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### Studies on the Carotenoids. III. Distribution of Pure Pigments between Immiscible Solvents<sup>1,2</sup>

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Most methods for the determination of carotene depend upon a partition between two immiscible solvents for its separation from other pigments. Peterson<sup>4</sup> has discussed such methods from a historical and critical viewpoint. Many methods in use today employ 85 to 90% methanol or ethanol and a hydrocarbon as the two phases. Following the work of Clausen and McCoord<sup>5</sup> who used diacetone alcohol in the determination of carotene in blood, Hegsted, Porter, and Peterson,<sup>6</sup> Zimmerman, Tressler and Maynard,<sup>7</sup> and Beadle

and Zscheile<sup>8</sup> have employed aqueous diacetone alcohol as the hypophasic solvent.

Despite the wide use of such partition methods in the carotene determination, very little quantitative work has been done with pure pigments. Clausen and McCoord<sup>5</sup> determined the distribution coefficients for "carotene" and "xanthophyll" between hexane and aqueous solutions of methanol, ethanol, and diacetone alcohol. The method developed from these studies was based upon a single distribution between hexane and a 100:14 solution of diacetone alcohol and water.

The determination of the carotenoids in corn grain is complicated by the presence of relatively large amounts of cryptoxanthol. Efforts to include this pigment in an analytical method have been based on the degree of adsorption of the pigment by calcium carbonate<sup>9</sup> or magnesium carbonate.<sup>10</sup> However, no attempt is usually made

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(4) W. J. Peterson, *Ind. Eng. Chem., Anal. Ed.*, **13**, 212 (1941).

(5) S. W. Clausen and A. B. McCoord, *J. Biol. Chem.*, **113**, 89 (1936).

(6) D. M. Hegsted, J. W. Porter and W. H. Peterson, *Ind. Eng. Chem., Anal. Ed.*, **11**, 256 (1939).

(7) W. I. Zimmerman, D. K. Tressler and L. A. Maynard, *Food Res.*, **6**, 93 (1940).

(8) B. W. Beadle and F. P. Zscheile, *J. Biol. Chem.*, **144**, 21 (1942).

(9) L. O. Buxton, *Ind. Eng. Chem., Anal. Ed.*, **11**, 128 (1939).

(10) G. S. Fraps and A. R. Kemmerer, *ibid.*, **13**, 806 (1941).

to separate the cryptoxanthol from the final "carotene" solution. Since cryptoxanthol is intermediate in structure between  $\beta$ -carotene and zeaxanthol, it should have solubility properties intermediate between the two. It has adsorption properties between those of  $\beta$ -carotene and zeaxanthol.<sup>11</sup>

The results of a quantitative study of the distribution of three representative carotenoid pigments between solvent pairs are presented here for three different pairs of solvents. The pigments  $\beta$ -carotene, cryptoxanthol, and zeaxanthol are representatives of three types of carotenoids, those with hydrocarbon, monohydric alcohol, and dihydric alcohol structures, respectively. All have the same system of double bonds. The solvent pairs studied were hexane and aqueous solutions of each of three alcohols: methanol, diacetone alcohol, and 2-methyl-2,4-pentanediol.

### Experimental

**Materials.**—The pigments were from preparations which have been described previously.<sup>12</sup> They were isolated by chromatography and were recrystallized until constant absorption coefficients were obtained following successive crystallizations. Specific absorption coefficients in liters/g. cm. were as follows:  $\beta$ -carotene 258 at 4500 Å. and cryptoxanthol 246 at 4515 Å. in hexane solution and zeaxanthol 248 at 4520 Å. in ethanol solution. The absorption spectra reported there were used as standards for photoelectric spectrophotometric analyses of all solutions. Diacetone alcohol was purified by distillation under reduced pressure; commercial 2-methyl-2,4-pentanediol was used without further purification. Other solvents were purified as previously described.<sup>12</sup>

**Procedure.**—For  $\beta$ -carotene and cryptoxanthol the following procedure was used. A sample of approximately 1.5 mg. of the pigment was dissolved in 1 liter of hexane and a 25-ml. aliquot was added to 25 ml. of each hypophasic solvent in a separatory funnel. The mixtures were shaken several times and allowed to stand in the dark at room temperature for ten minutes before the phases were separated. The volume of each phase was noted. The epiphase was washed three times with water, made to 25 ml., and the pigment concentration determined.

For zeaxanthol a different method was used. An unweighed sample of the pigment was dissolved in ethyl ether and aliquots were evaporated to dryness at room temperature under reduced pressure. The pigment residues were dissolved in 25 ml. of the particular alcohol-water solution to be studied, added to 25 ml. of hexane and equilibrated as above. The more highly colored phase was selected and its pigment was transferred through ether to ethanol for determination.

The concentration of the pigment in the phase not measured was taken as the difference between the measured

concentrations in the original solution and in the measured phase. Concentrations in each phase were then corrected for volume changes in the partition.

The methanol, diacetone alcohol and 2-methyl-2,4-pentanediol solutions were made up by the addition of water to the alcohol and are reported in per cent. by volume. The methylpentanediol was studied because it is so closely related structurally to diacetone alcohol. It was hoped that it might give results superior to those obtained with diacetone alcohol, which on standing develops a yellow color that interferes with the determination of carotenol concentration.

### Results

Figure 1 shows the effect of the water content of the alcoholic solvent upon the partition ratios (ratio of concentration in epiphase to concentration in hypophase) of the three carotenoids for each of the three solvent pairs. The dotted lines represent continuation of the curves to experimental points beyond the limit of the graph.

TABLE I  
PARTITION COEFFICIENTS

Pigment	Solvent pair		
	Hexane-90% methanol	78.5% diacetone alcohol	Hexane-70% methyl-pentanediol
$\beta$ -Carotene	54	>100	>325
Cryptoxanthol	29	40	8
Zeaxanthol	0.12	0.15	0.04

The values in Table I which were selected from Fig. 1 give the optimum water content of the hypophasic solvent for the best separation of zeaxanthol from a solution containing carotene, cryptoxanthol, and zeaxanthol.

TABLE II  
PARTITION COEFFICIENTS

Pigment	Solvent pair		
	Hexane-98.0% methanol	92.5% diacetone alcohol	Hexane-92.0% methyl-pentanediol
$\beta$ -Carotene	32.5	54	19
Cryptoxanthol	1.98	1.1	0.54

The data in Table II were selected to indicate the optimum water content of the alcoholic solvent for the separation of carotene from cryptoxanthol in the absence of dihydroxycarotenes.

As an application of the solubility data, the carotenoids of three samples of corn grain were subjected to a separation based on their differential solubilities in hexane and 78.5% diacetone alcohol (see Table I). This resulted in two solutions—one containing carotene plus cryptoxanthol in hexane and the other the dihydroxy carotenols in diacetone alcohol. The former, after washing with water, was extracted by 92.0% 2-methyl-2,4-pentanediol, which removed most of the cryp-

(11) L. Zechmeister, "Carotinoide," Berlin, 1934.

(12) F. P. Zscheile, J. W. White, Jr., B. W. Beadle and J. R. Roach, *Plant Physiol.*, in press.

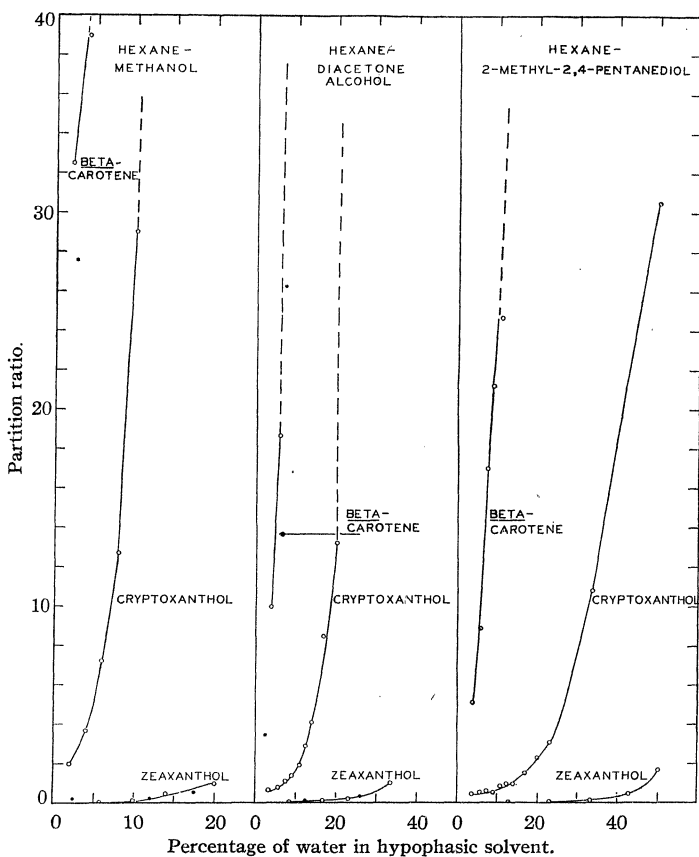


Fig. 1.—Distribution of carotenoids between hexane and aqueous solutions of three alcohols. The data of Clausen and McCoord<sup>6</sup> are plotted as solid circles.

toxinanthol (see Table II). The solutions were then examined chromatographically to determine their qualitative composition. Quantitative determinations on the eluted pigment from each fraction of the adsorption column were made spectrophotometrically. No carotene or cryptoxanthol was found in the dihydroxycarotene fraction, no dihydroxycarotene in the carotene fraction, and only traces of dihydroxycarotene in the cryptoxanthol fraction. The degree of separation of carotene from cryptoxanthol in this second extraction is presented in Table III.

TABLE III  
DEGREE OF SEPARATION OF CAROTENE FROM CRYPTOXANTHOL

Sample	% of total carotene in carotene fraction	% of total cryptoxanthol in cryptoxanthol fraction
1	88	90
2	82	82
3	80	74

## Discussion

Agreement with the distribution values of Clausen and McCoord<sup>6</sup> is good.

It appears that diacetone alcohol and methanol are of equal practical value in the separation of the carotene plus monohydroxycarotene from the dihydroxycarotene. The figure shows that the diacetone alcohol values change less rapidly than the methanol values with a change in the water concentration. This is of importance in application of these data to cases in which the water content of the system is not known accurately.

In the absence of cryptoxanthol, there is little practical difference between the solvents for the separation. When this pigment is present, strict control of the water content of the system is necessary if methanol is used. 2-Methyl-2,4-pentanediol is not satisfactory for this separation since the partition coefficient for cryptoxanthol does not increase rapidly enough with respect to that for zeaxanthol to permit the selection of an advantageous concentration.

The separation of carotene from cryptoxanthol by solvent partition was not as complete as was the separation previously discussed, since the partition coefficients are not so advantageous. Since the partition coefficient of cryptoxanthol is less than unity in the case of 92% methylpentanediol, this solvent is preferred.

Peterson<sup>4</sup> has pointed out that efforts to separate carotene from cryptoxanthol by the extraction of petroleum ether extracts of yellow corn with diacetone alcohol have been unsuccessful. The use of adsorbents to separate the two pigments is not easily adapted to a routine procedure because it requires testing and standardization of each new lot of adsorbent. The possibility of loss of pigment (which Peterson has reported to average 16%)<sup>4</sup> also discourages the use of chromatographic methods in a routine analytical procedure. Fraps and Kemmerer<sup>10</sup> have recently

described a chromatographic method for the separation of carotene from cryptoxanthol. Their losses averaged 3%; in 10% of their columns it was 10-18%.

The degree of the separation as shown in Table III is of the order that would be expected from the relative values of the distribution coefficients. Error in quantitative analysis caused by the incomplete separation would be nearly compensated when the two pigments are present in corn in approximately equal proportions.

#### Summary

1. A quantitative study was made of the distribution of  $\beta$ -carotene, cryptoxanthol, and zeaxanthol between hexane and various aqueous solutions of three alcohols—methanol, di-

acetone alcohol, and 2-methyl-2,4-pentanediol.

2. In the absence of monohydroxycarotene, partition of carotene-dihydroxycarotene mixtures between hexane and diacetone alcohol solutions (94.5 to 77.0% diacetone alcohol by volume) should be satisfactory for the separation of these two classes of pigment.

3. In the presence of cryptoxanthol, extraction by 78.5% diacetone alcohol gives satisfactory separation of the carotene plus cryptoxanthol from the dihydroxycarotenes.

4. Extraction of a hexane solution by a 92.0% solution of 2-methyl-2,4-pentanediol in water gives fair separation of cryptoxanthol from carotene, as shown by analysis of corn grain pigments.

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