Hydrolysis Study of Carotenoid Pigments of Paprika (*Capsicum annuum* L. Variety Lehava) by HPLC/Photodiode Array Detection†

Yitzhak Ittah,* Joseph Kanner, and Rina Granit

Department of Food Science, Agricultural Research Organization, P.O. Box 6, Bet Dagan 50250, Israel

A method of separating and identifying unsaponified pigments extracted from paprika (*Capsicum annuum* L. var. Lehava) is described. It is based on high-pressure liquid chromatography (two serially connected C_{18} columns with an empirical gradient) coupled with a photodiode array detection method. Well-separated groups of peaks, corresponding to free and mono- and diesterified carotenoids and β-carotene was observed. The relative changes of these various groups during saponification were monitorable owing to slow transesterification by sodium methoxide at room temperature. Unlike potassium hydroxide hydrolysis, this procedure is very convenient and nondestructive to labile carotenoids, e.g., β-carotene.

Red pepper fruits (*Capsicum annuum* L.) are used commonly in the preparation of various dehydrated products, such as ground pepper (paprika), red pepper flakes, and oleoresin, a solvent-extracted fraction from the fruit especially utilized as a food colorant (Bauernfeind, 1973; Emodi et al., 1976). Color is considered the most important criterion in paprika quality. The presence of highly conjugated double bonds confers to carotenoids their intense coloration. Ketocarotenoids, e.g., capsanthin, capsorubin, and cryptocapsin, are unique to red pepper fruits. However, only the two xanthophylls, capsanthin and capsorubin (Philip and Francis, 1971), contribute to the red color, whereas β-carotene and zeaxanthin are responsible for the yellow-orange color. During the ripening process, xanthophylls accumulate in the fruits, mainly as mono- and diesters of fatty acids. Evaluation of the carotenoid concentration in the fruits has been achieved in the past by measuring the absorbance of a benzene extract (Fekete et al., 1976). Separation and identification have been realized by TLC and column chromatography (Vinkler and Richter, 1972; Buckle and Rahman, 1979) and recently also by the use of HPLC (Philip and Chen, 1988). For many reasons, saponification of the samples is recommended since it provides a less complex mixture of compounds, rendering a relatively simpler chromatogram. However a great deal of information ought to be lost by such a treatment, viz. ratios between esterified and free carotenoids, on one hand, and between mono- and diesters, on the other hand. These ratios probably determine the distribution of the carotenoids in the cell according to the lipophilic character. Recently, Biacs et al. (1989) developed a solvent system for HPLC capable of separating unsaponified extract of paprika fruits into three groups of compounds according to their relative lipophilic character. Nonesterified pigments, capsanthin and capsorubin, eluted first, followed by mono- and diesterified pigments. β-carotene, being a nonhydroxylated polyene, eluted between the two groups of esters.

The main purpose of this study was to separate and identify the most representative paprika pigments using HPLC in conjunction with a photodiode array detection method and to monitor the alkaline hydrolysis by using sodium methoxide as the slow acting reagent at room temperature.

MATERIALS AND METHODS

Materials. All chemicals used were of ACS grade and purchased from Merck (Darmstadt, Germany). All solvents used in chromatography were of HPLC grade. Pepper fruits (*C. annuum* L. var. Lehava) were selected for this study. Fresh fruits were dried, under the complete absence of light, at 45 °C for 8 h and then ground to give a fine powder. Sodium methoxide was freshly prepared from absolute methanol and sodium; the dry white powder, obtained after evaporation of excess methanol, was kept under argon and light-free conditions.

Pigment Extraction. In the complete absence of light, pigments of paprika were extracted by shaking 50 mg of powder for 10 min with 50 mL of acetone. The mixture was left to stand at room temperature for 4 h. The shaking–standing was repeated twice. An aliquot (5 mL) was filtered, evaporated to dryness, redissolved in 200 μL of chloroform, passed through a 0.45-μm filter, and injected (25 μL) into the HPLC column.

Hydrolysis Procedures. *With Potassium Hydroxide.* Paprika powder (500 mg) was suspended in a 2% BHT solution in 7.5 mL of absolute ethanol, and a 60% KOH aqueous solution (1.25 mL) was added. This suspension was stirred at 60 °C and under nitrogen for 25 min and chilled immediately thereafter in ice for 10 min. Water (5 mL) was then added, and the pigments were extracted repeatedly with 5-mL portions of hexane (5 mL) until no color could be observed in the extract. The combined hexane extracts were washed with water (25 mL) and dried over anhydrous sodium sulfate. An aliquot for HPLC injection was prepared as aforementioned.

With Sodium Methoxide. Paprika powder (10 mg) was suspended in absolute methanol (15 mL), and 50 mg of sodium methoxide added. The flask was sealed tightly and left at room temperature under stirring and in the absence of light. The progress of hydrolysis was monitored by taking samples (1 mL) periodically. Hydrolysis was stopped by adding water (5 mL) and solid ammonium chloride until pH 4.5 was attained. The mixture was worked up as for the potassium hydroxide hydrolysis, and an aliquot for HPLC injection was prepared as above.

Chromatographic Methods. High-performance liquid chromatography (HPLC) was conducted on a Hewlett-Packard HP 1090 liquid chromatograph equipped with a 1040 HP diode array detector. Photodiode array measurements of spectral properties for the individual peaks (from 260 to 540 nm) were determined at the upslope, apex, and downslope. The fitting of the three spectra indicated the degree of peak purity. A serially connected end capped octadecylsilane column (Merck RP-18e, 3.4 × 250 mm, 5-μm particles) and an octadecylsilane column (Merck RP-8, 3.4 × 125 mm, 5-μm particles) were used for HPLC separations.

* Author to whom correspondence should be addressed.

† Contribution 3637-E from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, 1992 series.

0021-8561/93/1441-0899$04.00/0

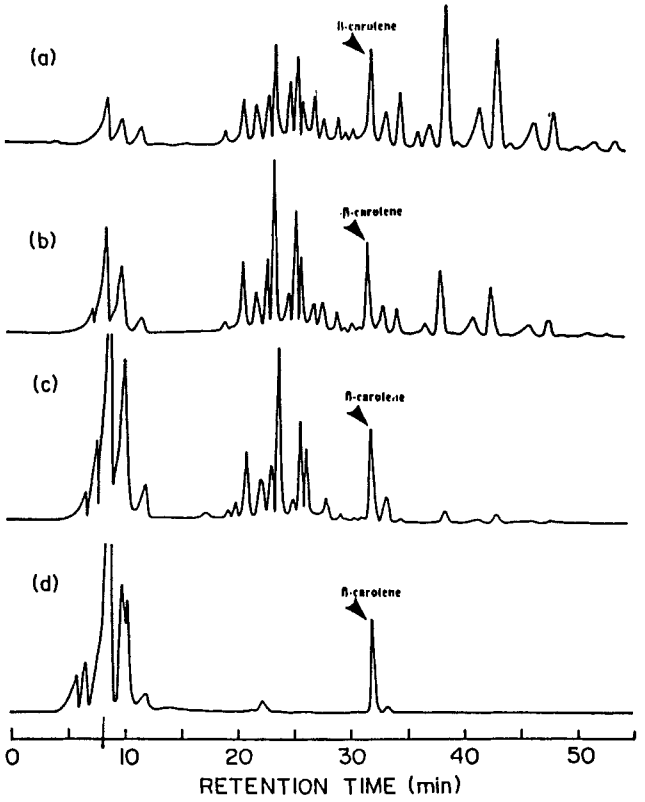

Figure 1. Examples of HPLC separation of pigments extracted from paprika at different stages of saponification with sodium methoxide: (a) without saponification; (b) after 1 h; (c) after 4 h; (d) after 48 h.

A guard column (RP-18, 1 cm) was also used. The pigments were eluted with a mixture of solution A (40:60 v/v, acetonitrile–2 propanol) and solution B (water) at a flow rate of 0.80 mL/min, using a gradient (14% to 0% solution B in 40 min). The best separations were obtained with the following empirical gradient:

time, min	0	1	3	4	8	10	12	19	20	29	30	39	40	60
% soln B	14	12	12	10	10	8	6	4	2	2	2	0	14	14

The peaks were detected at 462 nm.

RESULTS AND DISCUSSION

The diversity of carotenoids from different sources, e.g., paprika and tomato, makes their separation and identification a real task and a challenge for the analyst. The identification of each peak has always been a long and tedious process that has included the separation of each compound by column chromatography or TLC, its identification by other methods, and its injection into an HPLC column. The assignment of each peak from the extracted pigments had then been made by comparison with the corresponding retention times, assuming that the separation was complete and that one peak represented one compound. Now, using a diode array detector, peak assignments and purities are made instantaneously by taking the spectrum of each peak during its elution. A simple comparison with published data on known carotenoids allows almost total identification.

The chromatogram of the pigments contained in an unsaponified extract of paprika fruit is shown in Figure 1a. Over 40 peaks were detected at 462 nm, all having the characteristic UV–visible pattern of carotenoids. A fairly good fitting of the spectra taken for each peak indicates its high purity and therefore the efficiency of the chromatographic method we used. The relatively poor compounds, probably the nonesterified pigments, elute first

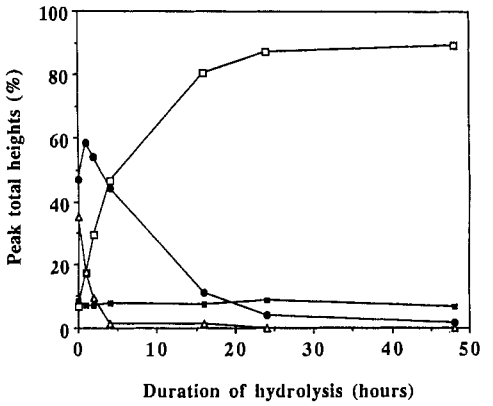

Figure 2. Changes in the content of different fractions of pigments extracted from paprika during saponification with sodium methoxide: (□) free pigments; (●) monoester fraction; (△) diester fraction; (■) β-carotene.

in a well-separated group, followed sequentially by the less polar monoesters, β-carotene, and finally the nonpolar diesters. Different chromatograms, obtained at various stages of the saponification, are shown in Figure 1. The first three peaks (Figure 1a) were identified as belonging to capsanthin, capsorubin, and lutein, in order of decreasing polarity. In the unsaponified extract, the relative amount of these three nonesterified pigments is quite low. But while the peak identified with lutein did not change, the two others increased gradually as the hydrolysis progressed. It seems, therefore, that in paprika lutein exists probably in its nonesterified state. Furthermore, additional polar peaks built up during the saponification.

Figure 2 shows the progress of the hydrolysis over the course of 2 days. The diesters appear to be very sensitive to alkaline conditions; they undergo hydrolysis quite rapidly and, after 4 h, disappear completely. The monoester fraction increases during the first hour and then gradually declines for the next 47 h until completion. The monoester fraction is constantly provisioned from the diester fraction. These observations suggest that, in vivo, the diester fraction appears to play the role of a reservoir from which mono- and/or disaponified products can easily form. Such a mechanism might confer to the carotenoid the hydrophilic character needed to reach various sites having different polarities in the living system. Unlike the partially destructive conditions of hydrolysis using potassium hydroxide, under our mild conditions (sodium methoxide at room temperature), β-carotene remains stable. With potassium hydroxide a chromatogram very similar to Figure 1d was recorded. However, the peak for β-carotene accounts for half the value obtained under our mild conditions. Therefore, we suggest our method for large-scale extractions of β-carotene from plants: after hydrolysis, a simple chromatography procedure (e.g., silica column with a hexane–methylene chloride mixture) should supply this important carotenoid in a highly purified state. The UV–visible pattern of capsanthin was later encountered up to nine times (five in the monoester region and four in the diester region) during the chromatography of the unsaponified mixture of the pigments, whereas the pattern of capsorubin was encounted only four times (two in each ester region). As expected, the UV–visible pattern of lutein was not encountered in the mono- or diester fraction. Although their composition was not determinated, it appears clearly that many different fatty acids participate in the esterification of capsanthin and capsorubin, as previously reported (Philip et al., 1971; Biacs et al., 1989). These results show that the red color of paprika is mainly due to capsanthin esters and, to a much

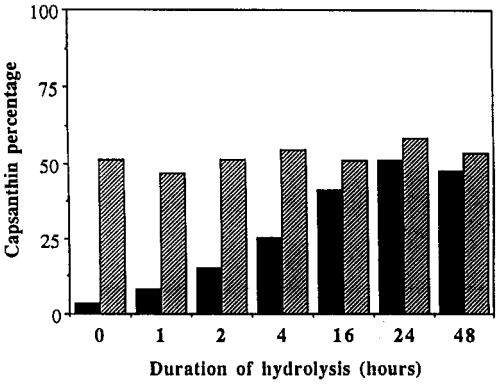

Figure 3. Changes in the percentage of capsanthin during saponification with sodium methoxide: (■) from total peaks; (▨) from polar fraction peaks.

lesser extent, capsanthin itself. When the hydrolysis was completed, the peak of capsanthin accounted for about half the total signals of the chromatogram (Figure 3). Nevertheless, the percentage of capsanthin within the polar fraction remained constant.

In conclusion, we propose here new conditions of separation and identification for unsaponified pigments extracted from paprika. An easy and nondestructive slow method of hydrolysis of esterified carotenoids allowed us to study the changes in the content of these red pepper species for paprika throughout the entire process.

We are currently focusing our research on the carotenoid composition of new cultivars of red pepper for paprika, using this new method of separation, especially for the quantification of the pigments responsible for the red coloration, e.g., capsanthin and capsorubin.

LITERATURE CITED

Bauernfeind, J. C. Direct and indirect coloring with carotenoid food colors. In *IFT: World Directory and Guide*; IFT: Chicago, IL, 1973; p 96.

Biacs, A. P.; Daood, H. G.; Pavisa, A.; Hadju, F. Studies on the carotenoid pigments of paprika (*Capsicum annum* L. var Sz-20). *J. Agric. Food Chem.* **1989**, *37*, 350–353.

Buckle, K. A.; Rahman, M. M. J. Separation of chlorophyll and carotenoid pigments in capsicum cultivars. *J. Chromatogr.* **1979**, *171*, 385–391.

Emodi, A.; Scialpi, L.; Antoshkiw, T. Water-Dispersible, optically-clear carotenoid colors. *Food Technol.* **1976**, 58–60.

Fekete, M.; Kozma, L.; Huszka, T. Determination of the total red and yellow pigment content of seasoning paprika without chromatography. *Acta Aliment.* **1976**, *15*, 319–328.

Philip, T.; Francis, F. J. Oxidation of capsanthin. *J. Food Sci.* **1971**, *36*, 96–97.

Philip, T.; Chen, T. S. Quantitative analyses of major carotenoid fatty acid esters in fruits by liquid chromatography: Persimmon and papaya. *J. Food Sci.* **1988**, *53*, 1720–1722.

Philip, T.; Nawar, W. W.; Francis, F. J. The nature of fatty acids and capsanthin esters in paprika. *J. Food Sci.* **1971**, *36*, 98–102.

Vinkler, M.; Richter, K. A thin layer chromatographic method to determine the pigment content components in the pericarp of paprika. *Acta Aliment.* **1972**, *1*, 41–45.

Received for review October 14, 1992. Revised manuscript received March 4, 1993. Accepted March 9, 1993.