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An isocratic reversed-phase HPLC separation of the stereoisomers of the provitamin A carotenoids (α- and ß-carotene) in dark green vegetables

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An isocratic reversed phase HPLC assay was developed for monitoring the isomers of α- and ß-carotene in dark green leafy vegetables using a mixture of isomers obtained from the light catalysed oxidation of all-trans α- and ß-carotene in the presence of iodine. The method was applied to Italian spinach, spring cabbage and cowpea leaves. The isomers were identified by the elution order from thin layer chromatography, the absorption spectral data and comparison with published results. The three main isomers of ß-carotene found to be dominant in the samples analysed were all-trans, 9-cis and 13-cis ß-carotene, and occurred in average proportions of 85, 11 and 4%, respectively, in fresh samples.

INTRODUCTION

Although there are over 500 known carotenoids only a small fraction are provitamin A active, of which about ten are found in vegetables (Chandler & Schwartz, 1987). Carotenoids exist in nature in the more stable all-*trans* configuration, although some *cis* isomers exist naturally in some fruits and vegetables (Khachik *et al.*, 1986; Rodriguez-Amaya & Tavares, 1992). The compound possessing highest vitamin A activity and occurring most abundantly in fruits and vegetables is ß-carotene. Although α-carotene contributes of the order of 30% of the total carotenoids of carrots it occurs only in low concentrations in other fruits and vegetables (Bauernfeind, 1972; Schwartz & Patroni-Killam, 1985).

Cis–trans isomerisation may occur giving a quasi-equilibrium mixture of *cis/trans* isomers consisting mainly of mono-*cis* and di-*cis* forms (Zechmeister, 1962). Increased temperature, exposure to light or induction by the presence of iodine or acids cause the formation of isomers. During food processing procedures such as cooking, drying and canning some of the *trans*-carotenes are converted to either *cis* isomers which have lower provitamin A activity or oxidative products which are either provitamin A inactive or have lower provitamin A activity (Sweeney & Marsh, 1971; Chandler & Schwartz, 1987 Chandler & Schwartz, 1988; Rodriguez-Amaya & Taraves, 1992; Ogunlesi & Lee, 1979; Simpson, 1983). These processes result in high losses of provitamin A activity in the affected foods.

The various functions and uses of carotenoids have promoted wide interest in the development of methods for the separation of different carotenoids and their isomeric forms. In addition to their provitamin A activity and their use as food colourants and antioxidants, carotenoids have medicinal applications as in the treatment of photosensitivity diseases and possibly in the prevention of cancer (Khachik *et al.*, 1986; Petterson & Jonsson, 1990; Saleh & Tan, 1991; Rodriguez-Amaya & Tavares, 1992). Many HPLC methods have been reported for carotenoid measurements, but only a few have addressed themselves to the separation of geometric isomers. Complete separation of the isomers has been achieved with the help of normal phase columns (Vecchi *et al.*, 1981; Tsukida *et al.*, 1982; Khachik *et al.*, 1986; Chandler & Schwartz, 1987, Chandler & Schwartz, 1988; Petterson & Jonsson, 1990), and some degree of separation with reversed phase chromatography (Quackenbush & Smallidge, 1986; Quackenbush, 1987; Craft *et al.*, 1990). Tsukida *et al.* (1982) separated 17 isomers of ß-carotene using a column packed in their laboratory with lime while Vecchi *et al.* (1981) separated 12 ß-carotene isomers using an alumina column. Although these methods proved superior in the separation of geometric isomers, they are associated with some shortcomings such as being sensitive to polar solvents and being variable (Petterson & Jonsson, 1990).

The separation of carotenoid isomers with reversed-phase (RP) chromatography has been achieved with columns packed with mainly C_{18} materials, especially

wide-pore, which have shown adequate selectivity (Quackenbush & Smallidge, 1986; Craft *et al.*, 1990). However, most of the methods employing RP columns separate only few isomers, notably the 9-*cis* and 13-*cis* while others either do not separate or appear as a shoulder on the ß-carotene peak (Quackenbush, 1987; Khachik *et al.*, 1986). Craft *et al.* (1990) separated five *cis* isomers of ß-carotene and tentatively identified the main isomers as 7-*cis*, 9-*cis*, 13-*cis* and 9, 13-di-*cis* ß-carotene. Although the use of RP columns has not been able to resolve a wide range of geometric isomers as completely as lime and alumina columns, careful selection of the mobile phase is likely to improve resolution (Wise & Sander, 1985). RP columns are commercially available and are routinely used in the separation of many compounds.

The development of an HPLC method which can be used to routinely separate all the isomers of both α- and ß-carotene present in food is imperative in assessing the fate of these compounds during processing and/or storage. Because the *cis* isomeric forms of the provitamins are less potent the need for their separate quantification in vitamin A assay has been increasingly accepted. Also, the need for vitamin A intervention strategies in developing countries with programmes aimed at promoting preservation and distribution practices which could prevent loss of carotenes, and revision of the vitamin A food composition tables emphasise the requirement for the assay of individual provitamin A isomers. The study therefore reports a reproducible reversed-phase HPLC procedure able to separate the isomers of α- and ß-carotene which could be used on a routine basis to assay these compounds in fresh and processed vegetables. Appreciable levels of the isomers are formed during various food processes and the amount of each isomer coupled with their relative biological activities as vitamin A precursors should be used for accurate nutritional content measurements.

MATERIALS AND METHODS

Materials and reagents

Spring cabbage (*Brassica oleracea* L. var. *acephala* L.) and Italian spinach (*Spinacia oleracea* L.) were obtained from local markets. Cow pea leaves (*Vigna unguiculata* L.) were grown in a temperature controlled greenhouse. The standards of α-carotene (type V) and ß-carotene (type IV) were obtained from Sigma Chem. Co. (UK).

Methanol, dichloromethane and water used for chromatography were HPLC grade (Fisons Scientific Apparatus, Loughborough, and Rathburn Co., UK) and all other solvents were Analar grade (Vickers Laboratories, West York, UK). All chemical reagents used were of Analar grade and were purchased from either British Drug House (BDH) Ltd, or Fisons, UK.

HPLC

The HPLC system consisted of a high pressure pump (series 300) from Applied Chromatography Systems (UK) connected to a 250 mm × 4.6 mm (i.d.) stainless steel reversed phase column (Vydack TP-201 5 μm particle size) and guard column from Chrompack (Middelburg, The Netherlands). Samples were introduced into the column through a 10 μl Rheodyne injection valve (model 7125) and the separated compounds monitored at 450 nm using 0.05 absorbance units full scale detector sensitivity by a variable wavelength detector (Varian UV-50). The chromatograms were recorded by a Servogor 460 recorder using a chart speed of 12 cm/min.

HPLC mobile phase

The mobile phase for HPLC separations was prepared by mixing methanol, dichloromethane and water in the ratio 79:15:6 for the mixture of α- and ß-carotene isomers, and in the ratio 80:15.2:4.8 for sample extracts. The mobile phase ratio was changed for the sample extracts which contained only ß-carotene isomers since they separated well at lower retention times and thus analysis time was reduced. The mixture was always freshly prepared and degassed by passing helium gas for 5 min before use. The mobile phase was pumped at a flow rate of 0.8 ml/min when using the ratio 79:15:6 and 1.0 ml/min for the ratio 80:15.2:4.8, and the separation done at room temperature.

Spectrophotometry

Ultraviolet-visible (UV-VIS) spectra were recorded in petroleum ether solution with a Pye Unicam spectrophotometer, model SP 8-100 (Cambridge, UK). The concentration of standard solutions were confirmed using Cecil Digital Spectrophotometer (Cambridge, UK).

Preparation and extraction of vegetables

The fresh raw samples were thoroughly washed under tap water and then destalked and all inedible parts removed before being shredded. The samples were blanched in boiling water for 3 min (spring cabbage and cowpea leaves) or 1 min (Italian spinach). After blanching, samples were divided into two portions; a smaller one for analysis and a larger part for both freeze-drying and simulated solar-drying. Analysis of blanched samples could be done immediately or samples kept frozen until use.

Twenty-five grams of the blanched samples were homogenised by blending with 50 ml of water containing 0.5% ascorbic acid for 5 min. Five grams of the resultant mixture was extracted with 50 ml of acetone–petroleum ether mixture (3:2) containing 0.5% butylated hydroxytoluene (BHT) by shaking at moderate speed for 10 min. The mixture was carefully decanted into a separating funnel and the residue re-extracted until it was colourless (three extractions were found to

be sufficient). The combined extracts were saponified by adding 25 ml of a saturated solution of KOH in ethanol to the mixture, shaken lightly and allowed to stand for 15 min before being washed with 100 ml of 10% sodium chloride solution followed by three portions of 100 ml of distilled water to remove acetone. The extract was then dried over anhydrous sodium sulphate, evaporated to near dryness in a rotatory evaporator at 30°C and the vacuum broken with nitrogen, and the residue dissolved in a mixture of methanol and dichloromethane (9:1) containing 0.5% (BHT) to a final volume of 100 ml. Due to the risk of oxidation and isomerisation of the carotenes during sample preparation, the extraction was performed in dimmed lights at room temperature with aluminium foil around the glassware.

Samples for drying were divided into two portions; one for freeze-drying and another for simulated solar-drying. Before freeze-drying, samples were blast frozen for 1 h at −30°C and then dried with a laboratory freeze drier (S B Freeze Driers Limited, Folkestone, UK) to a constant vacuum pressure of 50 mm Hg.

Simulated solar-drying samples were spread in wire-mesh trays in loads of less than 2.0 kg m^{-2} and allowed to dry for 6–8 h in a simulated solar dryer designed and constructed in the department of Food Science, University of Leeds. The nature and the working conditions of the dryer will be reported elsewhere.

For dry samples, 0.5 g of the finely ground material was used for extraction. Five ml of distilled water was added to the sample, allowed to stand for 10 min and then extracted following the above procedure. The extracts were filtered through a membrane filter (0.44 μm pore size) before being injected into the HPLC column. All samples were extracted and analysed in duplicate. Peak height measurement was used for calculations. All-*trans* α- and ß-carotene isomer were identified by comparison of their retention times with the standard compound and by spiking the samples with the standards under varying solvent conditions.

Isomerisation

The preparation of isomers was carried out by the photo-isomerisation method given by Zechmeister (1962). 1.0 ml of 0.2% iodine solution was added to 100 ml of 0.01 mg/ml solution of all-*trans* ß-carotene or α-carotene in petroleum ether. The mixture was shaken and allowed to stand for 30 min exposed to room radiation. Iodine was then removed by washing with two portions of 100 ml of 10% sodium thiosulphate solution followed by a similar portion of water. The petroleum ether solution was dried over anhydrous sodium sulphate and concentrated by evaporation at 30°C on a rotary evaporator. The solution was then run on TLC plates and HPLC column to separate the isomers.

TLC separation

The separation and collection of individual isomers was done by the method given by Schwartz & Patroni-Killam (1985) although with some minor modifications. The chromatographic plates were prepared from calcium hydroxide ground in a mortar into a fine powder and sieved to pass through 80 μm pore size. Calcium hydroxide was made into a slurry with water (5:12 w/v, calcium hydroxide–water) and spread on glass plates (20 × 20 cm) to a thickness of 0.25 mm. The plates were then allowed to dry for 24 h and then stored in an enclosed chamber equilibrated to 44% relative humidity with a saturated solution of potassium carbonate for at least 2 days or until use.

The plates were developed with 1.2% acetone in petroleum ether by a two-dimensional method to increase the theoretical plates to the level necessary for separation. Each direction required about 2 h to run. When the direction was being changed, the plates were dried rapidly for less than 1 min in a stream of nitrogen. The separated compounds were identified by their position on the TLC plate, their absorption spectra characteristics and the comparison with literature information (Zechmeister, 1962; Tsukida *et al.*, 1982; Schwartz & Patroni-Killam, 1985; Petterson & Jonsson, 1990; Craft *et al.*, 1990).

RESULTS

Separation of α- and ß-carotene isomers

Photoisomerisation of individual all-*trans* α- and ß-carotene solutions produced several isomers each. Fig. 1 shows the chromatogram obtained from a mixed solution of photoisomerised isomers of α- and ß-carotene when separated using a mobile phase consisting of methanol–dichloromethane–water in the ratio 79:15:6. The separation was achieved after various combinations of the mobile phase were tested for their ability to separate the isomers. Mobile phase mixtures containing methanol, acetonitrile, dichloromethane and water (with methanol dominating in all cases) were tested but

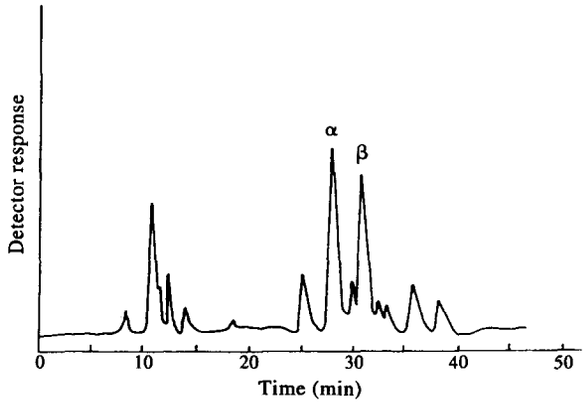

Fig. 1. Chromatogram of the separated isomers of a mixture of α- and ß-carotene solution. Conditions: reversed phase column (Vydack TP-201, 5 μm particle size); mobile phase, methanol–dichloromethane–water (79:15:6); flow rate, 0.8 m/min; detection, 450 nm; α, all-*trans* α-carotene; ß, all-*trans* ß-carotene.

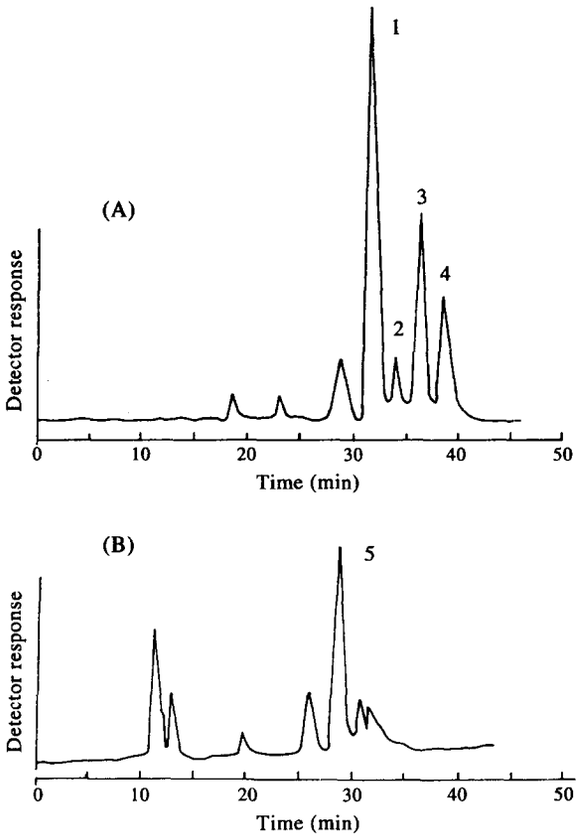

Fig. 2. Chromatogram of the separated individual isomers of α- and ß-carotene solutions. Conditions same as in Fig. 1. (A) ß-carotene solution; (B) α-carotene solution. 1, all-*trans*-; 2, 7-*cis*-; 3, 9-*cis*; 4, 13-*cis*-ß-carotene; 5, all-*trans*-α-carotene.

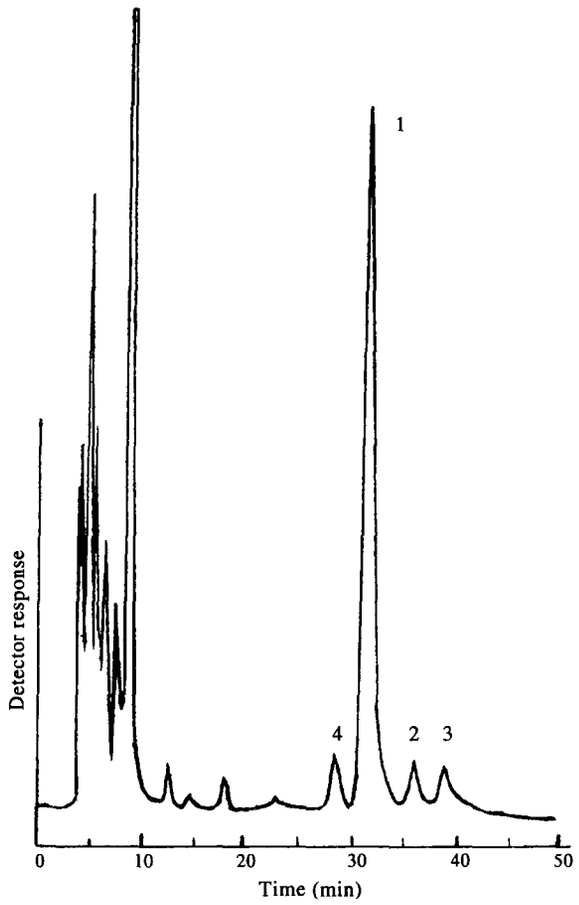

Fig. 3. Chromatogram of the separated provitamin A compounds in green leafy vegetables. Conditions same as in Fig. 1. 1, all-*trans*-ß-carotene; 2, 9-*cis*-ß-carotene; 3,3-*cis*-ß-carotene; 4, all-*trans*-α-carotene.

the presence of acetonitrile in the mixture had no significant effect on the selectivity. The amount of dichloromethane and water in the tertiary solvent system was varied in order to effect separation. Although increasing the water content in the mobile phase enabled separation to occur by increasing the retention times of some compounds in the column, peak tailing resulted. Tailing was minimised by increasing the amount of dichloromethane to a solution containing the minimum amount of water which effected separation. This reduced the retention times without compromising the separation. Dichloromethane was also found to improve the analysis by narrowing the peaks and hence enhancing separation.

These conditions also separated well the isomers for the individual all-*trans* α- and ß-carotene solutions after isomerisation (Fig. 2), and the provitamin A compounds in green leafy vegetables in which the main isomers of ß-carotene were dominating (Fig. 3). When the water content in the mobile phase was less than 4.5% the separation of one *cis* ß-carotene isomer from the all-*trans* α-carotene isomer was not complete. Similarly, some of the late eluting *cis* isomers of α-carotene interfered with the early eluting ß-carotene isomers. However, the separation of these isomers can be achieved by increasing the water content in the mixture, but tailing is compromised.

When excess iodine was used during photoisomerisation of ß-carotene and the mixture exposed to light over a longer period (45–60 min), ß-carotene degraded into various compounds which eluted very early (having lower retention times), suggesting that they might have been the oxygenated derivatives. These compounds did not separate on the TLC plate but remained close to the baseline.

A TLC method was used to isolate and collect major isomers of ß-carotene in order to identify them. Fig. 4 illustrates a typical plate obtained, indicating the identified isomers. The order of elution of these compounds was the same as that reported by Chandler & Schwartz (1987) and Schwartz & Patroni-Killam (1985). The adsorption affinity of carotenoids and their isomers is a

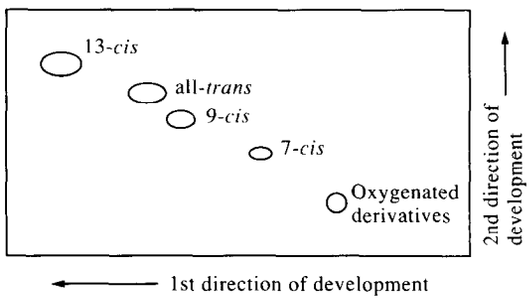

Fig. 4. Two-dimensional TLC chromatogram of ß-carotene isomers from iodine catalysed photoisomerisation.

function of the number and the position of the *cis* double bonds (Davies, 1976). Whereas poly-*cis* carotenes are adsorbed much less strongly than all-*trans*, mono-*cis* and di-*cis* forms, the adsorption affinities of the mono-*cis* isomers is influenced by the molecular shape due to the position of the *cis* double bond. The most bent isomer (a central mono-*cis* isomer) has the least adsorptivity while the peripheral mono-*cis* carotenes have greater affinities than those of the corresponding all-*trans* forms (Zechmeister, 1962; Davies, 1976; Tsukida *et al.*, 1982). The compounds were isolated by removing the appropriate spots from the TLC plate and dissolving in petroleum ether followed by concentration at reduced pressure prior to spectral analysis. It was observed that equilibration of the plates to 44% relative humidity was critical to achieving optimal resolution (Schwartz & Patroni-Killam, 1985). Plates had to be run in two dimensions to obtain the required number of theoretical plates for separation.

To determine whether *cis* isomers of the ß-carotene were formed on the chromatographic plate during the isolation process, a standard solution of all-*trans* ß-carotene was chromatographed. Only one spot was obtained, indicating that isomerisation of all-*trans* ß-carotene did not take place.

The isomers were identified by their behaviour in the UV-VIS absorbance spectra, the *Q*-ratios and the elution order from a TLC plate. The spectral properties of carotenoids and their isomers were comprehensively given by Zechmeister (1962). The first probable means of identifying the *cis*/*trans* isomers is through the 'λ_{max} shift' and the presence or absence of '*cis* peaks' in the absorption spectra. All-*trans* ß-carotene shows a strong absorption in the visible region between 400 and 500 nm and three maxima or two and an inflection appear, with the middle peak having the highest intensity. Although the same scenario applies in the *cis* isomers but with less intensity (lower extinction coefficienct) they absorb at shorter wavelength than the all-*trans* form. The wavelength difference, in nm, between the location of λ_{max} (maximum absorption wavelength) of an all-*trans* compound and that of one of its *cis* isomers is termed the λ_{max} shift. The λ_{max} shift of mono-*cis* isomers and di-*cis* isomers is about 2–5 nm and 10 nm, respectively, and that of poly-*cis* isomers may absorb at up to 50 nm below the all-*trans* carotene (Davies, 1976). The *cis* peak is a new absorption maximum in the near-ultraviolet region (between 330 and 350 nm) of the spectrum, that arises in some of the products formed during isomerisation. Central mono-*cis* carotenes have higher *cis*-peak but with the least intense absorption in the visible region than the peripheral mono-*cis* carotenes. All-*trans* carotene and poly-*cis* carotenes are more or less flat in the *cis* peak region.

Figure 5 shows the absorption spectra of the major isomers of ß-carotene isolated from TLC plates. Isomer 1 gave a spectrum with the same features as that obtained from all-*trans* ß-carotene standard and hence represents the original isomer. The other spectra had a hypsochromatic λ_{max}. shift and characteristic *cis* peaks

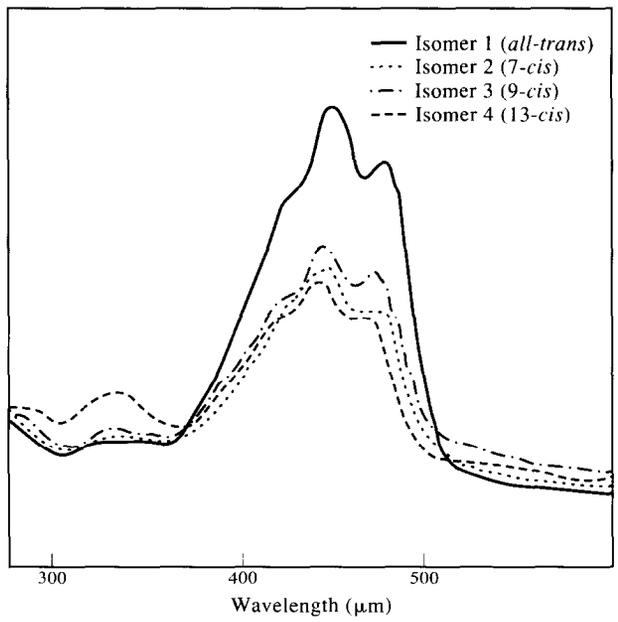

Fig. 5. Overlay of the absorption spectra of ß-carotene isomers isolated from TLC.

at 330–350 nm region. The λ_{max}. shift of all isomers was less than 10 nm, indicating that they were mono-*cis*. Since the isomer with a high *cis* peak represents a central mono-*cis* isomer, and that with the smallest *cis* peak represents a peripheral mono-*cis* isomer 2 was thus identified as the 7-*cis*, isomer 3 as 9-*cis*, and isomer 4 as 13-*cis*. Chandler & Schwartz (1987) used only the absorption maxima of the visible region to identify the 9-*cis* and 13-*cis* isomers, while Tsukida *et al.* (1981) similarly identified the isomers and then structurally elucidated their configuration using ^{1}H- and ^{13}C-nuclear resonance spectroscopy.

Petterson & Jonsson (1990) also used absorption maxima but further identified majors isomers of ß-carotene from spectral characteristics using the relative intensities of peak maxima in the visible region by setting the highest maximum peak (middle peak) to 100% and measuring other peaks relative to it. The peaks on the lower wavelength of the middle maximum peak (absorption maximum No. II in their article), though mere inflections, show different absorption intensities in all isomers. The intensity for all-*trans* isomer was the same as that of 9-*cis* isomer and was higher than for other isomers, reported to be 10% higher than for 13-*cis* and 15-*cis* isomers. Similarly, the relative intensity of the peak on the higher wavelength of the middle peak (maximum No. IV in their article) for 9-*cis* isomer was the same as that of all-*trans* but was lower for other isomers (13-*cis* and 15-*cis*). When they calculated the difference of the relative intensities between peaks No. IV and the depression that leads to the middle highest peak, all-*trans* isomer had 7%, 9-*cis* isomer 11% and all other ß-carotenes had only 1 or 2%. The Petterson and Jonsson procedure was applied to the data from this study. The results showed a similar trend including their absorption profile of 9-*cis* and 13-*cis* isomers being

Table 1. UV-VIS characteristics of the major isomers of ß-carotene

HPLC		Experimental data			Literature data[a]		
Isomer	Peak no.	λ_{max} (nm)	*cis* peak (nm)	*Q*-ratio	λ_{max} (nm)	*cis* peak (nm)	*Q*-ratio
All-*trans*	1	448	336	15.8	450	337	17.4
7-*cis*	2	445	335	13.6	446	337	16.1
9-*cis*	3	444	338	9.6	445	338	11.5
13-*cis*	4	440	332	2.3	442	336	2.8

[a]Values in hexane (Tsukida *et al.*, 1982)

comparable to what was observed. The relative intensities of the first peak (on the lower wavelength) for all-*trans* and 9-*cis* isomer were nearly similar (75% for all-*trans* and 77.8% for 9-*cis*) but higher for others (84.2% for 7-*cis* and 79.6% for 13-*cis*). The difference between the intensity of the valley from the highest maximum peak and the peak that leads from it (third peak) was 4.0% for all-*trans*, 5.2% for 9-*cis* while other isomers had less than 1%. The relative intensities of the third peak were almost similar for all-*trans* (86.9%) and 9-*cis* (88.2%) but lower for 7-*cis* (84.4%) and 13-*cis* (80.8%).

The *Q*-ratios, the absorbance at the maximum wavelength/absorbance at the *cis* peak, for the isomers of ß-carotene were calculated from the spectra and the results compared with published data (Tsukida *et al.*, 1982). The results obtained are shown in the Table 1. The spectral measurement by Tsukida *et al.* (1982) was recorded in pure *n*-hexane while it was recorded in petroleum ether in this study. Though there is often some difference in the absorption spectra of carotenes measured in different solvents, there was good agreement of results in the isomers with that of Tsukida *et al.* (1982). The *Q*-ratio of mono-*cis* isomers is a function of the molecular shape whereby the isomers with the most bent shape and hence the most pronounced *cis* peak had the least *Q*-ratio. Thus 13-*cis* ß-carotene isomer which is nearly a central mono-*cis* has the lowest *Q*-ratio. Similar identification was done by Quackenbush (1987), Craft *et al.* (1990), Petterson & Jonsson (1990) and Saleh & Tan (1991).

Analysis of α- and ß-carotene compounds in dark green leafy vegetables

Typical chromatographs obtained from the extracts are shown in Fig. 6. Three ß-carotene isomers were resolved in all the extracts studied, as expected for green vegetables (Sweeney & Marsh, 1971) except the cowpea leaves extract which contained all-*trans* α-carotene. All-*trans* α-carotene was confirmed by spiking the sample with a standard solution and varying the solvent conditions. However, Pepping *et al.* (1988) was not able to detect the presence of α-carotene in cowpea leaves. The ß-carotene isomers were found to correspond to the predominant *cis–trans* isomers found in iodine catalysed isomerisation of the ß-carotene solution and were identified as all-*trans* (isomer 1), 9-*cis* (isomer 2), and 13-*cis* (isomer 3). Due to the relatively few isomers in the extracts the mobile phase composition was modified to 200:38:12 (methanol–dichloromethane–water) to reduce the separation time without affecting the resolution.

Recovery studies were carried out in order to determine the efficiency of the extraction procedure. 0.2 g of a finely ground freeze-dried cowpea leaves and 2.5 g of the homogenised fresh spring cabbage were used. An amount of ß-carotene approximately equal to the expected amount in the sample was added to the samples in triplicate and then subjected to the extraction procedure. The recoveries obtained ranged from 96.4 to 103.5%. To determine the reproducibility of the extraction procedure, five portions of 0.2 g each of the dehydrated spring cabbage were extracted and the quantity of ß-carotene determined. The standard deviation of 0.78 and the coefficient of variation of 1.09% for all-*trans* isomer were comparable to those reported by Sweeney & Marsh (1971) and Rodriguez-Amaya *et al.* (1988) and indicates an adequately reproducible procedure (Table 2).

The possibility of the conversion of all-*trans* ß-carotene to *cis* isomers during the extraction process was investigated by subjecting a solution containing all-*trans* ß-carotene standard to the extraction process and obtaining a HPLC chromatograph of the resultant solution. The absence of the *cis* peaks indicated that there was minimal or no isomerisation during the extraction process.

The extraction procedure includes a saponification step meant to remove chlorophyll and fat-soluble substances, and hydrolyse carotenol esters (Davies, 1976; Rodriguez-Amaya *et al.*, 1988). The effect of the saponification step on the separation of the isomers from these and other compounds was investigated. Two sets of samples from the same batch were subjected to the extraction process with one set extracted without going through the saponification step. It was observed that chlorophyll and other compounds in the extract eluted earlier than the ß-carotene isomers. The separation of the ß-carotene isomers was therefore not affected by

Table 2. Reproducibility of the analytical procedure

Sample	% all-*trans*	% 9-*cis*	% 13-*cis*
Mean ($n=5$)	71.1	21.6	7.3
SD	0.78	0.56	1.30
CV (%)	1.1	2.6	17.9

the other compounds present in the unsaponified extract, nor was the nature and size of the peaks affected (Petterson & Jonsson, 1990; Rodriguez-Amaya *et al.*, 1988). Although Khachik *et al.* (1986) reported a 6.0% loss in all-*trans* and 15, 15′-*cis* ß-carotene fraction on saponification with 30% methanolic KOH under an atmosphere of nitrogen, Kimura *et al.* (1990) observed that cold saponifcation had minimal effect on ß-carotene, while hot saponification produced *cis* and oxidative products. The saponification step was however, maintained in this study in order to remove unnecessary peaks that may mask the identification of new compounds,

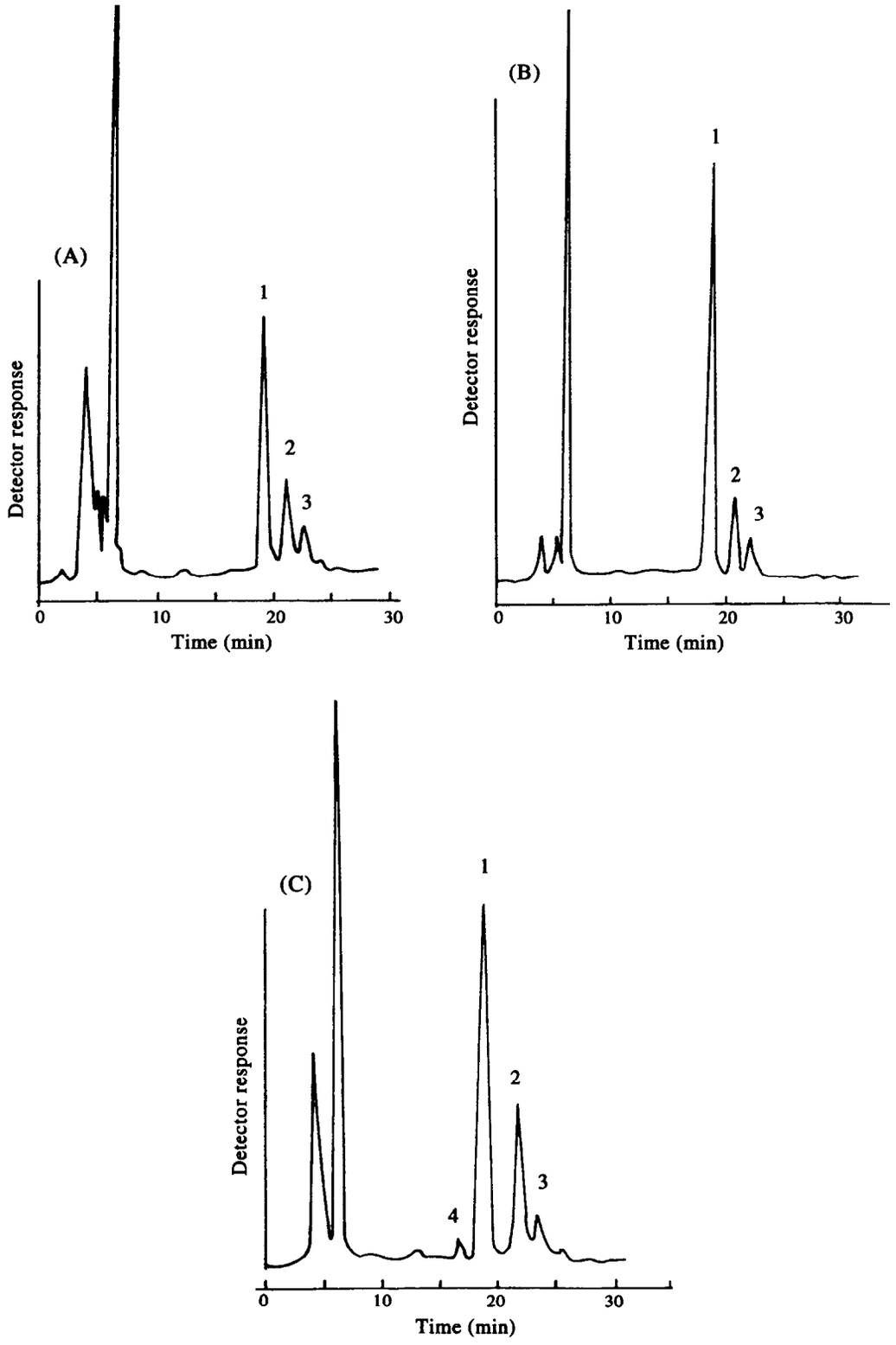

Fig. 6. Elution profile of extracts of dark green vegetables. Conditions same as in Fig. 1, except the mobile phase ratio was 80:15.2:4.8 and was pumped at the flow rate of 1.0 ml/min. (A) spring cabbage; (B) spinach; (C) cowpea leaves. 1, all-*trans*-; 2, 9-*cis*; 3, 13-*cis*-ß-carotene; 4, all-*trans*-α-carotene.

Table 3. Proportions of the major provitamin A isomers present in some fresh and processed green vegetables

Product	ß-Carotene			α-Carotene
	% all-*trans*	% 9-*cis*	% 13-*cis*	% all-*trans*
Cowpea leaves — fresh	81.2	8.2	3.5	7.1
—freeze-dried	80.4	9.2	4.1	6.2
—solar-dried*	81.0	8.9	3.8	6.3
Spring cabbage — fresh	85.4	10.2	4.4	—
—freeze-dried	83.0	12.8	4.2	—
—solar-dried	77.8	16.7	5.5	—
Italian spinach — fresh	83.4	11.8	4.8	—
—freeze-dried	78.6	15.2	6.2	—
—solar-dried	77.1	16.9	6.0	—

*Solar-dried samples were obtained by the simulated solar drying method indicated in the materials and methods section.

especially oxidation products formed during processing and storage (Goldman *et al.*, 1983; Chandler & Schwartz, 1988). This also enabled us to modify the mobile phase in order to reduce the retention time.

The relative amounts of the provitamin A isomers present in the analysed vegetables, expressed as the peak height percentage of the total peak height absorbance at 450 nm listed in Table 3. All-*trans* ß-carotene comprises over 80% of the provitamin A content in the fresh samples but decreases to about 77% after drying. This reduction in all-*trans* isomer is reflected in the increase in the amount of *cis* isomers, especially the 9-*cis* isomer; indicating that *cis* isomers were formed during drying. Sweeney & Marsh (1971) observed no formation of stereoisomers of carotene in freeze-dried carrots. The percentage retention of the *trans*-ß-carotene of some of the dehydrated products was greater in freeze-dried compared to the simulated solar dried products (Table 4).

DISCUSSION

The carotenoids are unstable to many processing procedures because of their conjugated system of double bonds. Drying and extrusion cooking are particularly destructive processing steps which promote oxidation (Simpson, 1983; Marty & Berset, 1990). The loss in carotene content in dehydrated products (Table 4) may be attributed to oxidative degradation of ß-carotene through a free-radical process, especially when samples are stored prior to analysis (Chandler & Schwartz, 1987). Maenda & Salunkhe (1981) reported that from 4.2 to 41.7% of the ß-carotene content of four types of tropical leafy vegetables remained after drying in different conditions.

Vitamin A is a major nutritional problem in developing countries, particularly among pre-school children and is often associated with protein-energy malnutrition, parasitic infestation and diarrhoeal diseases, which affect its normal absorption and metabolism (Lee & Lim, 1990). The major source of vitamin A in the diet of most communities in these countries is ß-carotene from plant foods. But the bioavailability of carotenoids as vitamin A depends on their biological conversion; the extent being affected by different conditions. Vitamin A activity has been assigned to carotenoids on the basis of animal bioassay, *in vitro* methods using ß-carotene 15,15′-dioxygenase with the formation of retinol, or inspection of the molecule for presumed activity (Simpson, 1983). However, during digestion the cleavage of the provitamin A carotenoids to retinoids is determined by the efficiency of the cleavage enzyme in the intestine. It has been observed that the enzyme in animals is more efficient than in human beings (Simpson, 1983). Thus the actual activity value when converted to retinol equivalent depends on the nature and amount of biologically active carotenoids, their state of isomerisation, their stability in the gastrointestinal tract and their digestibility, which is affected by the dietary fat, protein level, the presence of dietary antioxidants and vitamin A status (Simpson, 1983; Rodriguez-Amaya & Tavares, 1992; Scott, 1992). Some of the intervention strategy programmes for the prevention and control of vitamin A deficiency are concerned both with promoting conditions

Table 4. All-*trans* ß-carotene content and % retention (mean of triplicate) of some dark green leafy vegetables

Product	All-*trans* ß-carotene content (μg/g DM)		% retention
	Range	Mean[a]	
Cowpea leaves			
—fresh	859–906	883	100
—freeze-dried	545–778	70 079	
—solar-dried	481–646	553	62
Spring cabbage			
—fresh	789–826	808	100
—freeze-dried	456–750	68485	
—solar-dried	331–550	480	59
Italian spinach			
—fresh	1220–1275	1248	100
—freeze-dried	804–842	834	67
—solar-dried	680–721	711	57

[a]Mean of triplicate samples from same batch.
DM — dry matter.

for maximum intestinal retention of vitamin A precursors and promoting dietary intake of provitamins A from fruits and vegetables. Despite the fact that the amount which is bioavailable cannot be assumed to be the same as that calculated from analysed levels in foods reliable information on the types and concentrations of the various carotenoids found in fruits and vegetables eaten in these regions is essential as a result of the increased attention paid to the problem of vitamin A deficiency and xerophthalmia (Pepping *et al.*, 1988; Lee & Lim, 1990).

The carotenoid profile of vegetables is much more complex than previously recognised, with the proportion and forms occurring as ß-carotene varying considerably.

Changes in the structure of the provitamins in foods such as *cis–trans* isomerism or oxidative changes generally result in lowered activity. Reports from studies of provitamin A assay in foods have given conflicting results both because of the analytical complexities and the varying quantitative and qualitative composition in food samples due to variety, growing conditions and changes incurred during processing and storage (Kimura *et al.*, 1990; Rodriguez-Amaya & Tavares, 1992).

When the provitamin A content of several common fruits and vegetables is assayed by the HPLC method the values obtained are lower than those listed in food composition tables. These values were obtained from methodologies that did not discriminate against inactive carotenoids or separate the *cis* isomers, thus leading to overestimation (Simpson, 1983; Lee & Lim, 1991; Rodriguez-Amaya & Tavares, 1992). Notwithstanding the difficulties in its execution, isomer separation is necessary in the quantification of provitamin A content in foods. In some studies, overestimation of between 3 and 52% vitamin A activity has been reported in samples of tomatoes and tomato products when *cis* isomers were not separated (Rodriguez-Amaya & Tavares, 1992), while a decrease of 25–35% in concentrations of all-*trans* isomers which reflected 15% decrease in vitamin A value has been reported in thermal processing of carrots (Ogunlesi & Lee, 1979).

CONCLUSION

The investigation indicates that although in principle the analysis of stereoisomers of carotenoids is potentially very complex because of the large number of possibilities, in reality the analysis is simplified by the relatively small number of isomers formed even after extensive processing. The reported method was fast and could be used routinely to separate stereoisomers in leafy green vegetables. However, since the method was capable of resolving more isomers than required for leafy green vegetables, it may also be applicable to food systems presenting a wider range of isomers.

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