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DECAPRENO-β-CAROTENE AS AN INTERNAL STANDARD FOR THE QUANTIFICATION OF THE HYDROCARBON CAROTENOIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

FREDERICK KHACHIK* and GARY R. BEECHER
U.S. Department of Agriculture, Agricultural Research Service, Beltsville Human Nutrition Research Center, Nutrient Composition Laboratory, Building 161, BARC-East, Beltsville, MD 20705 (U.S.A.)
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SUMMARY

The application of decapreno-β-carotene as an internal standard in quantification of the hydrocarbon carotenoids extracted from raw carrots has been thoroughly examined. Decapreno-β-carotene is a C_{50} β-carotene that has most of the requirements of an internal standard and it can be commercially synthesized in high purity. An isocratic high-performance liquid chromatographic (HPLC) system has been developed that separated all-*trans*-α-carotene, all-*trans*-β-carotene, and its 15,15'-*cis*-isomer from this internal standard. Quantitative determination of the hydrocarbon carotenoids in carrots by HPLC using the internal standard technique gave values for α- and β-carotene similar to those obtained from α- and β-carotene standards alone.

INTRODUCTION

Numerous analytical methods for the separation and quantification of the naturally occurring carotenoids have been described in the literature. Most of the carotenoid data in food composition tables have been generated using AOAC analytical procedures which lack analytical specificity[1]. The use of new separation techniques and chemical instrumentation for generating detailed analytical information in regard to the carotenoid content of foods has become increasingly important. High-performance liquid chromatography (HPLC) has been shown to be the most efficient technique for the analysis of the complex mixture of plant carotenoids[2–7]. The separation and the analysis of carotenoids by HPLC minimizes the isomerization and decomposition of these light, heat, and air sensitive compounds. Accurate characterization of specific carotenoids in food is important in refining our understanding of the relation between ingestion of dietary fruits and vegetables and human cancer. The observed protective effect seen in epidemiologic studies to date has been attributed to β-carotene and, as a result, several human intervention studies using synthetic β-carotene have been initiated to confirm this hypothesis[8,9]. Since the preparation of carotenoid samples for HPLC analysis requires extensive extraction and work-up

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procedures that can be accompanied by various analytical errors, the use of an internal standard is very essential. Many examples have appeared in the literature of the separation and quantification of the plant carotenoids by HPLC; however, few of these have employed an internal standard. Stancher and Zonta[5] have employed azobenzene as an internal standard for quantitative determination of β-carotene and vitamin A in Taleggio cheese extract by HPLC. The light absorption of azobenzene and its HPLC retention time on a normal phase silica column are compatible with that of β-carotene. However, on a C_{18} reversed-phase column azobenzene elutes much faster than α- and β-carotene and is not suitable as an internal standard. Furthermore, azobenzene is not structurally close to carotenoids and is expected to have different solubility and chromatographic behavior, which may result in its limited application as an internal standard. β-Apo-8'-carotenal has been used as an internal standard in the separation and quantification of citrus carotenoids by reversed-phase HPLC[6]. We have also found that this compound is an appropriate internal standard for the quantification of the oxygenated carotenoids (xanthophylls) extracted from a number of fruits and vegetables[10]. Since the HPLC retention time of β-apo-8'-carotenal is much shorter than that of α- and β-carotene, this compound fails to serve as an internal standard for the quantification of the hydrocarbon carotenoids. The most interesting application has recently been reported by Driskell *et al.*[11], who have described a procedure for quantitative determination of β-carotene in human serum using (2*R*,2'*R*)-2,2'-dimethyl-β-carotene (Fig. 1) as an internal standard.

Although dimethyl-β-carotene has many attractive features as an internal standard, it is not stable more than two weeks at −70°C, as reported by the authors[11]. The preparation of small quantities of this compound requires elaborate synthesis, which also contributes to its lack of commercial availability[12,13]. We propose the use of decapreno-β-carotene (Fig. 1) as an internal standard for the quantification of the hydrocarbon carotenoids. We have shown that this compound has most of the requirements of an internal standard for the quantification of α- and β-carotene extracted from raw carrots. Decapreno-β-carotene is a C_{50} β-carotene that contains two isoprene units more than that of β-carotene and can be synthesized commercially in high purity.

EXPERIMENTAL*

Apparatus

A Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Hewlett-Packard 1040A rapid-scanning UV–VIS photodiode array detector. The data were stored and processed by means of a Hewlett-Packard 85-B computing system which was operated with a Hewlett-Packard Model-9121 disc drive and a Model 7470 A plotter. The chromatographic runs were monitored at 450 nm for α- and β-carotene and at 500 nm for the internal standard. The absorption spectra of the carotenoids were recorded between 200 and 600 nm as frequent as 1 scan/5 s (maximum scanning capability = 1 scan/100

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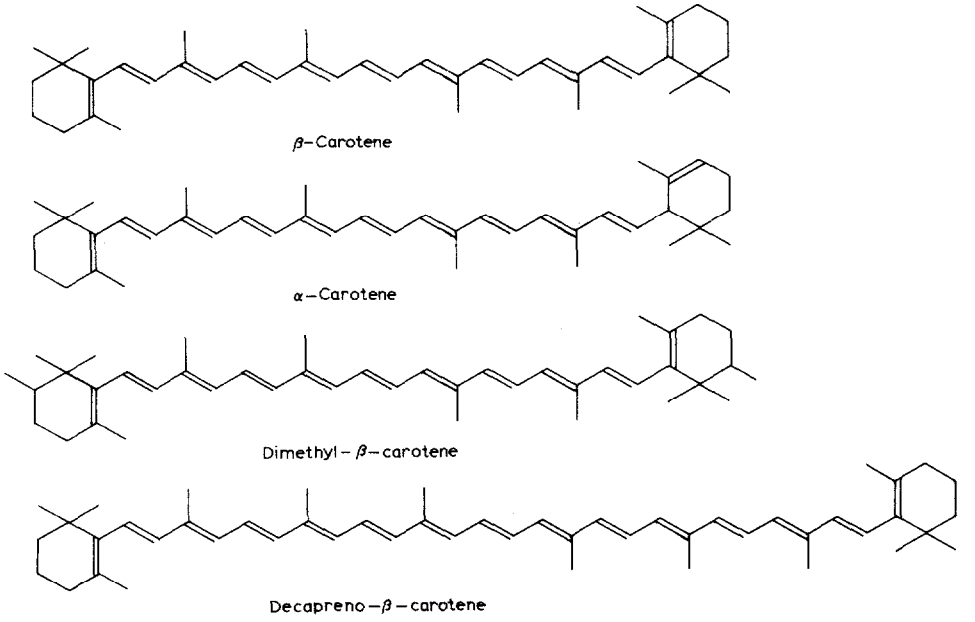

Fig. 1. Molecular structures of β-carotene, α-carotene, (2*R*,2′*R*)-2,2′-dimethyl-β-carotene, and decapreno-β-carotene.

ms). The HP-85B computer with a built-in integration program was used to evaluate the peak area and peak heights.

Column

Separations were performed on a stainless-steel (25 cm × 4.6 mm I.D.) Microsorb C_{18} (5-μm spherical particles) column (Rainin Instrument) which was protected with a Brownlee guard cartridge (3 cm × 4.6 mm I.D.) packed with Spheri-5 C_{18} (5-μm particle size). Analysis of food samples often required frequent changes of the guard cartridge and frequent reversed flushing of the pre-column with methanol and methylene chloride–hexane (1:1).

Reagents and material

The reference samples of α- and β-carotene (Sigma, St. Louis, MO, U.S.A.) were used without further purification. 15,15′-*cis*-β-Carotene was a gift from Hoffmann-LaRoche (Basel, Switzerland). An original sample of decapreno-β-carotene (internal standard) was obtained from Professor A. G. Andrewes but subsequently this compound was synthesized in large quantities in our laboratory. HPLC-grade solvents, methanol, acetonitrile, methylene chloride, and hexane (Fisher Scientific, Pittsburgh, PA, U.S.A.) were used without further purification. Carrots were purchased fresh from local supermarkets on the day of the analysis.

Chromatographic procedure

An isocratic system of methanol (22%, pump A), acetonitrile (55%, pump C), and methylene chloride–hexane (1:1) (23%, pump B) effected the separations of α-

and β-carotene and the internal standard in 23 min. The column flow-rate was 0.70 ml/min. Scans of the absorption spectra provided about 8 to 10 data points on each of the peaks as they were eluted from the HPLC column.

Extraction

The extraction procedure was similar to that employed by Bushway and Wilson[4] for the extraction of fruits and vegetables. The internal standard (5 to 6 mg) was added to a 10 g sample of carrots, 20 g of anhydrous sodium sulfate, and 1.0 g magnesium carbonate contained in a Waring blender. The resulting mixture was extracted with 150 ml of tetrahydrofuran at a moderate speed for 5 min. The extract was filtered *in vacuo* and the solid materials were re-extracted with tetrahydrofuran until the resulting filtrate was colorless (four extractions were sufficient to remove all the carotenes). The solvent was removed on a rotary evaporator at 30°C and the concentrated carrot filtrate (50 ml) was partitioned into petroleum ether (100 ml) and water (100 ml). The water layer was washed with petroleum ether several times and the resulting organic layers were combined, dried over sodium sulfate and evaporated to dryness. The residue was transferred to a 50-ml volumetric flask using hexane. Samples were injected (20 μl) in duplicate for the HPLC analysis.

Synthesis of decapreno-β-carotene

Decapreno-β-carotene has been synthesized by three different methods: the Grignard reaction that follows the building principle of $C_{21} + C_8 + C_{21}$ developed by Karrer and Eugster[14]; the enol ether condensation that was employed by Isler *et al.*[15] using a $C_{19} + C_{12} + C_{19}$ building scheme, and finally the condensation of vitamin A aldehyde with a Wittig compound prepared from a C_{10} diol. We have employed the last method that has been developed by Surmatis and Ofner[16] to prepare gram quantities of the decapreno-β-carotene, as shown in Fig. 2.

Vitamin A aldehyde (retinal) was purchased commercially (Aldrich) and the C_{10} Wittig compound (II) was prepared according to the method of Strong[17] and Surmatis and Ofner[16]. To effect the condensation to C_{50} β-carotene, the C_{10} Wittig (II) was added to a stirred solution of phenyllithium in ethyl ether, followed by the addition of vitamin A aldehyde. As methanol was added decapreno-β-carotene was obtained in 38% yield as a dark, violet crystalline solid which was recrystallized from methanol and methylene chloride. Alternatively, the Wittig compound of the acetylenic C_{10} diene (III) prepared in a similar manner as that of (II) was condensed with all-*trans*-retinal to form the dehydro C_{50} carotenoid (IV) in 42% yield. Selective reduction of (IV) followed by isomerization afforded all-*trans*-decapreno-β-carotene (V) in 69% yield. The UV–VIS absorption spectra of the product V were identical with the reported literature spectra of the decapreno-β-carotene[14] and that of an authentic sample of this compound provided by Professor Arthur G. Andrewes. The absorption spectrum of the decapreno-β-carotene (Fig. 3) in the HPLC solvent system had a maximum at 502 nm.

The internal standard was shown to be pure by plotting the peak ratios at various wavelengths. The purity of the internal standard was further ascertained by the evaluation of at least ten absorption spectra of this compound monitored by the rapid scanning detector. In all cases the absorption maximum remained at 502 nm and all spectra were superimposable.

Qualitative identification of the carotenoids

All-*trans*-α- and β-carotene were identified by comparison of their chromatographic retention times as well as their absorption spectra (Fig. 4) with those of the reference samples. 15,15'-*cis*-β-Carotene was identified from its absorption spectrum which contained a strong *cis*-peak in the near UV region at 334 nm (characteristic of the central *cis*-isomers of carotenoids)[18]. The chromatographic retention time and the absorption spectrum of the 15,15'-*cis*-β-carotene were also identical with that of an authentic sample of this compound gifted by Hoffman LaRoche.

Standard solutions and quantitative evaluation

Three stock solutions were prepared by dissolving 10 mg of all-*trans*-α-carotene, 12 mg of all-*trans*-β-carotene, and 37 mg of the internal standard in 100 ml of hexane. Aliquots of 1–3 ml of the stock solution of β-carotene were mixed with 5 ml of the stock solution of the internal standard into separate 10-ml volumetric flasks and the flasks were brought to volume with hexane. The standard solutions for α-carotene were similarly prepared by mixing 1–3-ml aliquots of this compound with 5 ml of the internal standard solution into separate 10-ml volumetric flasks. Samples were injected (20 μl) in duplicate onto the chromatograph and the peak areas of α- and β-carotene and the internal standard were integrated. The response factors of the individual carotenoids were also obtained from separate injections of their stock solutions at various concentrations. The standard curves were obtained by plotting the area ratio of α- and β-carotene to the internal standard *versus* the concentration

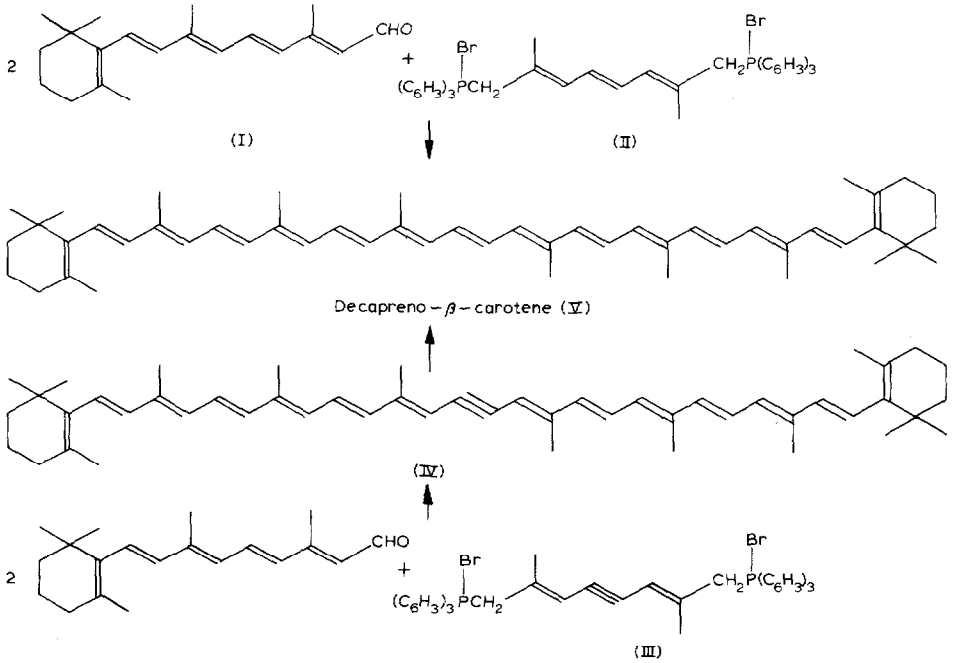

Fig. 2. Chemical synthesis of all-*trans*-decapreno-β-carotene according to the method of Surmatis and Ofner[16].

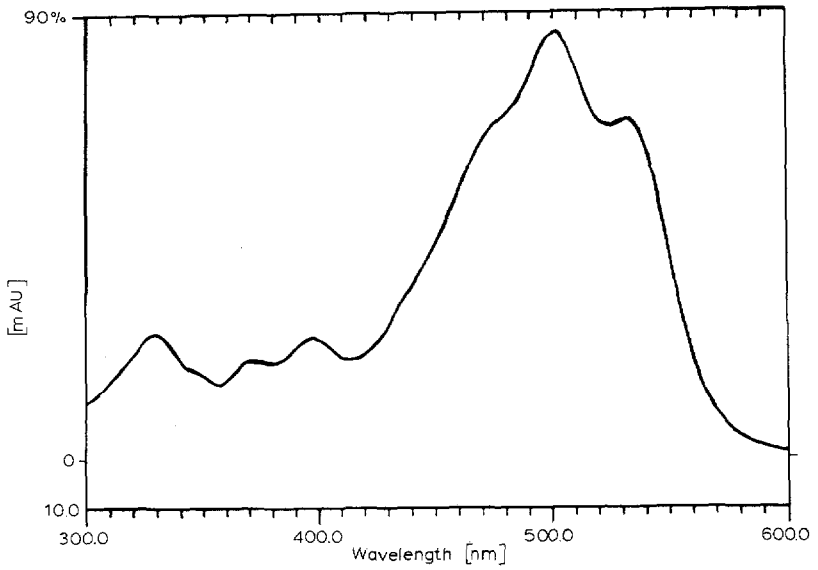

Fig. 3. Absorption spectrum of all-*trans*-decapreno-β-carotene (λ_{max} = 502 nm) in the HPLC solvent system methanol–acetonitrile–methylenechloride–hexane (22:55:11.5:11.5).

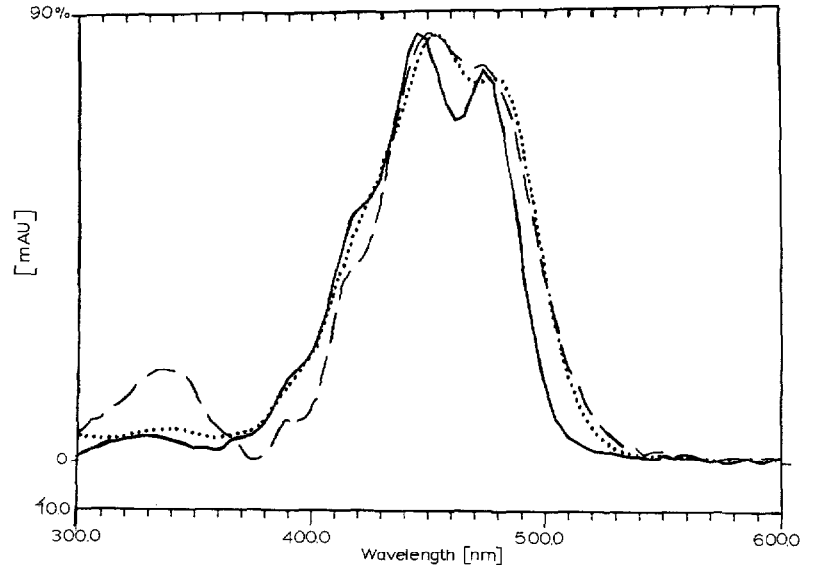

Fig. 4. Absorption spectra of all-*trans*-α-carotene (——) (λ = 446 nm), all-*trans*-β-carotene (.....) (λ = 454 nm), and 15,15′-*cis*-β-carotene (– – – –) (λ = 450 nm) in the HPLC solvent system methanol–acetonitrile–methylenechloride–hexane (22:55:11.5:11.5).

of the individual carotenes. The quantification of the hydrocarbon carotenoids in raw carrot extract was based on the evaluation of the area ratio of α- and β-carotene peaks to the area of the internal standard peak with the area ratios obtained from the standard curves.

RESULTS AND DISCUSSION

A typical chromatographic profile of a raw carrot extract containing decapreno-β-carotene (internal standard) is shown in Fig. 5. An isocratic system of methanol–acetonitrile–methylene chloride–hexane (22:55:11.5:11.5) has been employed to separate all-*trans*-α-carotene (peak A), all-*trans*-β-carotene (peak B), and 15,15′-*cis*-β-carotene (peak C, appearing as a shoulder on the all-*trans*-β-carotene peak) from the internal standard (peak D). Decapreno-β-carotene is the last eluting peak and it does not interfere with the α- and β-carotene peaks. For most of the other fruits and vegetables studied, particularly the green vegetables, the carotenoids that elute last on a C_{18} reversed-phase column are all-*trans*-β-carotene and its 15,15′-*cis*-isomer[19]. Therefore, the application of decapreno-β-carotene as an internal standard for the quantification of the hydrocarbon carotenoids is not limited to carrots and it can be extended to other fruits and vegetables. Decapreno-β-carotene has also been found suitable for the determination of α- and β-carotene in human serum[20]. There are several advantages that make this C_{50} β-carotene an attractive internal standard for the determination of α- and β-carotene. These are as follows: (a) it elutes after all-*trans*-α- and β-carotene on the C_{18} reversed-phase HPLC column; (b) it is not naturally occurring and can be readily synthesized on a large scale in high purity; (c) it has similar solubility behavior as that of α- and β-carotene and it can be added to the fruits and vegetables at the extraction stage; (d) it is stable relative to α- and β-carotene and it does not undergo degradation or stereoisomerization in the extracting solvents within the analysis time.

The recovery studies on decapreno-β-carotene indicated that the solubility behavior of this compound is very similar to that of α- and β-carotene. The average

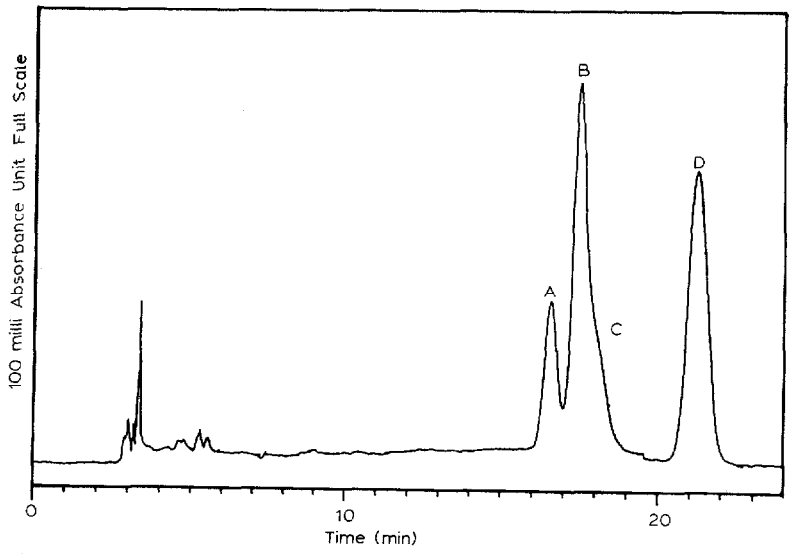

Fig. 5. HPLC profile of raw carrot extract containing all-*trans*-decapreno-β-carotene as an internal standard. Chromatographic conditions described in text. Peaks: A = all-*trans*-α-carotene, B = all-*trans*-β-carotene, C = 15,15′-*cis*-β-carotene, D = all-*trans*-decapreno-β-carotene (internal standard).

TABLE I

RECOVERY OF DECAPRENO-β-CAROTENE FROM CARROT EXTRACTS

Weight (mg) of internal standard added	*Weight (mg) of internal standard recovered*	*Recovery (%)**
5.2	5.0	96.2
5.5	5.3	96.4
5.8	5.7	98.3

* Relative standard deviation is 1.2%.

recovery of decapreno-β-carotene from carrot extracts was more than 96% (Table I). These results have been based on the weight of the internal standard before and after extraction as determined by the HPLC peak area of the internal standard.

The calibration curves obtained from a plot of the area ratios of α- and β-carotene peaks to that of the internal standard against the concentrations of these carotenes (Fig. 6) gave good linearity over a wide range of concentration and had a relative standard deviation of 3.3% for α-carotene and 3.4% for β-carotene. Since 15,15'-*cis*-β-carotene was not resolved and it appeared as a tailing shoulder on all-*trans*-β-carotene peak, the area corresponding to this *cis*-isomer was included in the integration of the all-*trans*-β-carotene. Therefore, in quantification of the total carotenoids in raw carrots, it has been assumed that the 15,15'-*cis*-β-carotene would exhibit a similar chromatographic response to its all-*trans*-isomer.

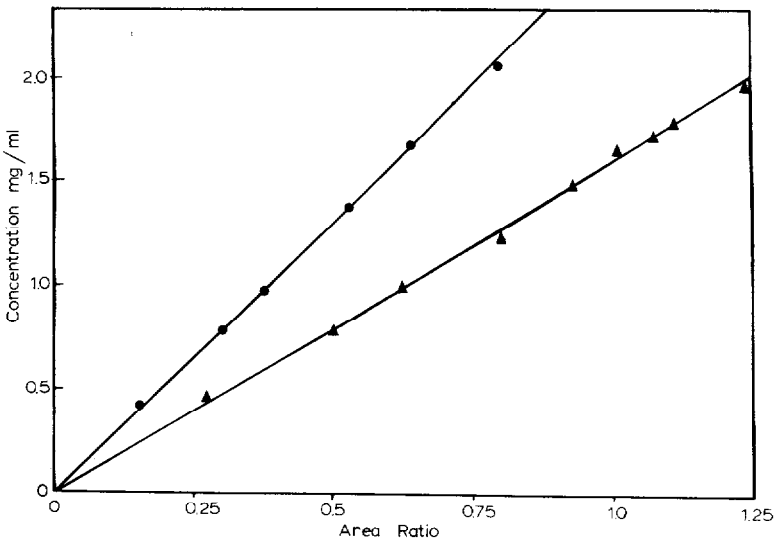

Fig. 6. Plots of the standard curves for α- and β-carotene. ●, Concentration of α-carotene (mg/ml) *versus* area ratio of α-carotene–internal standard, coefficient of variation 3.3%. ▲, Concentration of β-carotene (mg/ml) *versus* area ratio of β-carotene–internal standard, coefficient of variation 3.4%. [Coefficient of variation = (standard deviation/mean) × 100].

It is not clear at this point whether the presence of the *cis*-isomer of β-carotene in raw carrots is an artifact due to extraction and/or chromatography. There have been literature reports[21,22] that have described the separation of mono and di-*cis*-isomers of β-carotene by HPLC. These reports have indicated that the *cis*-isomers are stable enough in solvents such as hexane, acetone, and chloroform to allow their isolation and characterization.

The carotenoid content of five separate batches of raw carrots has been determined using the internal standard technique. The relative standard deviations for α-carotene, β-carotene, and total carotenes were 7, 2.5, and 3.6% respectively. The discrepancy within these data is consistent with the usually observed large variability of carrot samples. The quantitative determination of all-*trans*-α- and all-*trans*-β-carotene employing decapreno-β-carotene as an internal standard is in good agreement with the values obtained for these carotenes based only on the response factor of α- and β-carotene standard solutions (data not shown). The total carotene content of carrots using the internal standard was in the range of 16.44–17.92 mg/100 g which is consistent with the total carotene content of raw carrots reported by HPLC (6.6–18.4 mg/100 g)[4] and colorimetric methods (6–21 mg/100 g)[23].

The reproducibility of our results within the same series of samples indicates that the decapreno-β-carotene can be employed as an internal standard to generate reliable quantitative data on the hydrocarbon carotenoids. Since the HPLC analysis of carotenoids is usually accompanied by extraction and sample pretreatment where variable recoveries of these compounds may occur, we strongly recommend the use of an internal standard to compensate for various analytical errors. The accuracy of this approach is obviously dependent on the structure equivalence of the carotenoids of interest and the internal standard. We have shown that decapreno-β-carotene is a suitable internal standard for the quantitative determination of α- and β-carotene. However, the development of other internal standards for quantification of various classes of carotenoids may be necessary.

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