

Isocratic non-aqueous reversed-phase high-performance liquid chromatographic separation of capsanthin and capsorubin in red peppers (*Capsicum annuum* L.), paprika and oleoresin

M. Weissenberg*, I. Schaeffler, E. Menagem, M. Barzilai, A. Levy

Laboratory of Natural Products Chemistry, Department of Plant Genetics, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel

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Abstract

A simple, rapid high-performance liquid chromatography method has been devised in order to separate and quantify the xanthophylls capsorubin and capsanthin present in red pepper (*Capsicum annuum* L.) fruits and preparations made from them (paprika and oleoresin). A reversed-phase isocratic non-aqueous system allows the separation of xanthophylls within a few minutes, with detection at 450 nm, using methyl red as internal standard to locate the various carotenoids and xanthophylls found in plant extracts. 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 paprika products and oleoresin, and also for nutrition and quality control studies.

Keywords: Capsanthin; Capsorubin; Carotenoids

1. Introduction

The fruits of the genus *Capsicum* (Solanaceae) are used commercially as natural flavoring and coloring agents for foods and animal feeds, either as dried and finely ground powders (paprika), concentrated extracts (oleoresins), or as paste concentrates [1]. The bright red color of these preparations is related mainly to the presence of two oxygenated carotenoids (xanthophylls), capsorubin, (3*S*,5*R*,3'*S*,5'*R*)-3,3'-dihydroxy- α , β -carotene-6,6'-dione, and capsanthin, (3*R*,3'*S*,5'*R*)-3,3'-dihydroxy- β , γ -carotene-6'-one (I and II, respectively; Fig. 1), that contribute

65–80% of the total color of the ripe pepper fruits [2] in which they occur largely esterified with fatty acids as monoesters (26%) and diesters (54%), and also in free form (20%) [3]. Compound I occurs as a diester only [3].

Compounds I and II apparently are present only in the genus *Capsicum* [4] and accumulate in the wall of ripe fruits; therefore, pepper cultivars with high xanthophyll content and a thin pericarp would be preferred for industrial processing, since they could be dried and ground more easily [5]. Although the esterified form of xanthophylls is reported to have greater stability than the free form [6], the paprika preparations gradually deteriorate and lose color as a result of physical, chemical and enzymatic factors, as well as processing and storage conditions [5]. The

* Corresponding author.

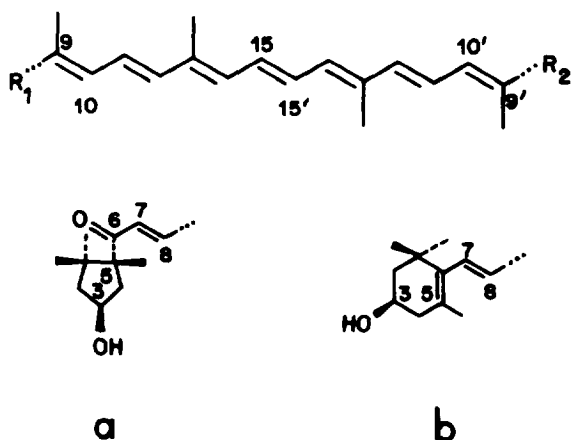


Fig. 1. The structures of capsorubin (I, $R_1=R_2=a$) and capsanthin (II, $R_1=b$, $R_2=a$).

liposoluble xanthophyll esters of red pepper are readily metabolized to safe and/or health-important metabolites, display provitamin A activity, and thus might act as chemopreventive agents for cancer [7,8]. They are known to have anti-ulcer properties [9].

As part of a study of the distribution and yield of compounds I and II in red pepper fruits and preparations made from them, analytical methods were considered for their estimation by a rapid and simple assay that would be suitable for large screening programmes and for plant selection and breeding. High-performance liquid chromatography (HPLC) is the method of choice, and previously reported procedures used both normal- and reversed-phase conditions, in either gradient or isocratic mode [5,6,10–22], with unprocessed or saponified extracts. Work with unsaponified extracts would reflect more accurately the xanthophylls' composition and occurrence in plant tissue, and would avoid the arguably destructive effect of saponification [9,11,19,23–26]. However, saponification is often preferred since it removes accompanying lipids and chlorophylls, and thus leads to a less crowded chromatogram. Furthermore, hydrolysis of the carotenol esters requires the use of authentic samples of the more common free xanthophylls, rather than of their corresponding esters – which are not generally available. We wish to report here a reversed-phase HPLC procedure for the separation and determination of I and II in paprika samples and products, in an isocratic non-

aqueous system, and involving mild saponification conditions. Previously reported isocratic procedures for estimation of I and II in red peppers usually employed mobile phase systems containing water [14–17], which could, however, induce on-column precipitation of carotenoids during elution (vide infra).

2. Experimental

2.1. Reference compounds and solvents

Capsorubin, capsanthin, canthaxanthin, lutein, zeaxanthin, cryptocapsin, β -cryptoxanthin and β -carotene samples, used as standards, were kindly provided by Dr. W. Schüep, Hoffmann-La Roche (Basle, Switzerland). A *Capsicum* oleoresin sample was kindly supplied by Avshalom Ltd. (Bet Kama, Israel) and a paprika food-grade preparation was purchased at a local market. Methyl red (4-dimethylaminoazobenzene-2'-carboxylic acid), of analytical grade (BDH, Poole, UK), was used as the internal standard. 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.

2.2. Plant material

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

2.3. 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. Reversed-phase columns (either Merck LiChrospher 100 RP-18, 5 μm , 25 \times 0.4 cm I.D., or Merck Superspher RP-18, 4 μm , 12.5 \times 0.4 cm I.D.) were used at ambient temperature and were protected with precolumns (Merck, LiChrospher 100 RP-18, 5 μm , 4 \times 0.4 cm I.D.). Chromatograms were monitored at 450 nm; the mobile phase was acetonitrile–2-propanol–ethyl acetate (80:10:10, v/v); the flow-rate was 0.8 ml/min; the pressure was 850–1050 p.s.i. and the recorder chart speed was 0.5 cm/min.

2.4. Pigment extraction

All extraction and saponification work was done in the dark or in subdued light. Dried and finely powdered paprika samples (100 mg) were repeatedly extracted under stirring at room temperature with diethyl ether (2 \times 20 ml, for 1 and 0.5 h, respectively), followed by methanol (3 \times 20 ml, for 1, 0.5 and 0.5 h, respectively) until colorless extracts were obtained. The suspensions were filtered on sintered-glass funnels and the combined filtrates were made up to 100 ml with methanol, in volumetric flasks.

2.5. Saponification

Aliquots (25 ml) of the extracts were treated with 5% methanolic potassium hydroxide solution (5 ml) and kept overnight at room temperature for complete saponification (TLC evidence). Water (25 ml) was then added and the mixture was extracted with diethyl ether (3 \times 15 ml) until colorless extracts were obtained. The combined ethereal extracts were washed with water (3 \times 15 ml) and brought to 50 ml with diethyl ether in a volumetric flask.

2.6. HPLC determination

Aliquots (2 ml) of the saponified extract were evaporated gently to dryness in a stream of nitrogen and the residue was 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 methanol after analysis and then was equilibrated with the mobile phase.

2.7. Standard solutions

Stock solutions of capsorubin (10 mg/100 ml) and capsanthin (20 mg/100 ml) were prepared in methanol. The other carotenoid standards were prepared in methanol, except for β -carotene which was prepared in petroleum ether (b.p. 40–60°C). Concentrations of the standard solutions were checked spectrophotometrically using the corresponding extinction coefficient reported values [27]. Aliquots (20–200 μl) were evaporated to dryness separately and/or together in a nitrogen stream and the residues were dissolved in the mobile phase (2 ml) and subjected to HPLC, as described in Section 2.6. Freshly prepared solutions of methyl red in methanol (10 mg/100 ml) were used as internal standard and aliquots (200 μl) were processed for HPLC in the same manner as done for standard carotenoids and xanthophylls.

2.8. Quantitation

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

3. Results and discussion

3.1. Sample preparation

Diethyl ether and methanol were used sequentially as extraction solvents in our experiments. Some solvents variously employed by other workers were avoided because of the reported risk of isomerization and/or undesirable side reactions: acetone (which would form aldol condensation artifacts with ketonic carotenoids [28] or polymerize in the presence of alkali, producing interfering oils [29]), chlorinated solvents like dichloromethane and chloroform (which might contain traces of hydrochloric acid that would lead to carotenoid losses [30]) and tetrahydrofuran (which might promote peroxide formation with consequent production of artifacts [31]). The extraction progress was followed visually by gradual fade-out of the extract's color.

3.2. Saponification

The optimal conditions for mild saponification of xanthophyll esters were achieved with 5% methanolic potassium hydroxide solution overnight at room temperature, as ascertained by TLC (see Section 2). Other conditions, including shorter reaction times or more concentrated alkaline reagents, amply documented in the literature [23–26], proved to be less convenient in our experiments.

3.3. Chromatographic conditions

Since the aim of our investigation was to develop a method suitable for routine screening of xanthophylls in paprika cultivars, we settled on an isocratic HPLC procedure, rather than solvent gradient systems, which are commonly used and require long equilibration periods between injections and the use of 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 capsorubin (I) and capsanthin (II), as no interfering peaks appear at similar retention times in the chromatogram of xanthophyll and carotenoid standards (Table 1, Fig. 2). Previous attempts to increase retention by the addition of water to the mobile phase [14–17] were avoided in our work because of the scant solubility of carotenoids in water and, consequently, the possibility of

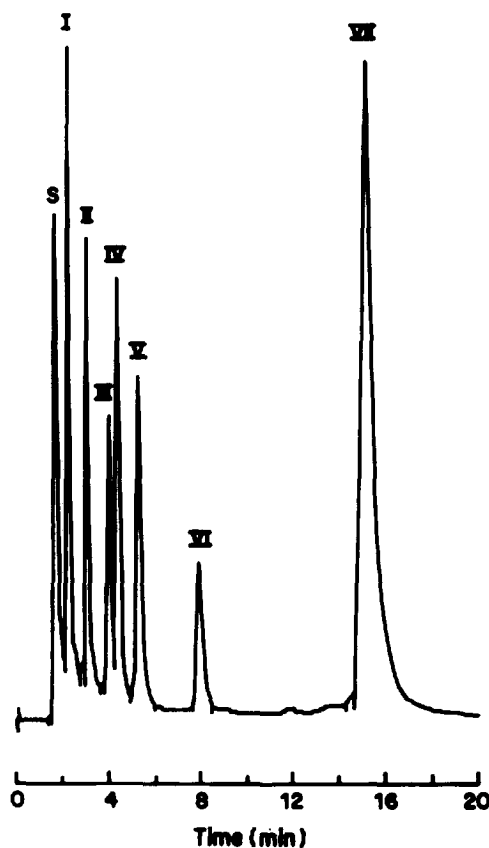


Fig. 2. Reversed-phase HPLC separation of carotenoids in a standard mixture on a 12.5-cm column. Peak identification: S= methyl red (internal standard); I=capsorubin, II=capsanthin; III= canthaxanthin+lutein; IV=zeaxanthin; V=cryptocapsin; VI= β -cryptoxanthin; VII= β -carotene.

Table 1
Retention time and elution solvents of capsorubin and capsanthin

Column length (cm)	Retention time (min) ^a		Mobile phase components (v/v/v) ^b		
	Capsorubin	Capsanthin	Acetonitrile	2-Propanol	Ethyl acetate
12.5	1.55	1.67	40	45	15
12.5	1.40	1.68	60	25	15
12.5	1.99	2.41	70	15	15
12.5	2.05	2.63	75	15	10
12.5	2.27	3.02	80	10	10
25.0	4.49	6.01	80	10	10
12.5	2.92	4.20	85	7.5	7.5
25.0	5.97	8.68	85	7.5	7.5
12.5	3.40	5.14	90	5	5
12.5	2.93	4.27	90	10	0

^a Average of ten runs; relative standard deviation was less than 3% for each reference compound.

^b Flow-rate, 0.8 ml/min.

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: For separation of I and II, the composition of the mobile phase appeared to be optimal at an acetonitrile content of 80–85% (Table 1). Increasing the acetonitrile content further improved the separation of I and II, but peak distortion was observed, presumably due to the formation of HPLC artifacts. Conversely, a decrease in the proportion of acetonitrile (to approximately 40%) allows virtual co-elution of I and II, which might have some practical value, should the total xanthophyll content be required. A standard mixture of reference carotenoids and xanthophylls was mostly resolved (within 15 min on the short column and 30 min on the standard column) by our HPLC method at a flow-rate of 0.8 ml/min. Under these experimental conditions, canthaxanthin and lutein ran together (Fig. 2), and capsorubin and capsanthin were resolved almost to baseline separation (within 3 min on the short column and 6 min on the standard column, Fig. 3).

The use of methyl red as the internal standard proved to be valuable, since it elutes slightly before

compounds I and II, in the region free of other carotenoid peaks (Fig. 2), and thus provides an additional parameter for the location of the usually meagre peak of I (Fig. 3). The samples were injected in small volumes (10 μ l) to avoid peak distortion and the production of artifacts [30].

3.4. Analysis of paprika cultivars and preparations

Typical elution profiles of paprika extracts are shown in Fig. 3. The identity of the capsorubin and capsanthin peaks (especially the former, which usually is quite faint) was confirmed by spiking samples with measured amounts of standard I and II solutions, and also by reference to the peak of the internal standard. Spiking experiments increased the peak heights of I and II, practically without altering their retention time. A linear regression was obtained of the peak area against concentration ($y=4.05x-6.51$, $r^2=1$, in the range of 10–100 ng of I; and $y=4.38x-35.2$, $r^2=0.94$, in the range of 10–80 ng of II). Standard curves based on peak areas were drawn for both I and II, and the amount of each compound in paprika cultivars and preparations was calculated accordingly. In routine analyses, the concentrations of I and II in plant samples were derived

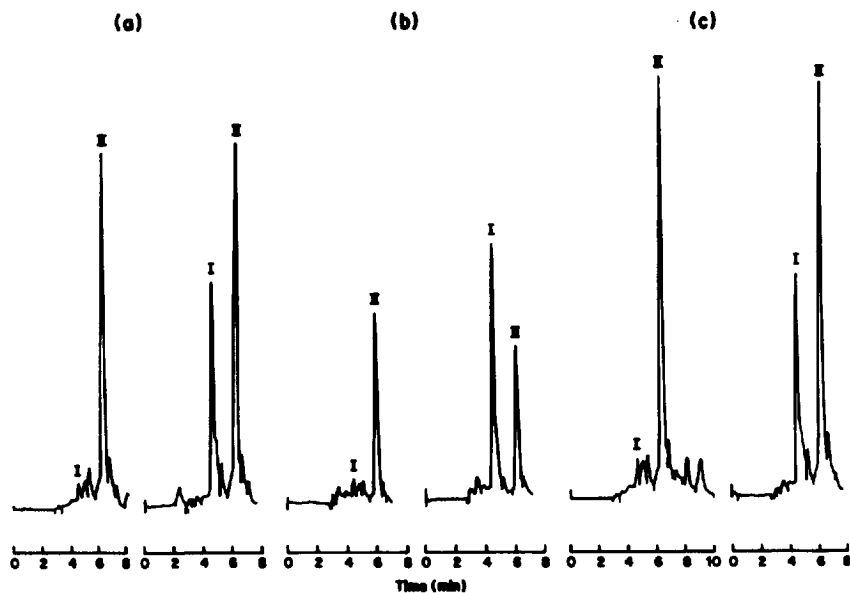


Fig. 3. Typical chromatograms on a 25-cm column of saponified extracts from (a) red peppers, (b) a paprika food-grade preparation, (c) *Capsicum oleoresin*, before and after spiking with capsorubin. Peak identification: I=capsorubin; II=capsanthin.

from peak areas, with the working standard solution as reference, aliquots of which were run before each series of several (5–7) samples. The detection limit was about 30 ng of either I or II. Recovery experiments were carried out in duplicate by the addition of selected volumes of the stock standard solutions of I and II to the saponification flask of the sample to be analyzed and by further processing as described above. Recovery of I and II from fortified plant extracts averaged $100.4 \pm 0.8\%$ for I and $100.1 \pm 1.9\%$ for II (Table 2). The reproducibility of analyses was estimated by the coefficient of variation of standard solutions of I and II in four successive assays performed on the same day (3.0 and 2.2%, respectively). Due to the extreme lability of these compounds, we saw no point in studying the reproducibility between assays at longer time intervals and we stress the importance of working only with freshly prepared extracts and standard solutions.

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 paprika preparations and oleoresin, and also for nutrition and quality control studies. Separation of xanthophylls 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. Likewise, methyl red was found to be a suitable internal

standard for locating all compounds. This method is currently in use in our laboratory for routine searches and taxonomic studies, the results of which will be reported elsewhere.

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Table 2
Recoveries of capsorubin and capsanthin in a spiked paprika sample

Xanthophyll	Determined in sample (ng)	Added	Calculated	Found ^a	Recovery (%)
Capsorubin	48.7	20	68.7	69.7	101.4
	46.0	40	86.0	88.2	102.5
	46.0	60	106.0	105.9	99.9
	46.0	80	126.0	126.4	100.3
	46.0	160	206.0	202.0	98.0
Capsanthin	545.2	200	745.2	731.9	98.2
	545.2	400	945.2	906.8	95.9
	572.6	600	1172.6	1195.9	101.9
	585.5	800	1385.5	1449.2	104.5

^a Each value represents the average of duplicate assays at each spiked level, analyzed on the same day.

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