotenoid *cis/trans* isomers.

2. CAROTENOIDS AND THEIR *CIS/TRANS* ISOMERS

2.1. Nomenclature

Carotenoids are composed of isoprene units linked to form a conjugated double bond system. The pigmentation of these compounds is the result of the chromophore created by the series of conjugated double bonds. Typically, carotenoids contain eight isoprenoid units bonded such that the units are reversed at the center of the molecule. With this arrangement, many carotenoids are symmetrical in nature. The two central methyl groups are arranged in a 1,6 position relative to each other with the remaining nonterminal methyl groups forming a 1,5 positional relationship [13]. The structure of lycopene, shown in Fig. 1, illustrates a typical example.

The basic acyclic structure of joined isoprene units can be modified by hydrogenation, dehydrogenation, cyclization or oxidation [14]. The oxygenated derivatives are known as xanthophylls, while the hydrocarbon carotenoids are referred to as carotenes. The nomenclature of carotenoids is based on the carotene backbone and because of the symmetrical structure, one half of the molecule is numbered 1–15 from the end to the center, with addi-

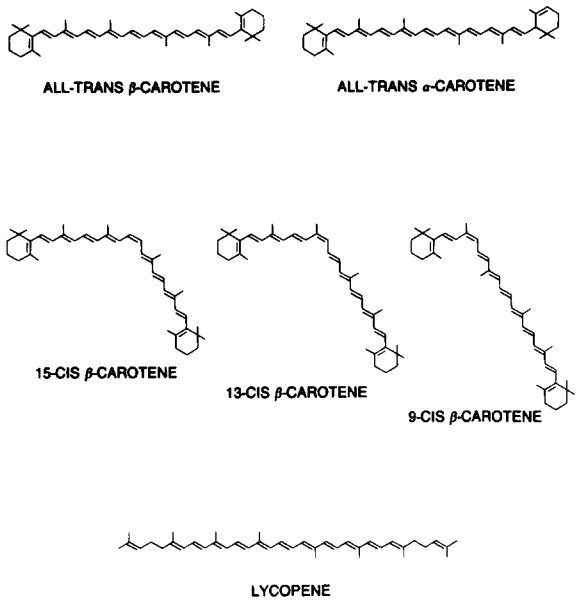

Fig. 1. Chemical structures of several carotenes.

tional methyl groups numbered 16–20. The other half is numbered in the same manner, only 1′–15′ and 16′–20′. Xanthophylls are widespread in nature, including hydroxy, methoxy, glycosyloxy, and carboxy derivatives [13] and these are often found esterified [15]. β-Carotene is a carotene which has undergone cyclization on either end to form two β-cyclohexene end-groups. β-Carotene is an example of a symmetrical carotenoid, while α-carotene is not, because it has a β-cyclohexene group on one end of the molecule and ε-cyclohexene group on the other.

Carotenoids undergo geometrical changes about the double bonds. Using the IUPAC nomenclature rules of carotenoids approved in 1974 [16], the stem name implies *trans*, thus *cis* configurations are specified by citing the specific bond as being *cis*. In Fig. 1, the β-carotene isomers are labeled with this terminology. These nomenclature rules will be used in this manuscript, instead of designating double bonds as *E* or *Z*. Isomers known to have at least one *cis* double bond but that are of uncertain configuration will be referred to as a *cis* isomer. The prefix "*neo*" has also been used to designate *cis* isomers of unknown configurations [17].

2.2. Historical background

While the existence of carotenes and xanthophylls was known prior to 1906, it was not until Tswett [18] developed column chromatography that much was known about the carotenoids. After its introduction by Tswett, open column chromatography became the principle technique for the separation of carotenoids. After Gillam and El Ridi [19] observed β-carotene separated into two zones with repeated adsorptions on an alumina column, investigations on the separation of geometrical isomers by column chromatography began. Polgár and Zechmeister [20] separated three major β-carotene bands, using a calcium hydroxide [$Ca(OH)_2$] packed column and a developer of petroleum ether with 1–5% acetone. Zechmeister [12] developed a nomenclature system to distinguish *cis* isomers of the same carotenoid separated chromatographically, but of unknown configuration. The *cis* isomers eluting prior to the all-*trans* carotenoid were referred to as *neo*-A, *neo*-B, *etc.*, while those isomers eluting after the all-*trans* compound are referred to as *neo*-U, *neo*-V, *etc.* [12]. The two main *cis* isomer bands for β-carotene were referred to as *neo*-β-carotene B and *neo*-β-carotene U by Polgár and Zechmeister [20]. Bickoff *et al.* [21] worked on improving the separation of these isomers, and found better separation with a developer of 1.5% *p*-methylanisole in petroleum ether.

Several treatments have been found that resulted in the formation of an equilibrium mixture of *cis–trans* isomers. These include: refluxing in organic solvents, melting of crystals, contact for prolonged periods with certain active surfaces, treatment with acids, and illumination of solutions (catalyzed by iodine) [12,14]. The equilibrium mixtures of isomerized carotenoids generally show two or three predominant *cis* isomers, which are referred to as the main or preferred isomers [12,14].

While the results of chromatographic separation revealed only several isomers, theoretically large numbers of possible geometrical configurations could occur because of the large number of double bonds in carotenoids (272 such isomers for β-carotene) [14]. The theory that best explains the limited number of preferred isomers was proposed by Pauling [22] in which two types of double bonds were described (hindered and unhindered). The methyl-substituted double bonds and the central double

bond in C_{40} carotenoids are generally unhindered from the formation of a *cis* configuration. The remaining double bonds are hindered, because the formation of the *cis* configuration is restricted by steric hindrance encountered between the methyl group attached to one of the adjacent carbons and a hydrogen attached to the other adjacent carbon. For β-carotene, only five double bonds are unhindered (position 9, 9′, 13, 13′, and 15–15′) [12]. Symmetrical carotenoids such as β-carotene have fewer possible configurations, because the formation of a *cis* double bond in the same position on either half of the molecule would yield the same structure; for example 9-*cis*-β-carotene and 9′-*cis*-β-carotene are the same molecule. Of the possible 20 configurations of β-carotene formed by *cis* isomerization about unhindered bonds, open column chromatography revealed 12 *cis* isomers with only two considered main isomers, *neo*-U and *neo*-B [12].

Spectroscopic properties were used by early chromatographers to distinguish between pigments and as a means to gain insight into possible structures. Absorption spectroscopy is still the major technique to identify and quantitate carotenoids [13]. The color of a carotenoid is produced by its absorption in the 400–500 nm region of the visible spectrum. Generally there are three maxima or two maxima and a shoulder, with the middle peak having the highest intensity [12]. One of the main determinants for the absorption spectra of these compounds is the number of conjugated double bonds. Longer chromophores have absorption maximum at longer wavelengths [13,14]. Modification from the basic acyclic structure can lead to wavelength shifts, such as shifts to longer wavelengths when carbonyl groups are added to the structure, or shifts to shorted wavelengths with the formation of ring structures [14]. The amount of fine structure in the spectral curves' shape and the intensity of absorption also vary among the carotenoids.

The absorption spectra of carotenoids are affected by the sample solvent. However, for a given solvent, the absorption spectra of an unknown carotenoid can be compared to spectra of known carotenoids to aid in identification. Reported absorptivities for given compounds are useful for the quantitation of the carotenoids. For example, the reported $E^{1\%} = 2592$ for β-carotene at 453 nm can be used to determine the amount of all-*trans*-β-carotene in a hexane solution [23].

As was found with different carotenoids, geometrical isomers of the same compound have different absorption spectra. In the visible region, the maxima shift to shorter wavelengths and the degree of fine structure decrease for spectra of *cis* isomers as compared to spectra of the compound in the all-*trans* configuration [12]. In the UV region, an absorption peak referred to as the *cis* peak is apparent for *cis* isomers, with the intensity of this peak varying with the position and number of *cis* double bonds [12]. For the mono-*cis* isomers of β-carotene, the intensity of the *cis* peak increases when the *cis* bond is located closer to the center of the molecule. Thus, 13-*cis*-β-carotene has a higher absorptivity in the *cis* peak region than does 9-*cis*-β-carotene. The UV–visible absorption spectra for β-carotene isomers, shown in Fig. 2, demonstrate the characteristic differences in absorption for carotenoid *cis*/*trans* isomers.

2.3. *Provitamin A activity of carotenoids*

While the importance of maintaining adequate levels of vitamin A in the body has long been known, in many parts of the world both clinical and "subclinical" vitamin A deficiencies occur [24]. It has been estimated that 500 000 new cases of corneal xerophthalmia occur yearly in parts of Asia [25]. In addition to these cases of individuals showing clinical signs of vitamin A deficiencies, studies

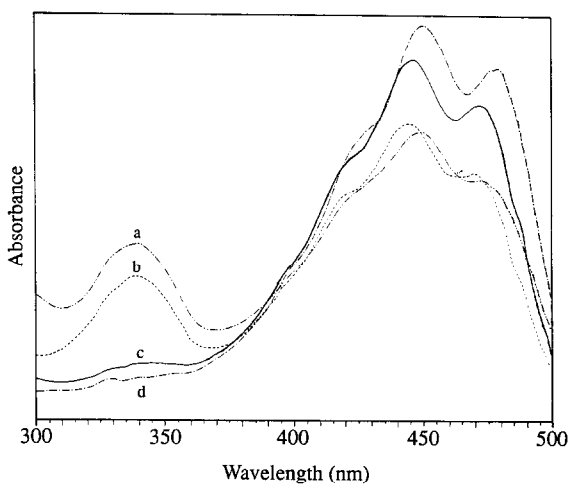

Fig. 2. Absorption spectra of (a) 15-*cis*-β-carotene, (b) 13-*cis*-β-carotene, (c) 9-*cis*-β-carotene and (d) all-*trans*-β-carotene. From O'Neil *et al.* [78].

have shown that vitamin A supplementation with children in Indonesia, India and South Africa can reduce mortality rates even in children without active xerophthalmia [26–28]. Because animal products are typically not accessible to those at risk for deficiency, provitamin A carotenoids derived from plant tissues are important in maintaining vitamin A levels.

Since it can be cleaved to theoretically yield two molecules of retinal, β-carotene has the highest provitamin A activity of the carotenoids. However, feeding studies suggest that the *cis* isomers of β-carotene have lower provitamin A activities when compared to the all-*trans* form [8,29–31]. Sweeney and Marsh [8] performed a vitamin A liver storage study of rats fed various carotene isomers. The rats fed *cis* isomers of β-carotene were found to have as low as 61% of the vitamin A liver storage compared to the amount of vitamin A stored by rats fed all-*trans*-β-carotene.

Most studies have shown that other provitamin A carotenoids have similar decreased vitamin A activity in the *cis* form. Sweeney and Marsh [8] found *cis* isomers of α-carotene also had lower vitamin A liver storage values in rats than all-*trans*-α-carotene. *Cis* isomers of cryptoxanthin and most *cis* isomers of γ-carotene have been shown to have lower provitamin A activity [32]. One exception is pro-γ-carotene, a penta-*cis* isomer of γ-carotene which was found to have a higher provitamin A activity in rats in comparison to all-*trans*-γ-carotene [33].

2.4. Extraction and separation of carotenoids

The conjugated double bond systems of carotenoids causes them to be particularly unstable compounds, especially sensitive to light, heat, oxygen, and acids [14]. Because of this instability, several precautions are necessary, when handling carotenoids. For example, laboratory experiments should be carried out in dim lighting, evaporation should be performed by rotary evaporation at reduced pressures with final stages of evaporation carried out under a stream of nitrogen, and samples should be stored in the dark, under nitrogen, at about −20°C [14].

The extraction procedures for carotenoids vary because of the wide variety of tissues and products containing these compounds. Detailed description of extraction procedures for various products have been discussed [14,34]. Carotenoids are fat soluble but because of the high moisture content of plant tissues, a preliminary extraction solvent miscible with water is generally necessary to allow for penetration of the solvent. Methanol is often used as an initial extractant [14,34]. Water-immiscible solvents can be used after the sample is dehydrated, however it has been found that more efficient extractants are composed of a slightly polar solvent in addition to the nonpolar solvent [34]. After extraction, the carotenoids are transferred to the nonpolar solvent by separating the liquid phases; adding water can facilitate this separation [34]. Saponification of carotenoid extracts may be necessary to remove neutral fats, chlorophylls, and chlorophyll derivatives. This procedure can be accomplished by exposure to potassium hydroxide saturated methanol [34].

Chromatographic methods are still used for the separation of carotenoids, however, newer methods have generally replaced open column methods. Taylor [35], Lambert *et al.* [36] and Tee and Lim [11] have written review articles on the various chromatographic methods for the analysis of carotenoids and retinoids. Thin-layer chromatography (TLC) has been found to be useful for qualitative analyses and as an aid in identification based on adsorption affinities [14]. Zakaria *et al.* [37] reviewed a wide variety of applications for TLC, including pigments. High-performance liquid chromatography (HPLC) offers several advantages over the TLC and open column methods for both analytical and preparative separations. Rüedi [38] discusses the usefulness of HPLC technologies for the analysis of carotenoids. Rodriquez-Amaya [10] has critically reviewed and compared open column and HPLC methods for the analysis of provitamin A carotenoids from plant tissues.

Many researchers have developed and used HPLC methods to qualitatively and quantitatively analyze carotenoids. Thompson and Maxwell [39] developed reversed-phase methods to determine β-carotene in several dairy products. Zakaria *et al.* [40] developed a reversed-phase method to separate carotenes in tomatoes. Similar reversed-phase methods have been used with other fruits and vegetables [41–44]. The US Department of Agriculture Human Nutrient Composition Laboratory has published extensively in the area of HPLC separation methods for carotenoids and carotenoid fatty

acid esters [45–48]. Beecher and Khachik [49], discussing the analysis of carotenoids in foods using HPLC methods, summarized much of this laboratory's developments in this area.

A more recent review by these authors surveyed the current literature on the separation, identification and quantification of carotenoids, which included developments in the analysis of human plasma as well as plant tissues [50]. Khachik *et al.* have since developed HPLC methods for the separation of plasma carotenoids [51]. Others researchers have also developed methods to quantitate specific carotenoids in serum and plasma [52–56].

3. ANALYSES OF β-CAROTENE *CIS/TRANS* ISOMERS

3.1. Historical background

After the separation of β-carotene isomers by open column chromatography methods [20,21], *cis* isomers were detected in extracts from several food products [57–59]. An analysis of a wide variety of both fresh and processed products was performed by Panalaks and Murray [60], using open column chromatographic methods. These authors found only small amounts of *neo*-β-carotene B and *neo*-β-carotene U in the extracts from most of the fresh and treated products, but increases in the relative amounts of *cis* isomers, especially *neo* B, were found for some canned food products.

While many TLC methods have been developed for the analysis of carotenoids [34,36], there are few reports on resolving β-carotene isomers. Sadowski and Wójcik [61] detected three *cis* isomers for both α- and β-carotene from TLC separations of bean leaf extracts. The method involved TLC plates of magnesium oxide that were developed with solutions of acetone in light petroleum. Schwartz and Patroni-Killam [62] developed a TLC method based on open column chromatography methods, that resolved three β-carotene isomers and four α-carotene isomers. The TLC plates were of $Ca(OH)_2$ and these were developed with 1.5% *p*-methylanisole in petroleum ether. *Cis* isomers were detected in a number of carotenoid extracts from fresh and processed vegetables, with the exceptions of fresh sweet potato and carrot extracts.

3.2. Development of modern HPLC methods and their use in plant tissue analyses: normal phase

Pressurized liquid chromatographic separations of β-carotene isomers began with the work of Sweeney and Marsh [63]. Based on open column adsorption chromatography, a 30 cm × 15 mm column was packed with a mixture of magnesium hydroxide and $Ca(OH)_2$ and an eluent of 1.5% *p*-methylanisole in petroleum ether was pumped through by nitrogen pressure. The principle isomers detected in extracts from green vegetables were all-*trans*-β-carotene and *neo*-β-carotene U, while extracts from red and yellow vegetables contained all-*trans*-β-carotene and *neo*-β-carotene B [63]. In 1971, these authors reported that thermal processing of vegetables increased the relative amounts of *cis* isomers detected [64].

The method of Sweeney and Marsh [63] was used by others to investigate the effects of processing. Lee and Ammerman [65] evaluated the effects of several canning processes on the β-carotene composition of sweet potatoes. The relative amount of *neo*-β-carotene B was found to increase significantly with canning. Contrary to results with other plant tissues, Gebhart *et al.* [66] found heat processing of peaches did not effect isomer composition. Ogunlesi and Lee [67] reported increases in *cis* isomer content, with concurrent loss of all-*trans*-β-carotene, when carrots were processed. More recently, Van der Pol *et al.* [68] reported increased relative isomer content after traditionally cooking Indonesian vegetables.

Vecchi *et al.* [69] developed an HPLC method based on adsorption chromatography, using an alumina (Al_2O_3) column with an eluent of hexane. The system was designed such that the hexane eluent had a controlled water content. Using NMR to identify geometrical configurations, they found 9-*cis*-β-carotene, 13-*cis*-β-carotene and 15-*cis*-β-carotene to be the main *cis* isomers in isomerized β-carotene preparations. Five minor isomers (mainly di-*cis*) were also isolated and identified. The 9-*cis* and 13-*cis* isomers were the main *cis* isomers in carrots and paprika, with none of the other isomers being detected at levels higher than 2% of the total carotene.

Tsukida *et al.* [70], using NMR, confirmed that the configurations of the *neo*-β-carotene B and *neo* β-carotene U from $Ca(OH)_2$ open column chroma-

tography were 13-*cis*- and 9-*cis*-β-carotene, respectively. Based on this open column method, Tsukida *et al.* [71] developed an HPLC method employing a $Ca(OH)_2$ column with a mobile phase of hexane containing 0.1–2.0% acetone. The authors identified nine *cis* isomers of β-carotene in thermally isomerized β-carotene. With this method, the sterically hindered 7-*cis*-β-carotene, which was thought to be too labile to isolate, was identified [72]. Later, Ca $(OH)_2$ columns of greater resolution were produced [73] and these allowed for the further identification and reassignment of certain di- and tri-*cis* isomers, using ^1H NMR. Fig. 3 illustrates this separation.

Chandler and Schwartz [74] using a $Ca(OH)_2$ HPLC column method similar to Tsukida *et al.* [71] reported on the isomeric composition of carotenoid extracts from fresh and processed fruits and vegetables. The main *cis* isomers of β-carotene detected were 9-*cis* and 13-*cis*. Chandler and Schwartz [75] used this method to evaluate the effects of various processing techniques on the β-carotene isomer composition of sweet potato. Severe heat treatments resulted in higher relative percentages of isomers (dehydration, 28.9%; and baking, 23.0%) as compared to milder processing techniques (canning, 17.2%; microwaving, 16.5%; and blanching

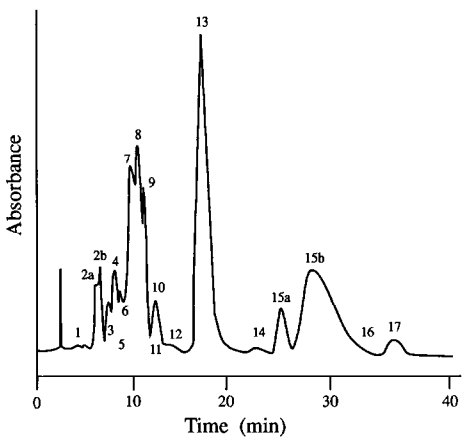

Fig. 3. Chromatographic separation of thermally isomerized β-carotene using $Ca(OH)_2$ packed column and mobile phase of 0.1% acetone in *n*-hexane. Peaks: 2 = 13,15-*cis*-β-carotene, 3 = 15-*cis*-β-carotene, 4 = 13,13′-*cis*-β-carotene, 7 = 13-*cis*-β-carotene, 8 = 9,15-*cis*-β-carotene, 9 = 9,13′-*cis*-β-carotene, 10 = 9,9′,13-*cis*-β-carotene, 12 = 7,13′-*cis*-β-carotene, 13 = all-*trans*-β-carotene, 14 = 7,9-*cis*-β-carotene, 15a = 9,9′-*cis*-β-carotene, 15b = 9-*cis*-β-carotene and 17 = 7-*cis*-β-carotene (the other peaks were not identified). From Koyama *et al.* [73].

10 min, 15.3%). As was seen by Lee and Ammerman [65], 13-*cis*-β-carotene (*neo*-β-carotene B) was the predominant isomer formed. Pettersson and Jonsson [76], using similar methods, reported fresh carrot juice did not contain significant *cis* isomers, while heat processing the juice resulted in the detection of several *cis* isomers. Thus, as was seen by earlier researchers employing the method of Sweeney and Marsh [63], these researchers using HPLC methods found that heat processing increases *cis* isomer content.

Most of the reported normal-phase separations of β-carotene isomers involved $Ca(OH)_2$ columns. The mobile phase usually contained small amounts of acetone in hexane, however, Craft *et al.* [77] preferred to use pentane as the main solvent. Sometimes a gradient of increasing acetone content is necessary to achieve the desired selectivity of earlier peaks yet elute the more retained isomers (*i.e.* 9-*cis*-β-carotene). For example, Tsukida *et al.* [71] started their elution with 0.1% acetone in hexane and finished with 2.0% acetone in hexane. $Ca(OH)_2$ columns are not commercially available. Therefore, slight differences in packing techniques and handling procedures for the $Ca(OH)_2$ result in column to column variability [78]. Thus, adjustments in mobile phase composition is often required to obtain optimum resolution for a particular column.

One concern with normal-phase methods is that the water activity of the stationary phase is thought to be important in the resolution of the isomers. This was first investigated by Bickoff *et al.* [21] for open $Ca(OH)_2$ column chromatography. It was reported that for optimum separation, a relative humidity in the range of 30–50% is desirable. Schwartz and Patroni-Killam [62] found that for TLC plates equilibration to a relative humidity (RH) of 44% was required to obtain optimum resolution. Chandler and Schwartz [74] also used Ca $(OH)_2$ equilibrated to a RH of 44% to pack HPLC columns. An increase in the separation of some minor *cis* isomers was found by O'Neil *et al.* [78] when the $Ca(OH)_2$ was hydrated first and then equilibrated to 44% RH. Another approach to control water activity was taken by Petterson and Jonsson [76]. $Ca(OH)_2$ was dried at 105°C and stored in a desiccator prior to packing HPLC columns. While there is a need to control water activity for Ca $(OH)_2$ column separations, the precautions re-

quired are not as elaborate as those employed for alumina column chromatography in a moisture-controlled isolated HPLC system [69].

The mobile phase or injection solvent can change the water activity of the stationary phase, and water needs to be removed, especially from sample extracts. This can be done by adding anhydrous sodium sulfate followed by filtration to remove the crystals and bound water [79]. Pettersson and Jonsson [76] reconditioned $Ca(OH)_2$ columns that may have adsorbed water by evaporating solvent with a stream of nitrogen, drying in an oven at 105°C, and flushing the column with a stream of pure nitrogen. On the other hand, it has been observed in our laboratory that when some $Ca(OH)_2$ columns lose the ability to resolve the *cis* isomers, adding water to the mobile phase (0.05%) often returns activity. Thus, it appears the loss of water from the surface of the stationary phase can also effect the separation.

3.3. Development of modern HPLC methods and their use in plant tissue analyses: reversed-phase

Several reversed-phase methods have been reported that separate geometrical isomers of β-carotene. Most of these methods involve the use of Vydac C_{18} TP columns (either a Vydac 201TP or a Vydac 218TP column). These columns are produced using trichlorosilanes, which is referred to as a polymeric synthesis of C_{18} reversed-phase columns. When monochlorosilanes are used, the column is said to be a monomeric column. These Vydac columns have a silica base that is spheroidal with a pore size of 300 Å and a surface area of 80 m^2/g. The wide bore is recommended for the separation of large bio-molecules. The low surface area results in a medium capacity reversed-phase column for the separation of very hydrophobic small molecules. The packing material of the Vydac 218TP column is endcapped with trimethylsilane after the C_{18} chain is bonded to the TP silica to prevent adsorption of polar compounds. No endcapping is performed for the Vydac 201TP column and thus is recommended for very nonpolar compounds.

Jensen *et al.* [80] resolved several β-carotene isomers employing a Nucleosil C_{18} column and a mobile phase of acetone–water (88:12). Analyzing solutions of all-*trans*-β-carotene photoisomerized in the presence of chlorophyll *a*, revealed the formation of three isomers, which were identified as 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene based on their absorption spectra and coelution with an authentic standard of 15-*cis*-β-carotene. The main isomers formed were 9-*cis*- and 13-*cis*-β-carotene, with the 9-*cis* isomer generally being the most predominant *cis* isomer. This method of separation was attempted in our laboratory, however, the 13-*cis* and 15-*cis* isomers coeluted and the 9-*cis* isomer eluted with all-*trans*-β-carotene [78].

Bushway [81] compared various reversed and normal-phase HPLC methods and concluded that Vydac C_{18} columns provided quicker and better separation of carotenoids. Several methods were found that separated at least three β-carotene peaks. Bushway [82], using the Vydac 218TP column and an eluent of methanol–acetonitrile–tetrahydrofuran (56:40:4), analyzed several carotenoid extracts from raw fruits and vegetables. Iodine isomerized β-carotene was analyzed and spectra of chromatographic peaks were used for peak identification. Both 15-*cis*- and 9-*cis*-β-carotene were detected.

Pesek and co-workers [83,84] employed similar methods to investigate the isomerization of β-carotene in organic solvents and during illumination of solutions. The mobile phase was slightly different being methanol–acetonitrile–tetrahydrofuran (58:42:1). They first investigated the effect of sample solvent on spontaneous isomerization [83]. All-*trans*-β-carotene was stored for 24 h at 25°C in the dark. Their results confirmed previous concerns that traces of HCl in chloroform may result in isomerization [9]. Both methylene chloride and chloroform resulted in a 20–30% loss in all-*trans*-β-carotene. On the other hand, the mobile phase solvents could be used without extensive isomerization ($\leqslant 5\%$ loss of all-*trans*-β-carotene). The kinetics of isomerization in the mobile phase at 45°C (in the dark) was also investigated [83]. After 4–6 days, an equilibrium mixture of isomers was reached, with all-*trans*-β-carotene constituting 67% of the β-carotene. The two *cis* isomers formed during isomerization were 9-*cis*-carotene and 13-*cis*-β-carotene. The rate of formation and equilibrium concentration of 13-*cis*-β-carotene was higher than that of 9-*cis*-β-carotene. Contrary to these results, when β-carotene was exposed to light (at 28°C), the photoisomerization resulted in the formation of more

9-*cis*-β-carotene than 13-*cis*-β-carotene [84]. However, it was noted that the rate of photodegradation under these conditions was much greater than the rate of photoisomerization.

Quackenbush and Smallidge [85] using the Vydac 201TP C_{18} column with a mobile phase of methanol–chloroform (90:10) resolved the *cis* isomers of β-carotene from the all-*trans* isomer. Quackenbush [86] investigated the β-carotene content of several fruits and vegetables, using a Vydac 201TP column and a gradient of methanol for 5 min followed by methanol–chloroform (94:6) or for isocratic analysis, methanol–chloroform (94:6). Fig. 4 is a chromatogram of an extract from a canned carrot sample, separated using the isocratic mobile phase. Spectral data and results from an analysis of iodine isomerized β-carotene were used to identify resolved β-carotene peaks. It was found that the 9-*cis*, 13-*cis*, and all-*trans* isomers were resolved. Results from analyses of plant extracts were similar to Sweeney and Marsh [63] with 9-*cis*-β-carotene being detected at relatively higher amounts in extracts from green vegetables and more 13-*cis*-β-carotene found in extracts from yellow vegetables. This author also found a larger percentage of *cis* isomers in processed products, in agreement with Chandler and Schwartz [74] and Sweeney and Marsh [64].

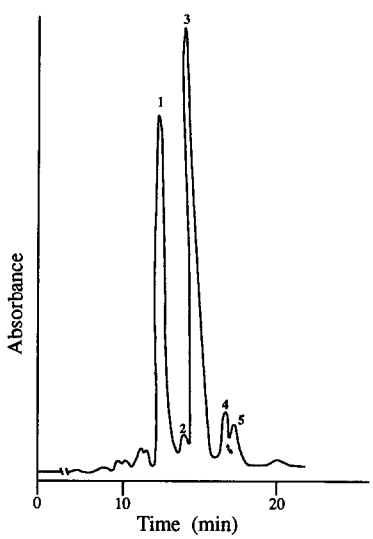

Fig. 4. Chromatographic separation of an extract from canned carrots using a Vydac 201TP C_{18} column and a mobile phase of methanol–chloroform (94:6). Peaks: 1 = all-*trans*-α-carotene, 2 = *cis*-α-carotene, 3 = all-*trans*-β-carotene, 4 = 13-*cis*-β-carotene and 5 = 9-*cis*-β-carotene. From Quackenbush [86].

Saleh and Tan [87] worked on optimizing a mobile phase to separate a number of carotenoid *cis/trans* isomers. A mobile phase of acetonitrile–methanol–methylene chloride (80:18:2) led to separation of several *cis* isomers of nonpolar carotenoids using a Zorbax column and also separated β-carotene isomers when a Vydac 218TP column was used. *Cis* isomers of β-carotene (identified as 9-*cis* and 13-*cis*-β-carotene) were detected from analyses of palm and carrot oils.

Another optimization of carotene *cis/trans* isomer separations was reported by Lesellier *et al.* [88]. An eluent of acetonitrile–methanol–methylene chloride (63:27:10) was used with a polymeric C_{18} column. As was found by others [77,81,85], the polymeric C_{18} column (Brownlee ODS RO 18) provided greater separation of the *cis/trans* isomers. It is interesting to note that the *cis* isomers eluted prior to the all-*trans* isomer on a monomeric Brownlee OD RP 18 column. This is in contrast to reports by others using monomeric columns, where the appearance of a *cis* isomer shoulder following the β-carotene peak was seen [45,89].

Polymeric C_{18} columns appear to offer the best reversed-phase separation of β-carotene *cis/trans* isomers, with Vydac C_{18} columns being the most often reported. It has been speculated that the wide bore of the polymeric columns allows greater interaction of the 33 Å long β-carotene molecule with the bonded phase [77,90]. Of the Vydac polymeric columns, the non-endcapped Vydac 201TP appears to offer greater resolution [78,85]. However, several researchers have had success in resolving the main *cis* isomers with endcapped columns [81,83,84,87]. An advantage of endcapped Vydac columns is short elution times [81].

One disadvantage of using polymeric C_{18} columns is that column to column variability tends to occur to a greater extent than for monomeric columns [91]. Resolution among the isomers with Vydac C_{18} columns has been reported to vary [78]. Another factor that can limit full resolution of the isomers on non-endcapped Vydac C_{18} columns is deposition of lipid material on the column. It appears that the insolubility of nonpolar compounds in a mobile phase of primarily methanol results in lipid material remaining on the column. This results

in decreased interaction of the carotenes with the stationary phase and loss of resolution [78]. In our laboratory, washing the columns frequently with methanol–chloroform (50:50) maintains or restores the original separation.

For the predominant methods employing normal-phase separations, NMR investigations were performed to identify the carotene isomers [69,73]. This is not the case for most reports of reversed-phase separations. The fact that positive identification is not always performed may result in the misidentification of certain isomers. The presence of a shoulder eluting after all-*trans*-β-carotene has been reported by a number of authors using monomeric C_{18} reversed-phase HPLC [45,46,89,92–94]. Speculations on the identity of this shoulder have been made based on coelution with standards or absorption spectra. However, this shoulder may contain a combination of isomers. For several monomeric C_{18} reversed-phase separations, standards of the main *cis* isomers (13-*cis*-, 15-*cis*-, and 9-*cis*-β-carotene) all eluted in the shoulder, demonstrating the lack of separation by these columns [78]. In addition to coelution problems with monomeric columns, several of the polymeric C_{18} reversed-phase methods have been reported to coelute 13-*cis*- and 15-*cis*-β-carotene [78].

The *cis* isomer eluting as a shoulder immediately following all-*trans*-β-carotene is observed when employing monomeric columns and has been identified as 15-*cis*-β-carotene in several fruit and vegetable samples [43,45,94,95]. These identifications were based on coelution with an authentic 15-*cis*-β-carotene standard. This standard has been readily available, because it is the precursor to all-*trans*-β-carotene in the organic synthesis of β-carotene [96]. Due to the accessibility of this *cis* isomer, while commercial standards of the other *cis* isomers were not attainable, the retention time of only this isomer could be confirmed. However, other researchers employing polymeric columns or $Ca(OH)_2$ columns have found that the 13-*cis* and 9-*cis* isomers are more prevalent in plant tissues [74,86]. Thus, the reports of the 15-*cis* isomer may in actuality include other isomers.

One advantage of reversed-phase chromatography is that additional handling to ensure complete removal of water from the sample is not required. However, in the case of the analysis of carotene, additional sample handling is often required. This is necessary because problems can arise when the sample solvent is not miscible with the mobile phase [97]. Nonpolar solvents [polarity (P') < 5] are often used for the extraction of carotenes. These solvents may not be miscible with relatively polar mobile phases (P' > 5), and this may lead to artifact formation [97]. From investigations in this laboratory, the method found to prevent this problem is to evaporate the nonpolar solvent, redissolve in ethyl ether and dilute with methanol to a proportion of 20:80 (P' = 4.6). This sequence of solvent transfers results in a carotene solution which is miscible with the mobile phase. This procedure adds extra handling, however, improved recovery and chromatographic separation is achieved.

While some of the reported reversed-phase separations utilize large amounts of acetonitrile in the mobile phase, it appears that using methanol as the primary solvent for carotenoid analysis results in greater selectivity [77]. In addition, low recoveries have been reported when acetonitrile was employed as the primary solvent. Craft *et al.* [77] reported only 74% recovery of β-carotene with an acetonitrile-based solvent; however, recoveries increased to 89% with a methanol-based mobile phase. Even though similar solubilities for β-carotene in methanol and acetonitrile were measured [98], between the two, methanol appears to be the solvent of choice to maximize sample recovery. On the other hand, the addition of small amounts of ammonium acetate (0.01%) to the mobile phase has been found to increase recoveries, when an acetonitrile-based mobile phase is used for carotenoids [99].

Temperature for optimum selectivity values was reported to be between 20 and 25°C for a polymeric C_{18} column with a mobile phase of acetonitrile–methanol–methylene chloride (45:45:10) [88]. It was found that the selectivity (α) for *cis*-α-carotene/α-carotene was inversely related to temperature, while α for β-carotene/α-carotene and *cis*-β-carotene/β-carotene decreased as temperature decreased. A column temperature of 20°C was also selected by Craft *et al.* [100] as the optimum for separating a mixture of carotenoids with a polymeric C_{18} column and a mobile phase of methanol–tetrahydrofuran (95:5).

3.4. Analyses of serum, plasma and human tissues

Bieri *et al.* [54] developed an HPLC method to detect additional carotenoids that had not been previously reported in plasma. The separation employed a C_{18} column with an eluent of acetonitrile–methylene chloride–methanol (70:20:10). A photodiode array detector was used in the method development and it was noted by these authors that the tail of the β-carotene peak contained 15-*cis*-β-carotene. This identification was based on its high absorbance at 340 nm. Sowell *et al.* [101], using a Microsorb C_{18} column and a mobile phase of ethanol–acetonitrile (1:1), detected a *cis* isomer of β-carotene in human serum. The β-carotene *cis* isomer was suggested to be 15-*cis*-β-carotene based on the previous report by Bieri *et al.* [54].

Jensen *et al.* [102] investigated the effects of ingesting *cis* isomers of β-carotene on serum levels in humans. A *cis* isomer peak was resolved from all-*trans*-β-carotene with a Vydac 201TP C_{18} column and an eluent of methanol–acetonitrile (90:10). The amount of all-*trans*-β-carotene was compared to *cis*-β-carotene in the serum of humans fed a β-carotene preparation from *Dunaliella* algae, a source of β-carotene that is high in the 9-*cis* isomer [103]. There was not a large increase in *cis*-β-carotene in the serum of these individuals.

Rushin *et al.* [104], in their analysis of human sera, also resolved *cis* isomers of β-carotene from the all-*trans* form. Two Vydac 201TP C_{18} columns connected in series were used with a mobile phase of methanol–chloroform–tetrahydrofuran (87:10:3). Standards of all-*trans*, 9-*cis* and 13-*cis*-β-carotene were isolated using a semi-preparative $Ca(OH)_2$ column separation and their identities confirmed using absorption spectra and ^{1}H-NMR. For serum samples, a collective *cis* isomer peak was separated from the all-*trans*-β-carotene, but individual *cis* isomers were not resolved. The standards of 13-*cis*- and 9-*cis*-β-carotene coeluted with this *cis* peak. Absorption spectra from serum samples suggested that 13-*cis*-β-carotene is the predominant *cis* isomer in human sera, however small quantities of other isomers may have been present in the serum extracts. The relative amount of *cis* isomers compared to all-*trans*-β-carotene in the serum is similar to that found for a mean value calculated from a variety of fruits and vegetables. These results indicate that the carotenes are likely absorbed as the geometrical configuration in which they were ingested.

Another method to resolve the *cis* isomers from all-*trans*-β-carotene was reported by MacCrehan and Schönberger [105]. These authors developed a multivitamin serum assay to quantitate retinol, α-tocopherol and β-carotene. A Vydac 201TP C_{18} column was used with a gradient of methanol–water–*n*-butanol (75:15:10) for 3 min followed by a linear increase to methanol–water–*n*-butanol (88:2:10) over 15 min. In contrast to Rushin *et al.* [104], a later article [90] identified the *cis* isomer peak as 9-*cis*-β-carotene.

Stahl *et al.* [106] were able to separate 13-*cis*- and 15-*cis*-β-carotene as one peak from all-*trans*-β-carotene for serum samples. These authors used a C_{18} endcapped column (E. Merck, Germany) with a mobile phase of methanol–acetonitrile–methylene chloride–water (7:7:2:0.16). This separation is illustrated in Fig. 5. Another isomer, 9-*cis*-β-carotene, was observed in many of the human tissues analyzed (liver, kidney, adrenal glands, and testes), however, it was not found in the serum samples. Since 13-*cis*-β-carotene and 15-*cis*-β-carotene were not resolved under these conditions, it is not clear whether 15-*cis*-β-carotene is a prevalent isomer in human plasma or tissues. Previous reports of 15-*cis*-β-carotene in serum [54,101] employed monomeric

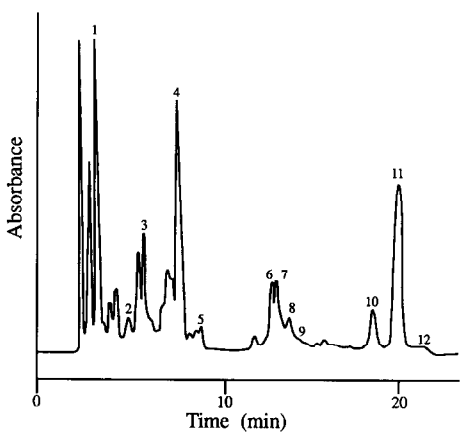

Fig. 5. Chromatographic separation of an extract from human serum using a C_{18} column with a mobile phase of methanol–acetonitrile–dichloromethane–water (7:7:2:0.16). Peaks: 1 = zeaxanthin, 2 = canthaxanthin, 3 = standard, 4 = cryptoxanthin, 5 = *cis*-cryptoxanthin, 6 = lycopene, 7 = 9-*cis*-lycopene, 8 = 13-*cis*-lycopene, 9 = 15-*cis*-lycopene, 10 = α-carotene, 11 = all-*trans*-β-carotene and 12 = 13-,15-*cis*-β-carotene. From Stahl *et al.* [106].

C_{18} column methods which have been shown to coelute the *cis* isomers of β-carotene [78].

3.5. Algal sources of carotenes

Ben-Amotz *et al.* [103] used an HPLC alumina column (Alox-T) with an eluent of hexane–methylene chloride (65:35) to analyze the algae species *Dunaliella salina* and *Dunaliella bardawil*. The authors reported that the algae accumulated approximately as much 9-*cis*-β-carotene as all-*trans*-β-carotene. Smaller quantities of other isomers were detected, with one isomer identified as 15-*cis*-β-carotene. Later, similar results were found using the method of Jensen *et al.* [102] employing a Vydac 201TP C_{18} column and a mobile phase of methanol–acetonitrile (90:10) [107]. A typical chromatogram illustrating the separation of the algal carotenoids is shown in Fig. 6. Watanabe and Hayashi [108] using a YMC pack ODS-A column and a mobile phase of acetonitrile–methanol–*n*-hexane–ethyl ether (70:15:10:5) also detected high amounts of 9-*cis*-β-carotene present in *Dunaliella* powder.

Ben-Amotz *et al.* [109,110] have been investigating the use of β-carotene from *Dunaliella* algae as a source of natural β-carotene. Rats and chicks fed β-carotene from *Dunaliella bardawil* satisfied the total requirements for retinol. Liver assays showed at least a ten-fold increase in β-carotene storage for both species when they were fed the algae source of carotene as compared to animals fed a diet containing synthetic all-*trans*-β-carotene [111]. The ratio of 9-*cis*- to all-*trans*-β-carotene in the liver was approximately the same as the algae β-carotene preparation. To determine if this greater storage level

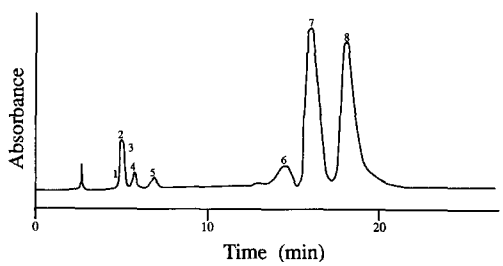

Fig. 6. Chromatographic separation of an extract from *Dunaliella bardawil* algae using a Vydac 201TP C_{18} column and a mobile phase of methanol–acetonitrile (90:10). Peaks: 1 = chlorophyll *b*, 2 = lutein, 3 = unidentified, 4 = zeaxanthin, 5 = chlorophyll *a*, 6 = α-carotene, 7 = all-*trans*-β-carotene and 8 = 9-*cis*-β-carotene. From Ben-Amotz *et al.* [110].

was an effect of the 9-*cis* isomer or the result of other components present in the algae, partially purified 9-*cis*-β-carotene was collected and fed to chicks [112]. Chicks fed 9-*cis*-β-carotene stored ten times more β-carotene in the liver than those fed synthetic all-*trans*-β-carotene. Greater storage of β-carotene was found to have no adverse effect on retinol levels in the liver. The authors speculated that the 9-*cis* isomer is more fat-soluble and more difficult to crystallize and thus, may increase the absorption and deposition of the β-carotene.

This work by Ben-Amotz and co-workers [111,112] indicates that 9-*cis*-β-carotene may be absorbed and deposited in the tissues. The results of Rushin *et al.* [104] also suggest that *cis* isomers are likely absorbed along with all-*trans*-β-carotene and circulate in the blood. However in humans, Jensen *et al.* [102] found little increase in the relative amount of *cis*-β-carotene in the serum of adults fed algae extracts high in *cis*-β-carotene.

4. ANALYSES OF OTHER CAROTENOID *CIS/TRANS* ISOMERS

4.1. Early HPLC methods

The number of reports concerning *cis* isomers of carotenoids besides β-carotene has not been as extensive. However, chromatographic separation of other isomers is beginning to be addressed and should lead to more reports regarding other carotenoids. One of the few earlier reports detecting *cis* isomers was by Matus *et al.*, in 1981 [113]. These authors used a multiple wavelength UV–visible detector to detect *cis* peak absorbances. *Cis* isomers of violaxanthin, lutein and capsanthin were separated from their all-*trans* forms using a 10-μm C_{18} Nucleosil column and a gradient of acetone–water (100:40 then 100:5). The source from which these *cis* isomers were extracted was a Canadian waterweed, *Elodea canadensis*.

Ruddat and Will [114], evaluating HPLC techniques for the analysis of carotenoids, detected *cis*-β-zeacarotene in an extract from a yellow strain of sporidia, and *cis*-lycopene and *cis*-ζ-carotene from a tomato extract. A gradient of 2-propanol and acetonitrile–water (9:1) from a ratio of 30:70 to 55:45 with a C_{18} Ultrasphere column was used for separation. Fractions were collected and absorption spectra were recorded to aid in identification of chromatographic peaks.

4.2. Analyses of canthaxanthin cis/trans isomers

Nelis *et al.* [115] detected relatively large quantities of *cis*-canthaxanthin in the encysted embryos of the brine shrimp *Artemia*. It was found that *cis*-canthaxanthin was found in only the ovaries, eggs and the hemolymph of reproductively active females. It was speculated that these isomers may play a specific role in reproduction or embryo development.

Because of these findings, Nelis *et al.* [116] investigated the possibilities of improving both normal-phase and reversed-phase separation of these isomers. The optimum reversed-phase separation occurred with a Zorbax C_{18} column and a mobile phase of methanol–acetonitrile–methylene chloride (50:41:9). However, the *cis* isomers eluted as one peak. Normal-phase separation with a silica column and a mobile phase of methylene chloride–2-propanol (99.3:0.7) enabled the detection of 13-*cis*-, 15-*cis*- and 9-*cis*-canthaxanthin. While this normal-phase method provided the best separation for qualitative investigations, the authors noted that extra handling required to transfer extracts to hexane for HPLC analyses and the contact with acidic silica resulted in a quantitative loss of *cis* isomers. Thus, it was recommended that reversed-phase be used for quantitative analysis and normal phase for qualitative investigations.

Hashimoto and Koyama [117] also separated canthaxanthin isomers by normal-phase chromatography. $Ca(OH)_2$ columns similar to those prepared for β-carotene separations [73] were employed. The mobile phase was benzene–hexane (95:5) instead of acetone in *n*-hexane, as is used for β-carotene separations. Using ^1H NMR, they identified three mono-*cis* isomers (13-*cis*-, 15-*cis*-, and 9-*cis*-canthaxanthin) and four di-*cis* isomers, isolated from heated canthaxanthin crystals.

Another reversed-phase separation was reported by Mayne and Parker [118]. They used a C_{18} Ultrasphere column with mobile phase of ethanol–water (95:5). One peak was identified as *cis*-canthaxanthin with other peaks as possible *cis* isomers of canthaxanthin. These three peaks were observed in analyses of canthaxanthin beadlet preparations. When these beadlets were fed to chicks, the same compounds were found in liver and membrane extracts, indicating that *cis* isomers are absorbed and deposited intact.

4.3. Development of HPLC methods and their use in plant tissue analyses

Chandler and Schwartz [74] detected *cis* isomers of α-carotene in canned carrots, however, because only small quantities were found and complete resolution was not achieved, these isomers were not unequivocally identified. Pettersson and Jonsson [76] also found α-carotene isomers to be present in heat processed carrot juice when using a $Ca(OH)_2$ packed column. Photodiode array detection was used to tentatively identify two of these isomers as 13-*cis*-α-carotene and 9-*cis*-α-carotene.

Quackenbush [86] performed iodine-catalyzed isomerization of α-carotene, lutein and lycopene. The resulting isomeric mixtures were separated using a Vydac 201TP column and a mobile phase of methanol and/or methanol–chloroform (94:6). Similar to β-carotene, the predominant isomers of α-carotene were 13-*cis*- and 9-*cis*-α-carotene. An additional isomer was found for lutein, which was identified as 13′-*cis*-lutein. Five *cis* isomers of lycopene were detected, however, due to insufficient data in the literature, there was no attempt to identify the configuration of these isomers.

Results from our laboratory have shown that with similar HPLC methods other carotenoid isomers are separated (unpublished data). Fig. 7 illustrates the separation of at least three *cis*-γ-carotene

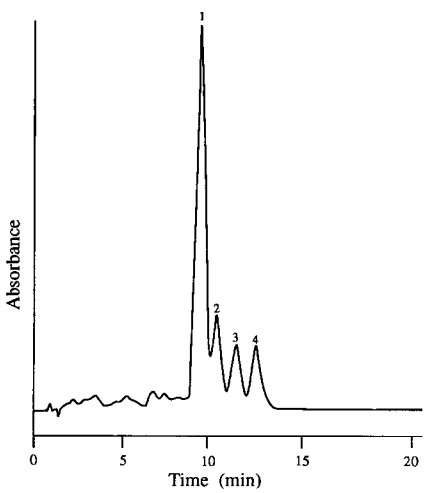

Fig. 7. Chromatographic separation of an extract from Indonesian oncom using a Vydac 201TP C_{18} column and a mobile phase of methanol. Peaks: 1 = γ-carotene and 2–4 = *cis*-γ-carotene.

isomers and all-*trans*-γ-carotene with an eluent of methanol and a Vydac 201TP C_{18} column. These isomers were found in oncom, which is an Indonesian food product of peanut presscake fermented by *Neurospora sitophila*. Additionally, several neurosporene and β-cryptoxanthin isomers have been separated with this procedure.

Saleh and Tan [87] developed a mobile phase that separated a number of *cis*/*trans* carotenoid isomers. For separating β-carotene isomers, the polymeric Vydac 218TP C_{18} column was most effective, however, for the more polar carotenoids, a Zorbax C_{18} column provided better resolution. The optimum solvent composition selected was acetonitrile–methanol–methylene chloride (80:18:2). Two isomers of lutein (13-*cis* and 9-*cis*) were found in carrot oil samples, along with *cis* isomers of γ-carotene, ζ-carotene, phytofluene, and phytoene. The analysis of palm oil carotenoids revealed *cis*-γ-carotene, *cis*-ζ-carotene and *cis*-phytoene, as well as four *cis* isomers of lycopene.

Khachik *et al.* [119] detected *cis*-phytoene and *cis*-phytofluene in dried apricots and peaches. These were detected with both a gradient elution monomeric C_{18} column (Microsorb) separation and an isocratic Vydac C_{18} column system. Both solvent systems included acetonitrile–methanol–methylene chloride–hexane (85:10:2.5:2.5). After 10 min of this mobile phase, the gradient separation linearly increased, over 30 min, to a composition of acetonitrile–methanol–methylene chloride–hexane (45:10:22.5:22.5). *Cis*-β-carotene and *neo*-γ-carotene were also detected in dried apricots, when the Vydac column system was employed. Using this isocratic separation, *neo*-lycopene was found in extracts of pink grapefruit. Employing the described gradient system, two *cis* isomers of lutein, *cis*-neoxanthin and *cis*-β-carotene were found in extracts of several raw and cooked green vegetables [48]. Also, *cis* isomers of phytoene, phytofluene and β-carotene were detected in extracts of tomato paste.

4.4. Analyses of serum, plasma and animal tissues

Sowell *et al.* [101] were able to obtain baseline separation between a *cis* isomer of β-carotene and all-*trans*-β-carotene, and two *cis* isomers of zeaxanthin and all-*trans*-zeaxanthin for extracts of human serum. A Microsorb C_{18} column with an eluent of ethanol–acetonitrile (1:1) was used for this separation. Shoulders following the all-*trans* form were identified as *cis* isomers of cryptoxanthin, lycopene and α-carotene.

Krinsky *et al.* [120] employed an Alltech Adsorbosphere HS C_{18} column and a gradient of solvent A [acetonitrile–methanol with 0.01% ammonium acetate (85:15)] for 10 min followed by solvent A–isopropanol (70:30) to separate carotenoids from human plasma. Several *cis* isomers of lycopene were partially separated, but these isomers were not identified. For both lutein and zeaxanthin, the 13-*cis* isomer was identified. It was noted that a peak appearing to be 9-*cis*-zeaxanthin was detected when an extract of corn meal was analyzed. However, this peak was too small to quantitate for the serum sample. Similar results were found for analyses of monkey plasma [121]. The authors noted that the 9-*cis* isomer of zeaxanthin was present in the diet, but not detected in plasma extracts.

Stahl et al. [106], in addition to reporting the detection of β-carotene isomers, found *cis*-cryptoxanthin and 9-*cis*-, 13-*cis*- and 15-*cis*-lycopene in serum samples and in several tissues from humans. These were identified using visible absorption spectra. The separation was a reversed-phase C_{18} separation with a mobile phase of methanol–acetonitrile–methylene chloride–water (7:7:2:0.16). Schmitz *et al.* [122], using a Vydac C_{18} column and a mobile phase of methanol–acetonitrile–water (88:9:3), also found *cis*-lycopene in human tissues (kidney, liver, and lung tissues). However, individual isomers were not separated. While Stahl *et al.* [106] did not find large amounts of 9-*cis*-β-carotene in serum, 9-*cis*-lycopene was found at nearly the level of the all-*trans* form. The ratio of 9-*cis*/all-*trans* for both carotenoids varied among the tissues.

Using two different HPLC systems, Khachik *et al.* [51] separated seventeen carotenoids from human plasma. Polar carotenoids were separated on a silica-based nitrile-bonded column with a mobile phase of hexane–dichloromethane–methanol–N,N-diisopropylenethylamine (74.65:25.00:0.25:0.10). The reversed-phase C_{18} separation employed a gradient of acetonitrile–methanol–dichloromethane–hexane (85:10:2.5:2.5) for 10 min followed by a linear gradient over 30 min to acetonitrile–dichloromethane–hexane–methanol (45:22.5:22.5:10). *cis*-3-Hydroxy-β,ε-carotene-3′-one, 9-*cis*-lutein, 9′-*cis*-lutein, 13-*cis*- and/or 13′-*cis*-lutein, and 9-*cis*-, 13-*cis*-

and 15-*cis*-zeaxanthin were reported to be detected in human plasma using the nitrile-bonded column. *cis*-5,6-Dihydroxy-5,6-dihydrolycopene, *cis*-2′,3′-anhydrolutein, *cis*-lycopene, *cis*-neurosporene, *cis*-β-carotene and *cis*-phytofluene were found when the reversed-phase system was employed. The identification of these carotenoids was based on UV–visible spectra and by comparison of retention times to thermally isomerized synthetic carotenoids. As was found by Krinsky *et al.* [120], the ratio of *cis* isomers to the all-*trans* isomer was smaller for lutein than for zeaxanthin and slightly more 13-*cis*-zeaxanthin than 9-*cis*-zeaxanthin was detected.

4.5. Supercritical fluid chromatography

Separation of the geometrical isomers of β-carotene and α-carotene with supercritical fluid chromatography (SFC) has been shown by Schmitz *et al.* [123]. Several β-carotene isomers were separated with a SB-cyanopropyl-25-column (25% cyanopropyl and 75% polymethylsiloxane cross-linked stationary phase). To resolve isomers of α-carotene, the authors used two SB-cyanopropyl-50-column (50% cyanopropyl and 50% polymethylsiloxane cross-linked stationary phase). For both of these separations, the mobile phase was supercritical carbon dioxide with 1% ethanol. Positive identification of the isomers has not yet been reported; however, the degree of separation achieved by these authors demonstrates the potential for the use of SFC to separate geometrical isomers.

Other researchers have been examining SFC for use in carotene separations, including an optimization varying temperature, pressure, and organic modifiers in the supercritical fluid [124]. An α-carotene *cis* isomer was resolved from all-*trans*-α-carotene as well as two *cis* isomers of β-carotene were separated from all-*trans*-β-carotene with a reversed-phase column. The analysis time was shorter than that achieved by the HPLC reversed-phase method previously reported by this group of researchers [88]. The density of carbon dioxide changed the efficiency of the separation but did not affect the selectivity. On the other hand, modifiers in the supercritical fluid affected the selectivity and not the efficiency. These authors also investigated the effects of stationary phase on the selectivity and retention of carotene when using carbon dioxide with methanol as a modifier [125]. Polymeric C_{18} columns were able to resolve the *cis* isomers of α and β-carotene from the all-*trans* isomers, but this was not achieved with monomeric columns. This conclusion is similar to results found with HPLC methods [77,81,85].

5. CONCLUSIONS

5.1. Separation of carotenoid isomers

For the separation of β-carotene isomers, most procedures employ either a $Ca(OH)_2$ or a Vydac C_{18} column. These two types of columns have been found to offer better separation of these isomers compared to other columns [77,78]. A comparison of HPLC methods for the analysis of plant tissues was reported by O'Neil *et al.* [78]. The reversed-phase method, selected as offering the best separation, employed a Vydac 201TP C_{18} column and the gradient elution conditions reported by Quackenbush [86]. The normal-phase $Ca(OH)_2$ column method was found to be less sensitive for quantitative measurements than the selected reversed-phase method, but offered greater selectivity for the carotenes. Thus, the procedure employing a $Ca(OH)_2$ column was selected as the preferred method for the analysis of β-carotene *cis*/*trans* isomers in plant tissues. Unfortunately, this method was found to be inappropriate for the analysis of plasma β-carotene isomers [104]. Of the HPLC methods discussed for the separation of β-carotene *cis* isomers in human plasma or tissues, the method of Stahl *et al.* [106] appears to offer the best resolution.

Chromatographic methods enabling the detection of *cis*-carotenoids usually involve reversed-phase separations. In general, polymeric C_{18} columns allow for the detection of *cis*-carotenes [78,86,87], while monomeric columns provide for some separation of certain xanthophylls [87,119]. However, for more complete separation of specific carotenoids, several normal-phase separations may be more appropriate. For example, $Ca(OH)_2$ columns could be used for α-carotene [76] and canthaxanthin separations [117]. Recently, an HPLC method employing silica-based nitrile columns [48] has shown potential for the separation of polar carotenoids and SFC methods [123–125] have resolved carotene isomers.

5.2. Limitations of HPLC methods

This review has presented a number of chromato-

graphic methods which enable the detection of carotenoid *cis* isomers. However, many of these separations do not afford baseline resolution of the *cis/trans* isomers being reported and identification of the *cis* forms may be tentative and unconfirmed. In addition, coelution of isomers may prevent the detection of other geometrical forms. An example of this situation, was the reports of 15-*cis*-β-carotene in the shoulder following all-*trans*-β-carotene when employing monomeric C_{18} columns. It was later found that 13-*cis*-β-carotene and 9-*cis*-β-carotene elute at the same retention time and thus, these isomers may have been present in the samples [78]. Even in systems that separate the main *cis* isomers of β-carotene, some of the minor isomers are not completely resolved [78]. Incomplete resolution is apparent with other carotenoids as well. Fig. 5 illustrates the detection of several *cis* lycopene peaks but also the lack of baseline separation for this difficult analysis [106].

Because there is a lack of commercial standards for geometrical isomers, care needs to be taken when identifying carotenoid isomers. UV–visible spectral information alone can not be used for positive identification, since coeluting isomers can lead to spectra representing a mixture of isomers. Additional means of identification should be used in interpreting HPLC separations, such as mass spectral [126] and ultimately NMR investigation, as was used by some investigators [71,104]. In spite of this, numerous papers have relied on only UV–visible spectra for their identification of carotenoids.

5.3. Current understanding of cis/trans carotenoid isomers

The main *cis* isomers detected in tissues and carotenoid preparations are the 13-*cis* and 9-*cis* forms. Some 15-*cis* carotenoids are found in larger quantities but most tissues contain only small amounts. Other forms, especially di-*cis* isomers have been detected in small amounts for some of the carotenoids (mainly β-carotene). The major causes of the occurrence of *cis* isomers in foods appears to be the result of their formation with heat processing. It appears *cis* isomers circulate in the plasma and are deposited in human tissues. It has been shown that *cis* isomers generally have lower provitamin A activity than the all-*trans* form. On the other hand, new studies indicate that 9-*cis*-β-carotene is more readily deposited in tissues relative to all-*trans*-β-carotene for several animals [111]. Thus, this isomer may play a role in increasing an animals ability to absorb larger amounts of carotenoids. However, it is interesting to note that 9-*cis* isomer forms of several carotenoids do not appear to be found in serum samples, but more often deposited in tissues. As has been postulated for all-*trans*-β-carotene, whether the isomeric forms have a protective effect against oxidation *in vivo* has not been determined. More studies into the metabolism and physiological consequences of *cis/trans* isomers in the diet are needed. However, further chromatographic method developments for the detection and quantitation of *cis/trans* carotenoid isomers will be required.

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