Rapid Isocratic HPLC Analysis of Beta-Carotene in Red Peppers (*Capsicum annuum* L.) and Food Preparations

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Key Words

Column liquid chromatography
β-Carotene
Capsanthin and Capsorubin
Red peppers

Summary

A simple, rapid high-performance liquid chromatography method has been devised in order to separate and quantify β-carotene present in red pepper (*Capsicum annuum* L.) fruits and food preparations. A reversed-phase isocratic non-aqueous system enables the separation of β-carotene within a few minutes, with detection at 450 nm. The selection of extraction solvents, mild saponification conditions, and chromatographic features, is evaluated and discussed. The method is proposed for rapid screening of large plant populations, plant selection, as well as for food products, and also for nutrition and quality control studies.

Introduction

The hydrocarbon carotenoid β-carotene (β,β-carotene, I; Figure 1) is a polyene, yellow-orange pigment found widely in plant tissues in the more stable E (all-*trans*) configuration. Its characteristic chromophore associated with the presence of eleven conjugated double bonds displays an absorbance maximum at approximately 450 nm, which makes it useful as a coloring agent for foods, cosmetics and pharmaceuticals, either as plant extracts and dehydrated powders, or in synthetic form [1]. In fact, β-carotene was the first synthetic carotenoid to be marketed (in 1954), and it has been classified by FAO/WHO in Class A under the label *E160 a* as acceptable for use in foods [1, 2]. In addition, β-carotene possesses provitamin A activity [3] and is a powerful antioxidant [4], thus being able to deactivate reactive chemical species such as singlet oxygen, triplet photochemical sensitizers, and free radicals that would otherwise damage DNA inside cells and could trigger cancer – inducing mutations [5, 6], and adversely affect specific immune functions [7]. However, early studies implicating dietary β-carotene with decreased incidence of certains forms of cancer [5, 8] have been challenged by recent trials which ruled out its relevance in reducing the incidence of lung cancer in male smokers [9–11]. Be that as it may, β-carotene is largely regarded as an important nutritional component and/or supplementing color additive in foods, and the need for monitoring it in various plant foods, biological tissues and food preparations has led to much activity in this area. High-performance liquid chromatography (HPLC) is the method of choice, and previously reported procedures using both normal- and reversed-phase conditions, in either gradient or isocratic mode, with unprocessed or saponified extracts, have been reviewed extensively [12–18]. Many of them require long retention times and mobile phase systems with gradient solvents, or containing water which could induce on-column precipitation of carotenoids during elution. We have stressed the importance of these points in a previous work on HPLC determination of capsanthin and capsorubin (II and III, respectively; Figure 1) in red peppers (*Capsicum annuum* L.) [19]. We present here an isocratic non-aqueous reversed-phase HPLC procedure for the rapid separation and quantification of β-carotene in plant samples and also in food preparations, suitable for routine determinations.

Experimental

Instrumentation

Chromatographic separations were done on a Tracor 985 liquid chromatograph equipped with a Model 970A variable-wavelength UV-Vis detector and a Model 951 pump. A Milton Roy LDC (I-10B) integrator was employed to record retention time and chromatograms, and to evaluate peak areas. A reversed-phase column (Merck Superspher RP-18, 4 μm, 12.5 × 0.4 cm I.D.) was used at ambient temperature and protected with a precolumn (Merck, LiChrospher 100 RP-18, 5 μm, 4 ×

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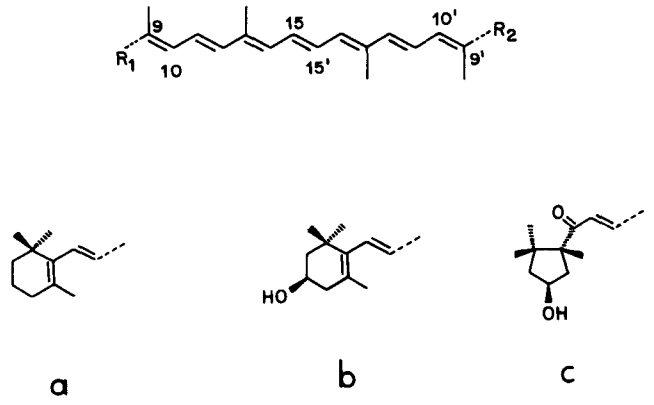

Figure 1
The structures of β-carotene (I, $R_1 = R_2$ = a), capsanthin (II, R_1 = b, R_2 = c), and capsorubin (III, $R_1 = R_2$ = c).

0.4 cm I.D.). Chromatograms were monitored at 450 nm; the mobile phase was acetonitrile-2-propanol-ethyl acetate (40:40:20, v/v/v); the flow-rate was 0.8 mL min^{-1}; the pressure was 850–1050 p.s.i.; and the recorder chart speed was 0.5 cm min^{-1}.

Materials

A β-carotene sample used as standard was kindly provided by Dr. W. Schüep, Hoffmann-La Roche (Basle, Switzerland). Various food-grade preparations were purchased at a local market. Solvents used for extraction were of analytical grade and those used for chromatography were of HPLC grade (Biolab, Jerusalem, Israel). Thin-layer chromatographic (TLC) examination of reference compounds, as well as of plant extracts, was performed using silica gel G (Merck) plates, and a 70:30 (v/v) mixture of petroleum ether (b.p. 40–60 °C)-acetone.

Plant Material

Fresh red pepper (*C. annuum* L. var. "Lehava") fruits, grown in an experimental field at the Bet Dagan Experiment Station of the Agricultural Research Organization, were dried in the dark at 45 °C under vacuum for 72 h, and then ground and sieved (1-mm mesh) to give a fine powder.

Pigment Extraction

All extraction and saponification work was done in the dark or in subdued light. Dried and finely powdered paprika samples (500 mg) were repeatedly extracted under stirring at room temperature with several portions of petroleum ether (b.p. 40–60 °C) until colorless extracts were obtained. The suspensions were filtered on sintered-glass funnels and the combined filtrates were made up to 100 mL in volumetric flasks. Commercially available food-grade preparations (soft drink, 20 mL; soup, 2 g; multivitamin supplement, one tablet, 1.4 g) were extracted with petroleum ether (2 × 10 mL) under stirring at room temperature, the organic phases were separated, washed with water (3 × 10 mL), brought to 25 mL with petroleum ether in a volumetric flask, and aliquots (10 mL) were evaporated gently to dryness in a stream of nitrogen.

Saponification (optional)

When required, aliquots (25 mL) of the extracts were treated with 5 % ethanolic potassium hydroxide solution (5 mL) and kept overnight at room temperature for complete saponification of xanthophyll esters (TLC evidence). Water (25 mL) was then added and the mixture was extracted with petroleum ether (3 × 15 mL) until colorless extracts were obtained. The combined extracts were washed with water (3 × 15 mL), brought to 50 mL with petroleum ether in a volumetric flask, and aliquots (2 mL) were evaporated gently to dryness in a stream of nitrogen.

HPLC Determination

The extracts obtained as described above were dissolved in mobile phase (1 mL), filtered through a 0.45-μm membrane disc (Schleicher and Schüll, Dassel, Germany) and injected into the chromatograph (injection volume, 10 μL). The column was regenerated by washing with 2-propanol after analysis, and then equilibrated with the mobile phase.

Standard Solutions

Stock solutions of β-carotene (0.25 mg mL^{-1}) were prepared in petroleum ether (b.p. 40–60 °C). Concentrations of the standard solutions were checked spectrophotometrically using the corresponding extinction coefficient reported values [20]. Aliquots (10–200 μL) were evaporated to dryness in a nitrogen stream and the residues were dissolved in the mobile phase (1 mL) and subjected to HPLC, as described above.

Quantitation

Standard calibration graphs were prepared for β-carotene by plotting peak area measurements at 450 nm *versus* concentration. Linearity, reproducibility and recovery were determined routinely.

Results and Discussion

Sample Preparation and Extraction Conditions

Although the presence of a relatively long chain of conjugated carbon–carbon double bonds makes β-carotene susceptible to light, air, heat, and acid degradation, no E-Z (*trans-cis*) isomerization is believed to occur during the extraction process [21–23], notwithstanding reports listing solvents able to induce it [21, 24]. Oddly, these concerns have been largely ignored by later works, as far as the choice of solvents for both extraction and HPLC mobile phases is regarded.

Table I. Retention time and elution solvents of β-carotene.

Column length (cm)	Retention time (min)[a]	Mobile phase components (v/v/v)[b]		
		Acetonitrile	2-Propanol	Ethyl acetate
25.0	27.53	80	10	10
12.5	13.48	80	10	10
12.5	9.58	75	15	10
12.5	8.77	70	15	15
12.5	5.28	60	30	10
12.5	4.43	50	35	15
12.5	3.54	40	40	20
12.5	2.94	30	50	20

[a]Average of ten runs; relative standard deviation was less than 3 % for the reference compound.
[b]Flow-rate, 0.8 mL min^{-1}.

Petroleum ether was used as extraction solvent in our experiments. Some solvents variously employed by other workers were avoided because of the reported risk of isomerization (hexane, ether, methanol [23], benzene, toluene [20]), and/or undesirable side reactions: acetone (which would form aldol condensation artifacts with ketonic carotenoids [25] or polymerize in the presence of alkali, producing interfering oils [20]), chlorinated solvents like dichloromethane and chloroform (which might contain traces of hydrochloric acid that would lead to carotenoid losses [21]), and tetrahydrofuran (which might promote peroxide formation with consequent production of artifacts [15]). The extraction progress was followed visually by gradual fade-out of the extract's color.

Saponification

Sample preparation for most β-carotene-containing food preparations does not necessarily require saponification, since this carotenoid is, of course, devoid of esterifiable hydroxy groups. In the case of plant samples, work with unsaponified extracts would reflect more accurately the xanthophyll's composition and occurrence in plant tissue, and could avoid the arguably destructive effect of saponification [15, 18, 20, 26–28]. However, saponification is often preferred since it removes accompanying lipids and chlorophylls, and thus leads to a less crowded chromatogram. If anything, cold (*i.e.*, ambient temperature) saponification should be preferred as it was found to have a minimal effect on β-carotene [22, 23].

In our work, the optimal conditions for mild saponification of xanthophyll esters were achieved with 5 % ethanolic potassium hydroxide solution overnight at room temperature, as ascertained by TLC (which, reportedly, does not induce by itself E-Z isomerization [23]). Other conditions including shorter reaction times or more concentrated alkaline reagents, amply documented in the literature [26–28], proved to be less convenient as far as complete hydrolysis and pigment stability are concerned.

Chromatographic Conditions

Since the aim of our investigation was to develop a method suitable for routine screening of β-carotene in paprika cultivars and food preparations, we settled on an isocratic HPLC procedure rather than solvent gradient systems which are commonly used and require long equilibration periods between injections, and additional equipment. As a result, the overall and per sample analysis time is sensibly reduced and lesser fluctuations afflict the retention time values. Reversed-phase chromatography provided satisfactory resolution of β-carotene, as no interfering peaks appear at similar retention times in the chromatogram of xanthophyll and carotenoid standards [19]. Regarding the composition of the mobile phase, examination of the reviewed literature [12–18] shows that most systems contain solvents that were demonstrated to display poor solubilization properties (acetonitrile and methanol) and insufficient inertness (methanol, chlorinated derivatives) towards β-carotene [24]. Typical eluent systems consisted of acetonitrile or methanol as a base solvent, and chloroform, dichloromethane, tetrahydrofuran or water were optionally added as modifiers to control the eluotropic strength [14, 29]. Previous attempts to increase retention by the addition of water to the mobile phase [13, 18] were avoided in our work because of the scant solubility of carotenoids in water and, consequently, the possibility of their precipitation on the column. Instead, addition of 2-propanol and ethyl acetate to acetonitrile afforded ternary non-aqueous systems that did improve the sample capacity, chromatographic efficiency and column use period.

Systematic modification of the mobile phase (Table I) undertaken to optimize the selectivity for β-carotene, led to a significant decrease in the proportion of acetonitrile (to approximately 40 %), the major part being now based on ethyl acetate (20 %) and 2-propanol (40 %) which are better solvents as far as solubilization and inertness are concerned. Under these conditions, β-carotene was resolved within 4 min at a flow-rate of 0.8 mL min^{-1} (Figure 2). The samples were injected in

small volumes (10 µL) to avoid peak distortion and the production of artifacts [30].

Analysis of Paprika Cultivars and Food Preparations

Typical elution profiles of plant and food extracts are shown in Figure 2. The identity of the carotenoid peaks was confirmed by spiking samples with measured amounts of standard solutions. Spiking experiments increased the peak height of I, practically without altering the retention time. A linear regression was obtained of the peak area against concentration ($y = 8.4\,x + 18.2, r^2 = 0.99$, in the range of 5–60 ng of I). Standard curves based on peak areas were drawn for β-carotene, and its amount in paprika cultivars and food preparations was calculated accordingly. In routine analyses, the concentrations of I in plant samples were derived from peak areas, with the working standard solution as reference, aliquots of which were run before each series of several (five-seven) samples. Further validation of the method was adduced by co-injection of extracts together with standards. The detection limit was approximately 30 ng of I. Recovery experiments were carried out in duplicate by the addition of selected volumes of the stock standard solution of I to the saponification flask of the sample to be analyzed, and by further processing as described above. Recovery of I from fortified plant extracts averaged 101.9 ± 1.4 % (Table II). The reproducibility of analyses was estimated by the coefficient of variation of standard solutions of I in six successive assays performed on the same day (3.8 ± 0.3 %). Due to the extreme lability of these compounds we saw no point in studying the reproducibility between assays at longer time intervals, and we emphasize the importance of working only with freshly prepared extracts and standard solutions.

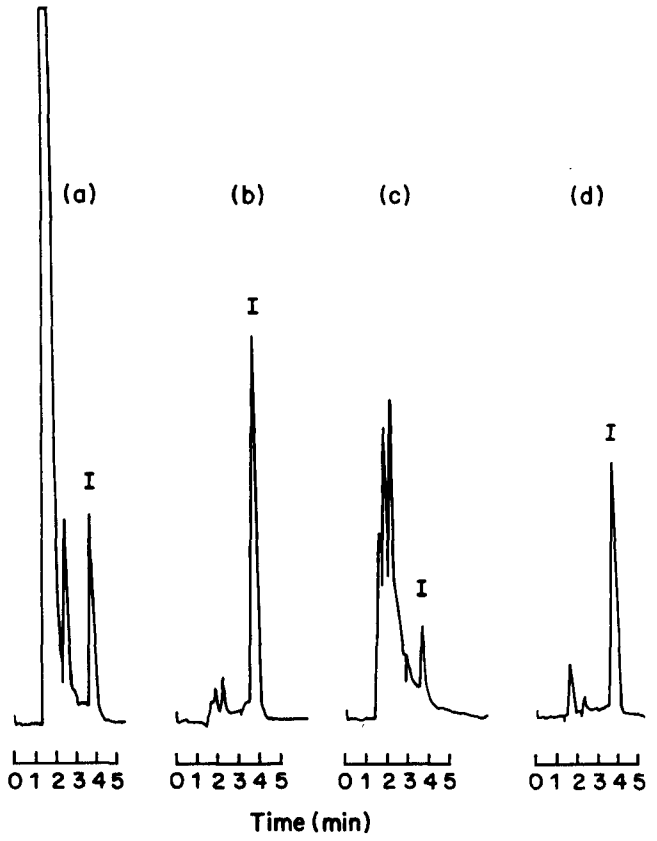

Figure 2
Typical chromatograms on a 12.5-cm column of extracts from (a) red peppers (after saponification), (b) soft drink, (c) chicken soup, (d) multivitamin supplement. Peak identification: I = β-carotene.

Examination of the chromatograms (Figure 2, a) suggests that the saponification step would be advisable in the case of plant samples, since it leads to simplified images of the actual mixtures. Contrariwise, in other food preparations such as soft drinks and multivitamins (Figure 2, b and d), β-carotene is smoothly resolved by usual extraction and subsequent submission to HPLC.

In conclusion, we propose that the HPLC procedure described can be reliably applied to the rapid screening of large plant populations, plant selection, as well as for food preparations, and also for nutrition and quality control studies. Separation of β-carotene was achieved within a few minutes by employing an isocratic non-aqueous reversed-phase system which conveniently avoids cumbersome and lengthy solvent gradients, and on-column solute precipitation by water. The formation of degradation products, artifacts and isomers is minimized due to the short elution time. Further applications, currently under investigation, include separation of the geometric E and Z isomers and/or the structural α, β, and γ isomers of carotene, which might be of interest for food processing and dietary considerations.

Table II. Recovery of β-carotene in a fortified red pepper sample.

Determined in sample	Added	Calculated	Found[a]	Recovery (%)
	(ng)			
23.4	20	43.4	44.4	102.3
23.4	25	48.4	48.1	99.3
23.4	30	53.4	55.6	104.1

[a]Each value represents the average of duplicate assays at each fortified level, analyzed on the same day.

Acknowledgements

We are grateful to Dr. W. Schüep, Hoffmann-La Roche AG (Basle, Switzerland) for generously making available a pure sample of β-carotene.

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Received; May 14, 1997
Revised manuscript
received: Jun 24, 1997
Accepted: Jul 18, 1997