CAROTENOIDS

Application of Countercurrent Distribution to Valencia Orange Juice Carotenoids

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The fractionation of carotenoids by means of countercurrent distribution in a glass Craig apparatus has been studied. By the use of two different solvent systems, a total of six fractions was obtained, which were identified as: *(a)* hydrocarbons; *(b)* monohydric alcohols; *(c)* diols; *(d)* and *(e)* diols containing in addition one or two cyclic ether groups, respectively; and *(f)* polyols containing more than two hydroxyl groups. Chromatography of fractions *(a)*, *(b)*, and *(c)* for Valencia orange juice constituents resulted in the identification of phytofluene, α-, β-, and ζ-carotenes, cryptoxanthin, zeaxanthin, and lutein. Another fraction was tentatively identified as hydroxy α-carotene.

SEPARATION AND IDENTIFICATION of the carotenoids of California Valencia orange juice have been part of a study of the deteriorative changes occurring on storage of orange juices and concentrates. Only a small part of the pigment mixture is hydrocarbon, the remainder being an unusually complex mixture of xanthophylls. Countercurrent distribution has been investigated as a means of fractionating this carotenoid mixture. Numerous types of compounds have been investigated so far by countercurrent distribution, such as amino acids, polypeptides, and fatty acids (*4*), but apparently the only study involving carotenoids has been by Lancaster *et al.* (*8*), who investigated the separation of chlorophylls A and B and carotene.

Chromatography is an indispensable tool in the separation of carotenoids. Partition between two solvents, such as petroleum ether and methanol containing 10 to 15% of water, is often used to separate the carotenoids into two fractions, carotenes and xanthophylls, preliminary to chromatography. Monohydroxy compounds such as cryptoxanthin occur in both fractions. It is possible to separate the carotenoids readily into three fractions by means of countercurrent distribution in a 200-tube glass Craig apparatus, with a solvent system consisting of petroleum ether and 99 or 100% methanol, and application of 99 or even somewhat fewer transfers. These fractions consist of compounds containing: (I) no hydroxyl group; (II) one hydroxyl group; and (III) two or more hydroxyl groups. With a solvent system consisting of petroleum ether, benzene, and 87% methanol, and with 150 to 200 transfers, separation into five fractions can be achieved. These fractions apparently consist of: (I + II) compounds containing not more than one hydroxyl group; (III A, III B, and III C) compounds containing two hydroxyl groups plus 0, 1, or 2 cyclic ether groups, respectively; and (III D) compounds containing more than 2 hydroxyl groups. Attempts to resolve fraction III D further by countercurrent distribution have been partially successful. The same solvent systems were also tested on two commercial xanthophyll preparations. Chromatography of the six fractions obtained from orange juice carotenoids showed two or more components in each fraction, or a total of at least 25 components, probably 7 of which are stereoisomers.

Countercurrent distribution is thus of value in effecting a preliminary separation of carotenoids into several fractions, but chromatography is still necessary for the final separation and purification. Countercurrent distribution does not appear promising as a means of separating similar pairs of compounds such as α- and β-carotenes, or zeaxanthin and lutein. In some cases two substances which occur together or close together in a chromatogram are found in different fractions on countercurrent distribution and are thus more readily separable. The relative position of the maximum (or the distribution coefficient) of a given substance on countercurrent distribution with a given solvent system is an important clue to the number of hydroxyl or other functional groups present and hence an aid in identification. This could be a valuable aid in differentiating substances with similar absorption spectra, such as neoxanthin, violaxanthin, and taraxanthin (*13*).

Apparatus

Preliminary experiments carried out in a 25-tube circular steel countercurrent distribution apparatus (*3*) indicated a partial separation of orange juice carotenoids into three fractions. Later a 100-tube glass apparatus (*1*, *2*) became available, which was subsequently expanded to 200 tubes.

Solvent Systems

Carotenoids could be readily separated into three fractions by distribution in a system of petroleum ether (boiling point 63° to 70° C.) and absolute methanol (commercial) (2.5 to 1 by volume). This system is rather sensitive to temperature and becomes homogeneous at about 26° C. Later it was found that petroleum ether and 99% methanol (1.8 to 1) was more satisfactory. It did not become homogeneous even at 45° C. and gave somewhat better separations of orange juice carotenoids into three portions. The two phases separated rapidly, only 1 hour and 45 minutes being required for a 99-transfer run. Petroleum ether and 98% methanol (1.68 to 1) was also a satisfactory system, but the separations were not as good as with the corresponding system using 99% methanol.

For the fractionation of xanthophylls, a 1 to 1 mixture of benzene and petroleum ether was found to be suitable. Runs were made with the 1 to 1 mixture of benzene and petroleum ether with 75 to 94.5% methanol. A good sepa-

ration of the xanthophyll fraction of saponified orange juice carotenoids into four parts was obtained when 87% methanol was used.

Materials

Most of the experiments were carried out on the lipide fraction of California Valencia orange juice. A few experiments were carried out with two commercial xanthophyll preparations—an oil manufactured from corn, and a concentrate prepared from alfalfa. Both unsaponified and saponified materials were used.

Orange juice was hand-reamed, screened, deaerated, canned in vacuo, and stored at −23° C. The cans were thawed when needed, 20 grams of filter aid was added per liter, and the juice was filtered on a precoated Büchner funnel. The filter cake was extracted with acetone in a fritted-glass funnel, the filtrate evaporated in vacuo, and the aqueous residue extracted with light petroleum ether (boiling point 30° to 60° C.) and finally with ethyl ether. This solution was washed with water, dried over sodium sulfate, evaporated in vacuo, and for runs with unsaponified material, the residue was dissolved in the upper phase of the solvent system being used.

For runs with saponified material, the residue was dissolved in a small volume of ethyl ether, and an equal volume of 20% potassium hydroxide in methanol was added (a homogeneous solution forms) and allowed to stand at room temperature overnight. The solution was then diluted with 5 volumes of water and extracted with ether. The ether extract was washed repeatedly with water, dried over sodium sulfate, and evaporated in vacuo, and the residue was dissolved in the upper layer of the solvent system being used.

Procedure for Countercurrent Distribution

In all runs reported, the fundamental procedure was used (*1–3*). After the lower layer of the solvent system had been added and distributed, tube 0 was entirely filled with lower layer and the excess decanted down to about tube 5 (to ensure that each tube was completely filled with lower layer when the upper layer arrived). For a 100-tube run, 7 to 10 transfers were usually made by adding successively 10-ml. portions of upper layer, containing no solute, to tube 0. (As the run progressed, a few of the highest-numbered tubes originally containing both upper and lower layers became homogeneous.) The material to be fractionated was then added successively in 1- to 5–10-ml. aliquots of upper layer. The latter number is preferable, as it usually results in smaller volume changes in the lower layer of the lower-numbered tubes. At the end of the run, the solutions were removed from the tubes by means of a glass syringe and transferred to numbered test tubes.

In order to measure the amount of carotenoid present, the solution in each, or every other, test tube was diluted with sufficient acetone to make it homogeneous and to a definite volume (25 or 30 ml., depending on the solvent system used). The depth of color was then measured in an Evelyn photoelectric colorimeter using filter 440. Further dilutions if necessary were made with acetone. The values obtained were calculated as β-carotene. When it is desired simply to separate the several fractions without quantitative color measurements, the points of minimum color between these fractions can be visually estimated rather closely.

Distribution

In Petroleum Ether–Absolute Methanol

Runs with 99 transfers were made separately on saponified and unsaponified lipide material obtained from orange juice, xanthophyll oil, and xanthophyll concentrate. In all six runs three distinct fractions were obtained, which were quite clean-cut in the saponified samples and somewhat less so in the unsaponified samples. The results with the orange juice samples are shown in Figure 1. The saponified orange juice lipides, xanthophyll oil, and xanthophyll concentrate samples were examined chromatographically. Fraction I was found to consist of hydrocarbons such as β-carotene, fraction II monohydroxy compounds such as cryptoxanthin, and fraction III polyhydroxy compounds such as zeaxanthin and lutein. In the unsaponified samples, fraction I was much greater in amount than in the saponified samples from the same material, indicating the presence of considerable amounts of fully esterified xanthophylls which accompany the hydrocarbons; fraction II also contained esters, probably with one free hydroxyl or other hydrophilic group or groups; fraction III probably contained little, if any, esterified xanthophylls.

The results of all six runs are summarized in Table I. The position of the peak of each of the three fractions is not given, but was used in calculating the partition coefficient and another value called N_{100}. This calculated value is the tube number of the peak for a hypothetical 100-transfer run. When the sample was added in several aliquots, a correction factor was applied to the number of transfers—for example, where a total of 99 transfers was made and the sample was added in 5 aliquots, the average or effective number of transfers is 97. There is some variation in the N_{100} values of a given fraction in the various materials tested, which may be due in part to differences in composition of the carotenoids present. This variation is somewhat greater than that found in other solvent systems which are not as close to the critical solution point at room temperature, but these variations are much smaller than the differences between the N_{100} values of the fractions containing two, one, or no free hydroxyl groups, respectively.

Petroleum Ether–99 And 98% Methanol

After the work reported above was completed, a comparison was made of the system of petroleum ether and absolute methanol with similar systems using 99 and 98% methanol. The material used was saponified xanthophyll concentrate. Because this was found to be rather low in fraction II, a sufficient amount of cryptoxanthin (obtained from orange juice) was added to make the amounts of fractions I, II, and III of the same order of magnitude for these two runs. The results are given in Table II. When 99%

Figure 1. Countercurrent distribution of Valencia orange juice carotenoids in petroleum ether–absolute methanol

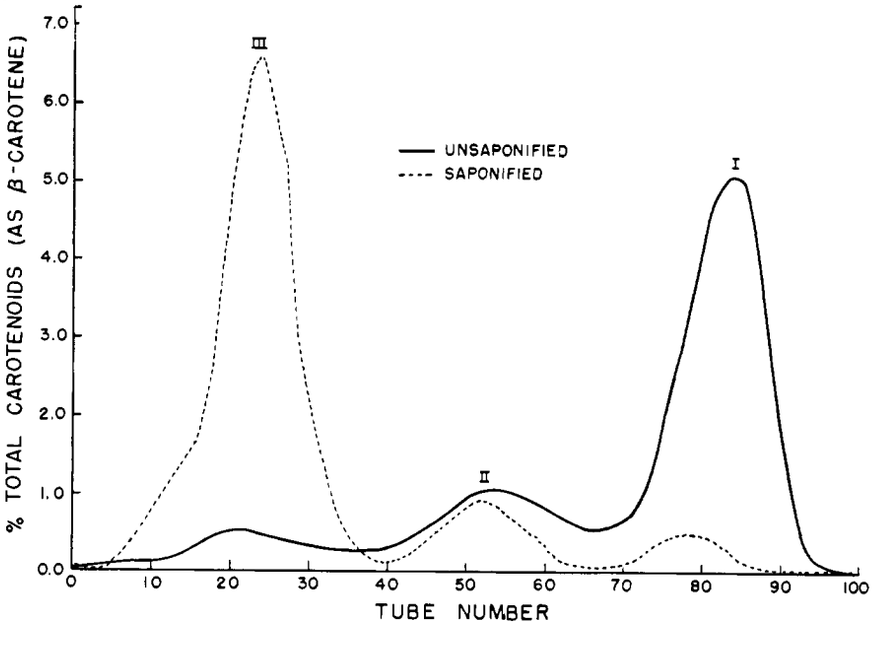

Table I. Countercurrent Distribution of Carotenoids in Petroleum Ether–Absolute Methanol

(99 transfers)

Material	Total Carotenoids (as β-Carotene), Mg.	N_{100}[a] and Partition Coefficient Fr. I	Fr. II	Fr. III	% of Total Carotenoids (Calcd. as β-Carotene) Fr. I	Fr. II	Fr. III
Orange juice lipides							
Unsap.	5.7	85 (5.7)	55 (1.22)	22 (0.28)	69	21	10
Sap.	27.7	79 (3.8)	53 (1.13)	24 (0.32)	6	12	82
Xanth. oil							
Unsap.	0.59	93 (13.3)	54 (1.18)	14 (0.16)	61	33	6
Sap.	1.69	86 (6.1)	50 (1.00)	15 (0.18)	6	41	53
Xanth. concentrate							
Unsap.	27.4	79 (3.8)	54 (1.18)	21 (0.27)	45	9	46
Sap.	24.8	81 (4.3)	48 (0.92)	20 (0.25)	42	6	52
N_{100}							
Av. unsap.		86 ± 5 (6.1)	54 ± 0 (1.18)	19 ± 3 (0.23)			
Av. sap.		82 ± 3 (4.6)	50 ± 2 (1.00)	20 ± 3 (0.25)			
Av. all		84 ± 4 (5.3)	52 ± 2 (1.08)	19 ± 3 (0.23)			

[a] N_{100} = tube No. of peak for a hypothetical 100-transfer run. Partition coefficients in parentheses. Partition coefficient = $\frac{N_{100}}{100 - N_{100}}$

methanol was used instead of absolute methanol, the N_{100} values of fractions I and II were increased 13 and 3, respectively, while that of fraction III was decreased 9. This signifies that a good separation of fractions I, II, and III could be obtained with considerably fewer transfers with petroleum ether and 99% methanol. With the corresponding system using 98% methanol, the N_{100} values of fractions I and II were increased 3 and 5, respectively, and fraction III was lowered 2. In this case the separation of fractions II and III was improved, and that of fractions I and II was less good than in the system petroleum ether–99% methanol.

In Petroleum Ether–Benzene-Methanol-Water The system of petroleum ether and methanol showed little promise in fractionating the xanthophylls containing two or more hydroxyl groups, at least 10 of which occur in orange juice. In order to increase the solubility of the xanthophylls in the upper layer, systems similar to the above, but containing benzene in addition, were investigated. When equal volumes of petroleum ether, benzene, and methanol were mixed, the system was homogeneous; it was necessary to add about 1.5% water (this corresponds to 95.5% methanol) in order to cause separation into two layers. This system is not satisfactory, as it is too sensitive to temperature changes; systems with higher water contents were more stable.

When 94.5% methanol was used, the carotenoids were separated into three fractions in a manner similar to that in petroleum ether–absolute methanol. With 93.7% methanol, a fourth fraction was separated, which apparently consisted of compounds containing three or more hydroxyl groups.

When the system of petroleum ether, benzene, and 87% methanol was used with saponified orange juice carotenoids, a 198-transfer run resulted in separation into five fractions (Figure 2); fractions I and II of Figure 1 now appeared as a single peak, while fraction III was split into four parts. Considerably more than 100 transfers were necessary to separate fractions III A, III B, and III C effectively. By means of chromatography, fraction III A was found to contain zeaxanthin and lutein only. Fractions III B and III C consisted of compounds containing, in addition to two hydroxyl groups, one and two cyclic epoxide or furan oxide groups, respectively. Fraction III D separated readily and apparently consisted of compounds containing three or more hydroxyl groups; the broad, nearly flat peak indicated a further partial separation of this fraction. All components isolated from this fraction also contained cyclic ether groups.

The same system was also used for saponified material from the two xanthophyll preparations (Table III); it was found to be unsuitable for unsaponified material, as slowly separating emulsions were formed. With the xanthophyll oil and xanthophyll concentrate preparations, a much smaller amount of carotenoid material was found in fractions III B, III C, and III D than with orange juice lipides, indicating the relative absence of compounds containing more than two oxygen atoms per molecule; for this reason the runs were not carried beyond 99 transfers. The values of N_{100} obtained for the various fractions showed somewhat less variation than with the system of petroleum ether and methanol (Table I).

Table II. Comparison of Fractionation of Carotenoids in Craig Apparatus with Petroleum Ether and 100, 99, and 98% Methanol

Methanol, %	N_{100}[a] Fr. I	Fr. II	Fr. III	ΔN_{100} Fr. I–II	Fr. II-III
100	79	54	21	25	33
99	92	57	12	35	45
98	95	62	10	33	52

[a] N_{100} = tube No. of peak for hypothetical 100-transfer run.

Runs were also made in the Craig apparatus with the system consisting of petroleum ether, benzene, and 87% methanol, with samples of zeaxanthin and lutein. The values of N_{100} obtained were 70 and 72, respectively, which are in excellent agreement with the values for fraction III A in Table III (70 to 72). This close agreement of the N_{100} values for zeaxanthin and lutein indicates that a very large number of transfers would be needed to separate those compounds by countercurrent distribution.

Attempts were made to separate fraction III D further by the systems of petroleum ether, benzene, and 81.6 and 75% methanol. In the former the peak was broadened somewhat; in the latter, separation into four fractions was indicated, but the run was carried to only 59 transfers because of difficulty with emulsions.

Chromatography

Method The solutions comprising each fraction were combined and evaporated in vacuo. The residue was dissolved in petroleum ether (fractions I and II) or benzene, and chromatographed on columns (12 × 200 mm.) of magnesia (Westvaco No. 2642) plus diatomaceous earth, 1 to 1 by volume. The tubes were filled with solvent, adsorbent was added, and the tubes were packed with compressed air. The carotenoid solution was then added, washed into the column with the solvent, and fractionally eluted with a series of eluents of increasing strength. For fraction I the eluent was acetone in petroleum ether, for fractions II and III, ethanol in petroleum ether or in benzene. The eluted fractions were evaporated in vacuo and the residue was dissolved in

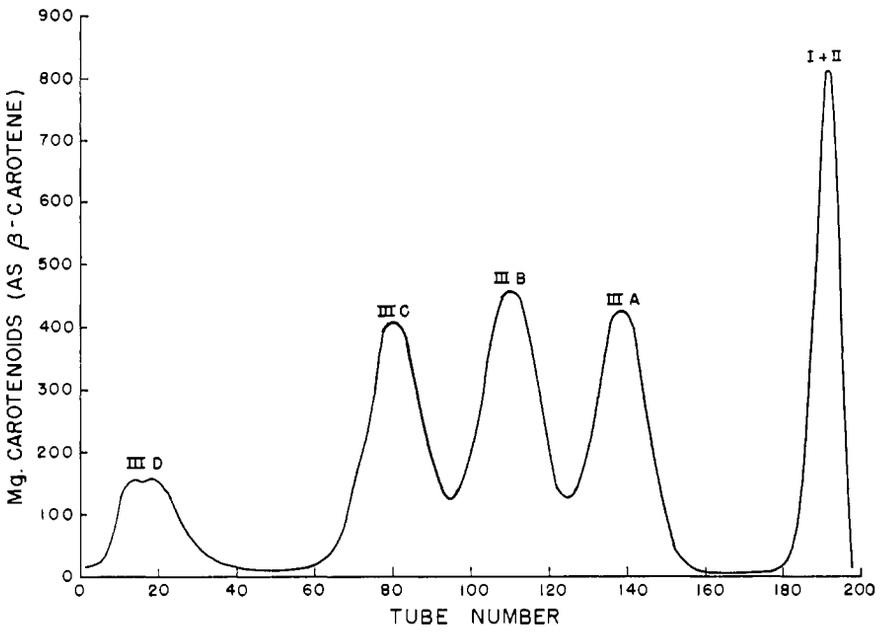

Figure 2. Countercurrent distribution of saponified Valencia orange juice carotenoids in petroleum ether–benzene–87% methanol

petroleum ether (fractions I and II), benzene (fraction III), or ethanol (fraction III D), and spectral absorption curves were run in a Cary recording spectrophotometer.

Saponified Xanthophyll Oil. Only fractions I, II, and IIIA were investigated chromatographically. Fraction I contained β-carotene with perhaps a small amount of α-carotene; fraction II consisted of cryptoxanthin accompanied by about half as much hydroxy-α-carotene (?); fraction III A contained zeaxanthin and lutein, the former perhaps five times as much as the latter.

Saponified Xanthophyll Concentrate. Fractions I, II, and III A only were investigated chromatographically. Fraction I consisted mostly of β-carotene; fraction II of cryptoxanthin and hydroxy-α-carotene (?) in similar amounts; fraction IIIA contained lutein and zeaxanthin, the former in 5 to 10 times the quantity as the latter.

Saponified Orange Juice Lipides

Fraction I. The fraction (I-1) just preceding the first colored zone had a brilliant green fluorescence with ultraviolet light and a spectral absorption curve which corresponded closely to that of phytofluene (*15*). The identifications of fractions I-2 and I-3 as α- and β-carotenes, respectively, were confirmed by mixed chromatograms with preparations of α- and β-carotenes which had been obtained by chromatographing a commercial carotene sample stated to be 90% β-carotene and 10% α-carotene. The amount of β-carotene obtained from orange juice was about three times that of the α-carotene.

Fraction I-4 was eluted just after β-carotene and gave a spectral absorption curve which corresponded closely to that of ζ-carotene (*11*). This fraction gave no color in the ether–hydrochloric acid test, which together with the position in the chromatogram would rule out aurochrome (*11*). A fraction with absorption maxima corresponding to fraction I-4 was found by Zechmeister and Tuzson (*16*), but was not identified. The authors' work indicated the presence of ζ-carotene and β-carotene in similar quantities in orange juice.

Fraction II. This fraction contained two carotenoid components, the spectral absorption curves of which corresponded closely to those of α-carotene and β-carotene. These fractions were eluted from a column much more rapidly than lutein and much more slowly than ζ-carotene. It was concluded that fraction II-2 was cryptoxanthin, which had been reported as a major constituent of the carotenoids of oranges, by Zechmeister and Tuzson (*16*). The other component (II-1) appears to be hydroxy-α-carotene, which has been reported several times (*5*, *10*, *14*), but apparently has not been crystallized and the structure elucidated. It might be either (or a mixture of both) of two compounds, depending on whether the hydroxyl group is on the α- or β-ionone ring of α-carotene. A similar fraction was found in orange peel by Zechmeister and Tuzson (*16*), but was not identified. In the authors' work cryptoxanthin appeared to be present in about three times the quantity of the hydroxy-α-carotene (?); the latter would account for about 3% of the total carotenoids.

Fraction II contained a considerable amount, relatively, of colorless material, which on chromatography was eluted out ahead of fraction II-1. This material readily crystallized in long needles from absolute alcohol and gave positive tests for sterols in the Solkowski and Liebermann-Burchard reactions. On recrystallization the melting point (uncorrected) was 136°, indicating it to be sitosterol, which was found in Valencia orange pulp by Matlack (*9*).

Fraction III A. This fraction consisted essentially of two components which from the spectral absorption curves were identified as lutein and zeaxanthin. These identifications were confirmed by mixed chromatograms with samples of

Table III. Countercurrent Distribution of Saponified Carotenoids in Petroleum Ether–Benzene–87% Methanol

Material	Total Carotenoids (as β-Carotene), Mg.	No. of Transfers	Fraction I-II	III A	III B	III C	III D	I-II	III A	III B	III C	III D
			N_{100}[a] and partition coefficient					% of total carotenoids (calcd. as β-carotene)				
Orange juice lipides	33.0	198	96 (24)	70 (2.3)	56 (1.27)	39 (0.63)	9 (0.09)	19	22	26	23	10
Xanth. oil (xanthophyll fr.)	0.86	99	95 (19)	71 (2.4)	...	44 (0.79)	10 (0.11)	3	73	..	12	13
Xanth. concentrate	21.6	99	97 (32)	72 (2.6)	...	43 (0.75)	11 (0.12)	52	45	..	2	2
Av.			96 ± 1 (25)	71 ± 1 (2.4)	56 (1.27)	42 ± 2 (0.72)	10 ± 1 (0.11)					

[a] N_{100} = tube No. of peak for hypothetical 100-transfer run. Partition coefficients in parentheses. Partition coefficient = $\frac{N_{100}}{100 - N_{100}}$

Table IV. Carotenoids Isolated from Saponified Orange Juice Lipides

(Fractions I, II, III A)

Fraction	Solvent	Wave Length of Maxima and Minima[a], Mμ					Identity or Tentative Identification
		Max.	Min.	Max.	Min.	Max.	
I-1	Pet. ether	367 (0.88)	359 (0.53)	348 (1.00)	339 (0.63)	332 (0.78)	Phytofluene
I-2	Pet. ether	474 (0.89)	462 (0.71)	446 (1.00)	427 (0.68)	422 (0.69)	α-Carotene
I-3	Pet. ether	478 (0.84)	468 (0.80)	450 (1.00)	.....	.....	β-Carotene
I-4	Pet. ether	425 (0.93)	414 (0.47)	400 (1.00)	387 (0.53)	378 (0.63)	ζ-Carotene
II-1	Pet. ether	474 (0.89)	463 (0.70)	445 (1.00)	427 (0.69)	422 (0.69)	Hydroxy-α-carotene[b]
II-2	Pet. ether	477 (0.85)	468 (0.81)	450 (1.00)	.....	.....	Cryptoxanthin
III A-1	Benzene	487 (0.88)	473 (0.71)	456 (1.00)	437 (0.68)	430 (0.69)	Lutein
III A-2	Benzene	490 (0.86)	480 (0.81)	463 (1.00)	.....	.....	Zeaxanthin

[a] Relative optical density in parentheses.
[b] Tentative identification.

lutein and zeaxanthin. The amount of zeaxanthin present appeared to be about double that of the lutein.

Fractions III B, III C, and III D. These three fractions were found to consist of five, six, and six components, respectively, of which 3, 3, and 1, respectively, appear to be stereoisomers of other components. The properties of these components, and the identification of some of them, will be the subject of a separate communication.

None of the components isolated from Fractions I, II, or III A gave a color in the hydrochloric acid–ether test, indicating the absence of epoxide or furanoxide groups. All of the components of fractions III B, III C, and III D gave blue colors; those of fraction III B were light blue, while those of fraction III C were deep blue, indicating the presence of one and two cyclic ether groups, respectively.

Discussion

Each of the six fractions obtained from saponified orange juice was resolved into two or more components, which were numbered I-1, I-2, I-3, etc., in the order in which they were eluted from the column. The eight components thus obtained from fractions I, II, and III A are listed in Table IV, together with their identity or tentative identification; the wave lengths of the maxima and minima of the spectral absorption curves, together with the relative optical densities at these points, are also given. The values for most of the fractions are in close agreement with those found for carotenoids isolated from corn seedlings by Moster *et al.* (*10*), such as α- and β-carotenes, OH-α-carotene (?), cryptoxanthin, lutein, and zeaxanthin.

A number of carotenoids have been previously found in oranges. The pigments of Sicilian orange peel and pulp were investigated by Zechmeister and Tuzson (*16–18*), who also studied the pigments of the Mandarin orange (*19, 20*). In the orange, β-carotene, cryptoxanthin, citraurin, zeaxanthin, lutein, and violaxanthin were identified, and tentatively, flavoxanthin. It was pointed out that only a small portion of the total carotenoids was carotenes, the remainder being an unusually complex mixture of xanthophylls. A number of fractions were not identified. Karrer and Jucker (*6, 7*) reported the presence also of citroxanthin in the peel. Natarajan and Mackinney (*12*) reported the presence in Valencia orange juice of phytofluene, α-, β-, and ζ-carotenes, and also probably lutein epoxide and flavoxanthin. Of the compounds found in the present work in fractions I, II, and III A, only hydroxy-α-carotene (?) was not previously reported, though it was apparently found by Zechmeister and Tuzson (*16*).

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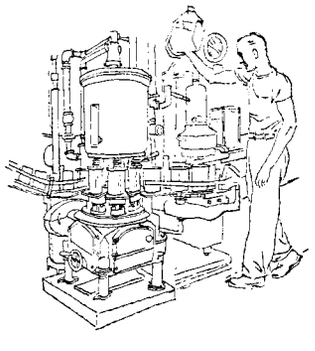

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