Separation of Paprika Pigments by HPLC

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The carotenoid pigments in paprika extract were separated by high-performance liquid chromatography (HPLC) without prior saponification and compared with saponified paprika extract. In the general order of elution on the C-18 reversed-phase column, four classes of compounds were recognized: the hypophasic xanthophylls, mono fatty acid carotenoid esters, epiphasic carotenoids, and bis fatty acid carotenoid esters. The major hypophasic xanthophylls are capsorubin, violaxanthin, capsanthin, antheraxanthin, and zeaxanthin. The major epiphasic carotenoids are cryptocapsin, cryptoxanthin, and β-carotene. The mono and bis fatty acid carotenoid esters reactive to acid treatment (contains epoxy group) and sodium borohydride treatment (contains carbonyl group) were identified.

The carotenoids form one of the most important groups of natural pigments and are to be found in all families of the vegetable and animal kingdoms (Isler, 1971). Various carotenoid extracts are now being used in the food industry to color foods and animal feeds. The natural pigments are more acceptable to consumers as they have always been present in natural foods and are readily metabolized. In addition, the metabolites are good for human health; the hydrocarbon carotenoids have provitamin A activity (Isler, 1971), and the oxygenated carotenoids or xanthophylls are possibly linked to a lower risk of cancer (Beecher and Khachik, 1984).

One carotenoid extract of commercial significance is paprika oleoresin. This spice extract is prepared from the dried ground fruits of *Capsicum annuum* or *Capsicum frutescens*, often referred to as red pepper. Most of the carotenoids in paprika oleoresins are esterified with fatty acids, which makes these pigments oil soluble. In the isolation and identification of the paprika pigments (see Table I) the ester linkages are broken (saponification) to make separations and analyses easier (Baranyai et al., 1982; Camara and Moneger, 1978; Davies et al., 1970; Curl, 1962). The structures of the major paprika pigments are shown in Table II.

The present paper describes an HPLC procedure in which the pigments in paprika oleoresin are separated without resorting to saponification. This HPLC assay would be useful for the detection of adulteration, i.e. the addition of synthetic canthaxanthin [used as internal standard [Baranyai et al. (1982)]] or β-carotene, determining the differences in pepper varieties (chemotaxonomy) and analyzing the qualitative differences in pigment composition attributable to environmental conditions.

PROCEDURE

Conditions. HPLC Chromatograph: Varian 5000, equipped with a variable-wavelength UV-50 detector and a Spectrophysics 4290 integrator. Column: Zorbax C-18 (4.6 mm × 25 cm), column can last over 500 injections if a guard column containing VYDAC RP packing (Varian) is used. Eluants: A, 75:25 (v/v) acetone–water; B, 75:25 (v/v) acetone–methanol. Gradient elution: 0% B to 65% B in 10 min, to 80% B in 30 min, to 100% B in 60 min. Flow rate: 1 mL/min. Wavelengths: 510, 480, 460, 428 nm. Sample injection: 10 μL (all samples diluted in acetone and filtered before injection). Sample preparation: Paprika oleoresin was prepared by exhaustive acetone extraction of ground paprika pods and solvent removal. (See ASTA method 11.0 with acetone as extraction solvent.) Saponification, acid treatment (*p*-toluenesulfonic acid), and sodium borohydride treatment are described in the literature (Baranyai et al., 1982; Matus et al., 1981).

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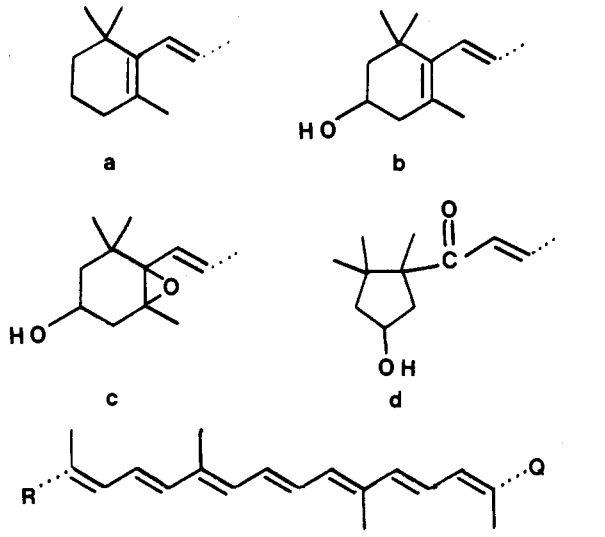
Table I. Relative Amounts (%) of Carotenoid Pigments in Saponified Extracts of Paprika as Reported in the Literature

	Curl (1962)	Davies (1970)	Camara (1978)	Baranyai (1982)
capsanthin	34.7	31.7	33.3	38.1
β-carotene	11.6	12.3	15.4	18.6
violaxanthin	9.9	9.8	7.1	7.9
cryptoxanthin	6.7	7.8	12.3	4.2
capsorubin	6.4	7.5	10.3	9.5
cryptocapsin	4.3	5.0	5.1	1.8
zeaxanthin	2.3	6.5	3.1	4.0
antheraxanthin	1.6	9.2	9.2	5.0
capsanthin epoxide	0.9	4.2	1.7	2.6

Table II. Structure of Identified Paprika Pigments



	R	Q		R	Q
capsanthin	b	d	cryptocapsin	a	d
β-carotene	a	a	zeaxanthin	b	b
violaxanthin	c	c	antheraxanthin	b	c
cryptoxanthin	a	b	capsanthin epoxide	c	d
capsorubin	d	d			

RESULTS AND DISCUSSION

The four end groups (**a**–**d**; see Table II) found in paprika carotenoids can be differentiated by means of simple chemical reactions and physical properties (Matus et al., 1981).

The nonoxygenated β-carotene end group **a** does not have an alcohol group to which a fatty acid can be linked. Therefore, when the pigments are treated with base, the

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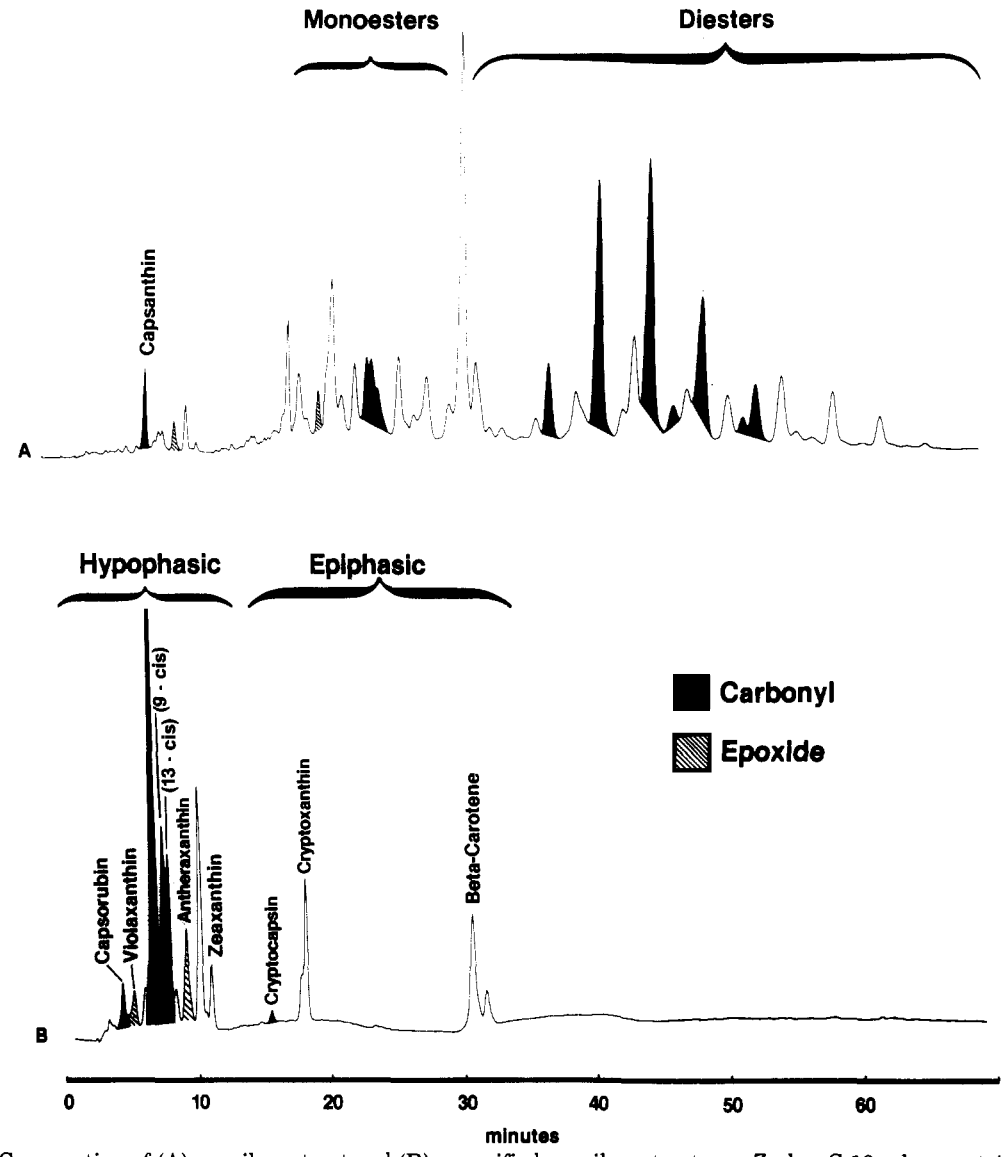

Figure 1. HPLC separation of (A) paprika extract and (B) saponified paprika extract on a Zorbaz C-18 column, at 460 nm. The solid peaks represent carotenoids containing a ketone group, and the hash-marked peaks represent carotenoids containing an epoxide group. See text.

pigment does not change its chromatographic properties. (The peak does not change in retention time or in relative area with saponification.) Also, this end group is less polar than the others, and when the saponified pigments are shaken with hexane–aqueous methanol, the epiphasic pigments (β-carotene, cryptoxanthin, cryptocapsin) stay in the hexane phase and the hypophasic pigments (dihydroxyxanthophylls) can be extracted into the methanolic phase.

Acid treatment converts the 5,6-epoxides in carotenoids having the end group **c** (see Table II; violaxanthin, antheraxanthin, capsanthin epoxide) into furanoid oxides (Matus et al., 1981; Isler, 1971), which have different retention times and absorption maxima.

Sodium borohydride treatment reduces the red conjugated polyene ketones in carotenoids having the end group **d** (see Table II; capsanthin, capsorubin, cryptocapsin, capsanthin epoxide) into the more yellow unconjugated alcohols (Matus et al., 1981; Isler, 1971) with a pronounced change in retention times and absorption maxima.

The bands of carotenoids in the chromatogram of the saponified extract (see Figure 1B) have been identified by comparison to the published HPLC assay (Baranyai et al., 1982) and the above described procedures. Capsanthin epoxide was not conclusively identified; however, it is believed to coelute with capsorubin.

The bands of carotenoid esters in the paprika extract (see Figure 1A) have been identified as to which group they belong, but individual peak identification has not been done. The solid peaks have at least one end group **d**, the hash-marked peaks have at least one end group **c**, and the uncolored monoester peaks probably have end group **b**. The remaining, unmarked diester peaks have both end groups containing an alcohol group onto which two fatty acids are attached.

Francis and co-workers assumed that capsanthin diester was capsanthin diluaurate (Philip et al., 1971). It can be seen in the HPLC diester region that capsanthin must be esterified to a family of fatty acids (probably the five largest solid peaks in Figure 1A). This complicates the identification of individual peaks as the fatty acids also must be identified.

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Tentative Identification of Humulene Diepoxides by Capillary Gas Chromatography/Chemical Ionization Mass Spectrometry

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Five isomers of humulene diepoxide, designated as A–E, were identified by capillary gas chromatography/mass spectrometry (Cap-GC/MS) operated in the pulsed positive ion–negative ion chemical ionization (PPINICI) mode. These isomers had a molecular weight of 236 and a molecular formula of $C_{15}H_{24}O_2$. An herbal/spicy flavor was detected from the mixture of these isomers. These diepoxides were found in selected hop essential oils and in both pilot-scale and commercial beers.

The "noble hop" flavor is generally regarded as one of the most desirable characteristics of beer. In order to achieve this goal, many American breweries import European hops such as Hallertauer mittlefruher, Northern Brewer, Saazer, and Tettnanger. Earlier studies showed that compounds responsible for hop-derived flavor do not come from the major hydrocarbon fraction, but instead from the minor oxygenated fraction of the hop oil (Howard and Stevens, 1959; Tressl et al., 1978a). Although the argument about the contribution of oxygenated compounds to hoppy aroma in beer has not been settled (Tressl et al., 1978b, 1983; Verzele and Sandra, 1981; Fukuoka and Kowaka, 1983), there is a general agreement that the oxidation products of α-humulene are particularly significant (Tressl et al., 1978a; Peacock et al., 1980; Peacock and Deinzer, 1981). The purpose of this study was to obtain oxidation products of α-humulene through chemical synthesis and to evaluate the contribution from each of these individual oxidation products to the overall beer flavor.

EXPERIMENTAL SECTION

Oxidation of Humulene. *m*-Chloroperbenzoic acid (MCPBA; Aldrich Chemical Co., Milwaukee, WI) was used as the oxidant. In several small portions, 5.8 g (27.1 mmol) of MCPBA was added to a chilled (−15 °C) methylene chloride solution containing 5.0 g (24.5 mmol) of α-humulene (Fluka Chemical Co., Hauppauge, NY). The mixture was allowed to stand overnight at 0 °C. Unreacted MCPBA and byproduct *m*-chlorobenzoic acid were removed by extracting the methylene chloride solution with 10% sodium hydroxide. The organic solvent was then removed under vacuum on a rotary evaporator. The crude product contained both humulene monoepoxides and diepoxides in a ratio of 4 to 1. When this crude oxidation product was reacted with another molar ratio of MCPBA, a quantitative conversion of humulene to its diepoxides was obtained.

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All crude oxidation products of α-humulene were analyzed by capillary gas chromatography/mass spectrometry (Cap-GC/MS).

Hop Essential Oils. Hops were harvested and stored frozen until analysis. Hop essential oils were isolated by the method of Lam et al. (1986a). All samples were analyzed by Cap-GC/MS.

Pilot Beers and Commercial Beers. Four pilot beers were made under identical conditions in a 1-barrel (120-L) pilot-scale brew kettle by a commercial brewery. Normal commercial malt and adjuncts were used. Wort preparation, fermentation, and lagering were performed under standard pilot brewery operations. Three pilot beers were made with one of the three European hop varieties: Hallertauer mittlefruher, Northern Brewer and Saazer. The raw hops were added 15 min before knockout. The hopping rate was adjusted to produce 30 bitterness units (BU) in the finished product. An unhopped reference brew was adjusted to 30 BU with isomerized α acids.

An American beer of 15 BU and European commercial beer of 40 BU were also chosen for this study.

The extraction and cleanup procedure reported by Lam et al. (1986b) was used to process all beer samples. All extracts were analyzed by Cap-GC/MS.

Cap-GC/MS. All compounds were identified and quantified by a Finnigan Model 4023 quadrupole mass spectrometer. Detailed instrument setup in electron-impact (EI) mode has been reported elsewhere (Lam et al., 1986b). When the mass spectrometer was operated in pulsed positive ion–negative ion chemical ionization (PPINICI) mode, methane was chosen as the reagent gas with a pressure of 0.75 torr at the ion source. A scan range of m/e 45–450 was selected.

RESULTS AND DISCUSSION

Products from chemical epoxidation of α-humulene are shown in Figure 1. Depending on the location and the relative configuration between the two epoxide rings, there could be 12 possible diepoxide isomers. In this study only five isomers were detected among the crude diepoxide mixture. They are temporarily designated isomers A–E

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