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CONVERSION OF β-CITRAURIN TO RETICULATAXANTHIN AND β-APO-8′-CAROTENAL TO CITRANAXANTHIN DURING THE ISOLATION OF CAROTENOIDS FROM *CITRUS**

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Key Word Index—*Citrus*; Rutaceae; carotenoids; β-citraurin; reticulataxanthin; citranaxanthin; β-apo-8′-carotenal.

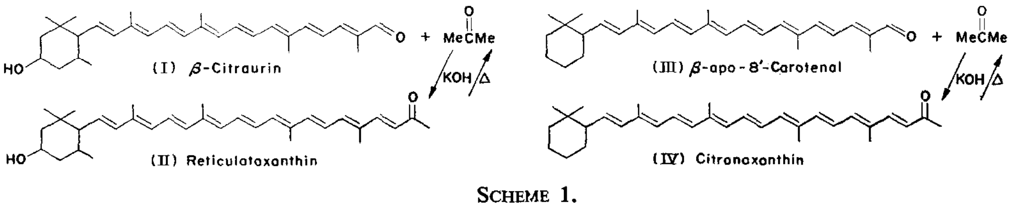
Abstract—Reticulataxanthin and citranaxanthin were found to be artifacts formed from β-citraurin and β-apo-8′-carotenal during the saponification of citrus carotenoids in the presence of small amounts of acetone. The reaction takes place at room temperature and under normal conditions of extraction. Based on these studies it was concluded that β-citraurin rather than reticulataxanthin is the main pigment contributing to the external reddish color of a number of citrus cultivars.

INTRODUCTION

Both β-citraurin (I) and reticulataxanthin (II) have been reported to be the primary pigment contributing to the reddish color of citrus fruits. β-Citraurin, isolated by Zechmeister and Tuzson[1] in 1936, has been widely reported in citrus and considered a major carotenoid in a number of varieties. Reticulataxanthin, a methyl ketone, was isolated and identified more recently.[2,3] It has also been found in a number of citrus varieties and is considered by Yokoyama *et al.*[4] to be the main pigment contributing to the deep red–orange color of several cultivars.

Recently, Schmidt *et al.*[5] found methyl keto artifacts of bacterial carotenoids were readily formed during saponification by aldol condensation, if a small amount of acetone was present. The reaction is reversible. Yokoyama *et al.*[3] have demonstrated the alkaline cleavage of reticulataxanthin when heated in ethanolic KOH to give β-citraurin and acetone.

Other carotenals will also condense with acetone to give their respective methyl ketones.[6] In this study, both reticulataxanthin (II) and citranaxanthin (IV) were readily formed from their respective carotenals in plant extracts and from pure materials.



Scheme 1.

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* Florida Agricultural Experiment Stations Journal Series No. 4938.

[1] Zechmeister, L. and Tuzson, P. (1936) *Ber.* **69**, 1878.

[2] Curl, A. L. (1962) *J. Food Sci.* **27**, 537.

[3] Yokoyama, H., White, M J. and Vandercook, C. E. (1965) *J. Org. Chem.* **30**, 2482.

[4] Yokoyama, H., Guerrero, H. C. and Boettger, H. (1972) In *The Chemistry of Plant Pigments* (Chichester, C. O., ed.), Academic Press, New York.

[5] Schmidt, K., Francis, G. W. and Liaaen-Jensen, S. (1971) *Acta Chem. Scand.* **25**, 2476.

[6] Yokoyama, H. and White, M. J. (1965) *J. Org. Chem.* **30**, 2481.

We[7] previously reported reticulataxanthin in citrus where extractions were made using acetone as a solvent. However, during the last 3 years, acetone has not been used as an extracting solvent and we have not found any trace of reticulataxanthin in any citrus cultivars. Therefore, we considered the possibility that reticulataxanthin was formed as an artifact in the presence of acetone.

The purposes of this work were to establish the conditions required for the condensation of acetone with carotenoids, to determine if reticulataxanthin and citranaxanthin were artifacts from citrus and to establish which pigments contribute most to the red–orange color of citrus.

RESULTS AND DISCUSSION

Comparison of Extraction Methods in the Formation of Artifacts

Yokoyama and White[8] and Yokoyama *et al.*[4] reported the peel of the Minneola, Sinton and Temple to be rich in reticulataxanthin and other methyl keto carotenoids. In an attempt to confirm these findings, Sinton fruit was collected from two trees near Orlando, Florida, believed to be the same trees from which these investigators obtained some of their samples. Minneola and Temple fruit were taken elsewhere in Florida. The peel was extracted according to the method of Yokoyama and White[8] using a blender with methanol and acetone. The filtered extract was mixed with an equal volume of ether and the ether layer separated with a solution of NaCl. The carotenoid mixture was then divided into two equal parts. Part 1 was saponified by adding an equal volume of 10% KOH in methanol. After 2 hr, the base was washed free of the pigments and the ether removed. The extract containing the carotenoids was chromatographed and the main red pigment was reticulataxanthin (Table 1) and a minor amount of citranaxanthin. These observations confirmed those of Yokoyama and White. However, in working with part 2 of these extracts the results were quite different. These extracts were thoroughly dried *in vacuo* to remove any acetone prior to saponification. When these samples were then chromatographed, the main red pigment was β-citraurin (Table 1) and the minor one β-apo-8′-carotenal. No reticulataxanthin or citranaxanthin could be detected. These tests suggested that the carotenals occur in nature while the methyl ketones are artifacts from the extraction procedure.

TABLE 1. RETICULATAXANTHIN OR β-CITRAURIN CONTENT OF CITRUS PEEL DEPENDING ON WHETHER THE EXTRACT WAS DRIED PRIOR TO SAPONIFICATION

Variety	Extract	Reticulataxanthin (μg/g)	β-Citraurin (μg/g)
Minneola	Not dried	1·7	—
	Dried	—	2·4
Sinton	Not dried	8·7	—
	Dried	—	9·5
Temple	Not dried	2·3	—
	Dried	—	3·5

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[7] STEWART, I. and WHEATON, T. A. (1971) *J. Chromatog.* **55**, 325.

[8] YOKOYAMA, H. and WHITE, M. J. (1966) *Phytochemistry* **5**, 1159.

The extracting solvents used in our laboratory for routine studies consist of methanol, 1,2-dichloroethane, hexane, and ethyl ether. Otherwise, the procedure for extracting the carotenoids is similar to that reported by Yokoyama and White.[8] A test was made to determine if reticulataxanthin would break down in our extraction method. A sample of the crystalline pigment was added to some tangerine peel in a blender and extracted. A chromatogram of the saponified mixture showed reticulataxanthin still intact, but none was found in a control sample run simultaneously where this carotenoid was omitted.

Aldol Condensation of Carotenals

Since the results indicated that acetone in the saponification mixture caused the formation of reticulataxanthin from β-citraurin, experiments were conducted to determine, more precisely, the conditions necessary for aldol condensation of carotenals. Our results showed that the formation of reticulataxanthin from β-citraurin depended not only on the amount of acetone during saponification but water was also necessary for a smooth reaction.

Crystalline β-citraurin was dissolved in ether and to various aliquots was added increasing amounts of acetone and an equal volume of 10% KOH in methanol. When the solvent mixture was dry, very little reticulataxanthin was formed but, instead the acetone reacted with β-citraurin to form a brown precipitate. However, when the ether containing the carotenoid was saturated with water, detectable amounts of reticulataxanthin were found when 0·05% of acetone was present in the mixture. With increasing amounts of acetone, increasing amounts of reticulataxanthin were formed until all of the β-citraurin was converted to the methyl ketone. Similar results were obtained starting with β-apo-8′-carotenal and converting it into citranaxanthin.

Unsaponified Pigments

The main red pigment (β-citraurin or reticulataxanthin) was found to occur in citrus peel as an ester. This was based on the difference in retention time and R_f values for the saponified and unsaponified forms. It was reasoned that the identification of the ester prior to saponification would establish which pigment occurs in the peel. The ester was isolated as a red, oily substance from Osceola peel. The absorption spectrum in hexane was 477, 451, 428 (sh) and that of the saponified pigment was 477, 452, 429 (sh). The spectra were similar to that of a mixed *cis–trans* isomer sample of β-citraurin. The chromatographic properties of the saponified pigment were similar to β-citraurin in every respect. The absorption spectra obtained were much different from those found for reticulataxanthin which was 493, 464, 444 (sh). We are not aware that the ester of reticulataxanthin has been reported.

Other Methyl Ketones

Yokoyama and White isolated citranaxanthin, sintaxanthin, and other methyl ketones from the peel of Sinton Citrangeqaut. These could be the condensation products of β-apo-8′-carotenal and β-apo-10′-carotenal both of which have been reported in citrus.[8–10] We have isolated β-apo-8′-carotenal from Osceola peel and characterized it to the extent of obtaining a high resolution mass spectrum of the compound. β-apo-10′-Carotenal was condensed with methyl ethyl ketone to give a product with absorption spectra similar to sintaxanthin (473, 447, 425).

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[9] Winterstein, A., Studer, A. and Ruegg, R. (1960) *Chem. Ber.* **93**, 2951.

[10] Thommen, H. (1962) *Naturwissenschaften* **49**, 517.

Acetone as a Reactant

Acetone, which is necessary for the aldol condensation reaction of carotenoids, is probably the most common solvent used for the extraction of plant pigments. However, the potential problem associated with the use of acetone in preparation of plant pigments and other biological material is frequently overlooked. Previously, we[11] have pointed out that acetone reacted with ammonia in plant extracts to produce diacetoneamine and triacetoneamine. Even when acetone is not used in extraction procedures, it may occur as a contaminant in other solvents. For example, it may be found in some commercial grades of methanol. Our results show that very small amounts of acetone can induce artifacts.

The observations put forth in this paper do not preclude the presence of the methyl keto carotenoids in nature. However, it is our belief that if they do occur in citrus, they are not common. The ease by which carotenals are condensed into methyl keto carotenoids raises serious doubt as to their existence in citrus fruit.

EXPERIMENTAL

Source of material. The fruits used in these studies were collected from various orchards in Central Florida during the months of October to March for three seasons. Many of these samples were taken weekly. The Sinton citrangequat was from the USDA citrus variety collection near Orlando, Florida and was sampled in January. Minneola tangelos were collected from several places in Florida and also from Riverside and the Lindcove Field Station, Exeter, California. The latter samples were taken in February and March.

Carotenoid extraction. The peel was removed from the fruit and frozen. The usual procedure was to extract a sample in a blender with an excess of $MgCO_3$ and MeOH–1,2 dichloroethane (1:1). The dichloroethane was washed with 10% $NaHCO_3$ prior to use. The homogenized sample was left overnight. The mixture was then filtered and the residue washed with hexane. To the extract was added an equal volume of Et_2O and the ether layer separated with a 10% NaCl soln. The excess water was removed with Na_2SO_4 and the ether evaporated on a rotary evaporator followed by further drying with a vacuum pump. The carotenoids were taken up in Et_2O and an equal vol of 10% KOH–MeOH was added. After 2 hr, the base was washed from the Et_2O and the extract dried. All procedures were carried out in N_2 where practical.

Chromatography. The thin layer procedure of Hager and Meyer-Bertenrath[12] was found to be an excellent means of separating the methyl keto carotenoids. High pressure liquid chromatography was used for quantitative measurements.[7] Aliquots were collected from various peaks for spectra studies. The column 13 × 0·64 cm was packed with a mixture consisting of 90% $ZnCO_3$ and 10% $MgCO_3$. A nonlinear gradient was generated by an aluminum gradient device previously described.[7] The solvent was as follows: Chamber 1, 70 ml hexane; Chamber 2, 69 ml hexane and 1 ml *t*-pentyl alcohol and Chamber 3, 55 ml hexane and 15 ml *t*-pentyl alcohol. In making quantitative studies, crystalline pigments were used for standards. The unsaponified pigments were separated on thin layer plates using $ZnCO_3$. The solvent consisted of 0·1 ml *t*-pentyl alcohol, 0·1 ml triethylamine, and 99 ml hexane. On occasions, it was found advantageous to increase the amount of *t*-pentyl alcohol and methylamine to 2 ml. The carotenoid esters were also separated with high pressure liquid chromatography. A column 50 × 0·64 cm was packed with a mixture of $MgCO_3$–infusorial earth (3:1, w/w). The solvent consisted of: Chamber 1, 90 ml light petrol.; Chamber 2, 89·9 ml light petrol., 0·1 ml *t*-pentyl alcohol; Chamber 3, 88 ml light petrol., 2·0 ml *t*-pentyl alcohol.

Identification of pigments. β-Citraurin. Isolation was made from a saponified extract of Robinson peel. A preliminary separation was made on a column filled with Sea Sorb 43-infusorial earth (1:1, w/w) using a solvent mixture starting with 1% EtOH in hexane. The large red band was collected and additional separations were made on a column packed with $ZnCO_3$-infusorial earth (1:3, w/w) starting with a solvent mixture of 0·5% *t*-pentyl alcohol in hexane. Fractions were collected and monitored by absorption spectra. By this means, the *trans* isomer was separated from the cis forms. The *trans* β-citraurin was crystallized from light petrol. and Et_2O. The visible absorption maxima in hexane were at 480, 453 and 428 (sh). There were no well-defined peaks in EtOH. A strong peak in the IR spectrum was observed at 1665 (conjugated carbonyl). The MS showed the parent peak at *m/e* 432·3067; Calc. for $C_{30}H_{40}O_2$:432·3027. The ester of β-citraurin was isolated first by passing an extract through a Sea Sorb 43 packed column. The red band was then put through a column packed with silica gel (Bio-Sil A 2-10 microns without binder)-infusorial earth (1:1, w/w). The separated product was a reddish oil with absorption maxima in hexane 477, 451 and 428 (sh). The oil was a mixture of isomers. Upon saponification, the pigment was identical to β-citraurin.

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[11] STEWART, I. and WHEATON, T. A. (1967) *Phytochemistry* **6**, 1587.

[12] HAGER, A. and MEYER-BERTENRATH, T. (1966) *Planta* **69**, 198.

Reticulataxanthin. This pigment was separated from tangerines extracted with acetone, otherwise the separation was similar to that reported for β-citraurin. Crystallization was from Et_2O and light petrol. The visible absorption maxima in hexane were at 493, 465, 444 (sh) with no definite peak in EtOH. The IR spectrum showed a peak at 1662 cm^{-1}. The compound had identical chromatographic properties as a sample kindly furnished by Dr. Yokoyama.

β-apo-8′-Carotenal. Isolated from Osceola peel using a column packed with Sea Sorb 43-infusorial earth (1 : 1, w/w). The starting solvent was light petrol. containing 0·1 % *t*-pentyl alcohol. The crystalline material gave visible absorption maxima in hexane at 480, 454 and 432 with no definite peak in EtOH. The IR spectrum showed a strong band at 1662 cm^{-1}. The MS showed the parent peak at *m/e* 416·3071, Calc. for $C_{30}H_{40}O$: 416·3078. The carotenal was identical with a synthetic sample.

Citranaxanthin. This pigment was not isolated in crystalline form. Studies were made on extracts of Sinton peel saponified with acetone and on synthetic crystalline β-apo-8′-carotenal similarily treated with acetone in 10 % methanolic KOH. Fractions taken from the high pressure chromatographic column gave an absorption spectrum in hexane of 493, 464. The TLC chromatographic properties were similar to a sample kindly furnished by Dr. Yokoyama.

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