Nature of the Vitamin A Activity of Annatto Seed (*Bixa orellana L.*)[1]

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INTRODUCTION

The annatto or *achiote*, as is locally known, *Bixa orellana L.*, is a tropical bush 4 to 6 feet high that produces pods containing a large number of small seed, usually brick-red in color and measuring about 0.3 cm. in diameter. The seed is covered by a thin sticky layer composed of fatty substances, bixin, and other pigments.

Cook and Axtmayer (*1*)[3] reported that the petroleum-ether extract of the whole unbroken annatto seed amounted to 1.5 to 2 percent of the weight of the whole seed and had a vitamin A activity of the order of 1,000 Sherman units per gram of oily extract. Although it did not contain bixin, it had a deep red-orange color due to other pigments.

The above authors reported positive qualitative carotenoid tests for this extract and stated that its absorption expectra suggested the presence of a substance "with an absorption band in the same region as that of carotene". Unfortunately, they did not document this statement with quantitative data.

Squibb, Guzmán, and Scrimshaw (*2*) observed vitamin A activity in the annatto seed and concluded that it is a good source of this vitamin for the rat. The high vitamin A activity of the petroleum ether extract of the seed has been confirmed by De Jesús and Lim (*3*). They reported values of about 1,300 international units per gram of oil. Diemair, Janecke, and Heusser (*4*) cromatographed on a calcium hydroxide column the bixin-free oily material and obtained six different zones that had the following absorption maxima:

Zone	*Maximum in mμ hexane*
1. Orange	454–455, 487
2. Red orange	453, 486
3. Rose red	452, 471–472
4. Orange	451
5. Brownish orange	420, 444
6. Light yellow	375, 400, 425

[1] A preliminary note on this investigation was published in *Proc. Fed. Amer. Biol. Soc., 18:* 544, 1959.

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[3] Italic numbers in parentheses refer to Literature Cited, pp. 267–8.

They suggested that zone 6 could be ζ carotene.

The present investigation was undertaken in order to determine the fate of the vitamin A activity exhibited by the petroleum ether extract of the annatto seed when chromatographed through a column of magnesia (MgO) and hyflo supercel, similar to the one used in the official carotene assay (*5*).

MATERIALS AND METHODS

The annatto seed used in this investigation were bought in the public market in Santurce, P.R., and belonged to the red-brick-colored variety.

Whole seed, in batches of about 800 gm., were repeatedly extracted by shaking at room temperature with different portions of petroleum ether, b.p. 30 to 60°C. until all the soluble pigments were removed. The extract thus obtained had a yellow-red color. The petroleum ether was evaporated under reduced pressure in a thin-film rotating evaporator heated to 45 to 50°C in a water bath.

After all traces of petroleum ether were removed, the residue left amounted to 2.2 percent of the weight of the original seed. All operations were performed using amber-colored glassware and in semidarkness to prevent alterations of the pigments by the action of light. The viscous oily residue obtained had a dark red-orange color.

Attached to the surface of the petroleum ether-extracted seed remained the bixin which was afterwards easily removed with chloroform. Part of the petroleum ether oily residue was dissolved in sesame oil in a proportion such that 0.05 ml. of the final solution was equivalent to 0.5 mg. of the petroleum ether extract. This sesame-oil solution was used in the feeding experiments to be described later. Sesame oil does not contain vitamin A or precursors.

Another portion of the petroleum ether oily residue was redissolved in petroleum ether and was used in the chromatographic separation of the different pigments. The column used for this purpose contained a 1:3 mixture of activated magnesia (MgO)[4] and diatomaceout earth hyflo supercel.[5] This mixture was carefully packed in tubes 15 cm. high by 2 cm. diameter, although the actual height of the packed column was only 11 cm. Twenty-five ml. of the petroleum ether solution containing approximately 0.5 gm. of the oily residue was chromatographed at a time. The column was then eluted with portions of a mixture of 3-percent acetone in petroleum ether. After this elution was performed other solvents were tried. The only one which effectively removed all the pigments that re-

[4] Micron brand, No. 2642, Westvaco Chlorine Products Co., Newark, Calif. General approval of such products (see also footnotes 5, 6, and 7) is not indicated.

[5] Johns-Manville, Box 60, New York 16, N.Y.

mained absorbed on the column after it has been eluted with 3-percent acetone in petroleum ether, was methyl alcohol.

It was found most convenient, after some preliminary trials, to remove the column and perform the final methyl alcohol extraction in an Erlenmeyer flask with shaking.

TABLE 1.—*Effect of various supplements on vitamin A-deficient rats*[1]

Supplements fed per day	Rats started on assay	Average food consumption per rat	Average gain or loss of weight per rat	Rats surviving the experimental period
	Number	*Gm.*	*Gm.*	*Number*
1 int. units vitamin A	6	169	−5	All
2 int. units vitamin A	5	145	+16	Do.
0.5 mg. annatto petroleum ether extract	3	144	+7	Do.
1.0 mg. annatto petroleum ether extract	12	199	+16	10
2.0 mg. annatto petroleum ether extract	3	265	+34	All
0.6 μg. B carotene	3	237	+29	Do.
1.2 μg. B carotene	3	270	+52	Do.
1 mg. bixin	4	35	−25	None
None (negative controls)	13	23	−32	Do.

[1] 4 weeks curative assay.

TABLE 2.—*Effect of eluate and methanol extract on vitamin A-deficient rats*[1]

Supplements fed per day	Rats started on assay	Average food consumption per rat	Average gain or loss of weight per rat	Rats that survived the experimental period
	Number	*Gm.*	*Gm.*	*Number*
1 mg. of eluate of 3-percent acetone in petroleum ether	6	146	−18	2
1 mg. of methanol extract	6	220	+26	All
Negative controls receiving 4 drops of sesame oil per day	3	19	−12	None

[1] 4 weeks curative assay.

By repeating the above procedure several times enough petroleum ether acetone eluate, as well as methyl alcohol extract were accumulated to allow the biological assays of these two fractions.

The rat assay was essentially that of the Pharmacopea (*6*). Twenty-eight-day-old rats, Wistar School of Tropical Medicine strain, raised on a low vitamin A diet and weighing about 60 gm., were placed on the vitamin A-free diet *at libitum*. In a period of 3 to 4 weeks they showed signs of vitamin A deficiency, *i.e.*, xerophthalmia, loss in weight, ataxia, etc. The animals were then divided into different groups containing nearly equal

numbers of males and females. All these groups received supplements, except those that served as a negative control. The supplements fed are shown in tables 1 and 2.

Vitamin A was fed as Aquasol.[6] The crystalline β carotene used contained 10 percent of α carotene.[7]

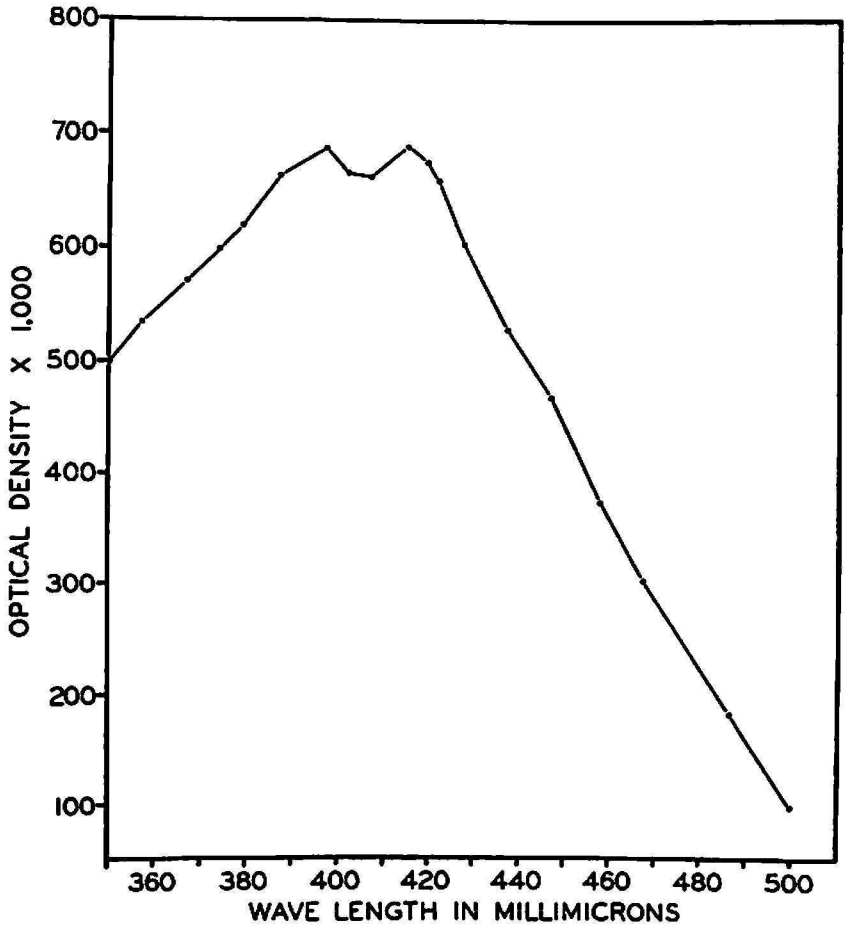

FIG. 1.—Absorption curve of the petroleum ether extract of annatto seed in n-hexane. Maximas are shown at 397 and 415 mμ.

RESULTS AND CONCLUSIONS

ABSORPTION CURVE OF THE PETROLEUM ETHER EXTRACT OF ACHIOTE SEED

The absorption curve of the petroleum ether extract of the annatto seed was determined in a DU Beckman spectrophotometer using n-hexane as solvent. The extract was evaporated under vacuum at room temperature, using a thin-film rotating evaporator. The residue was then taken again into n-hexane and diluted to a convenient concentration for use in the spectrophotometer. The resulting absorption curve did not resemble any

[6] U.S. Vitamin Corp. New York, N.Y.

[7] Nutritional Biochemicals.

one of the known provitamin A compounds. It showed two maximum peaks at 397 and 415 mμ respectively (fig. 1). It was also entirely different from that of bixin. The bixin used was prepared by extracting with chloroform the petroleum ether extracted seed. By slow evaporation of the chloroform extract crystalline bixin was deposited. The absorption curve of bixin was determined in ethanol solution (fig. 2) as it is insoluble in *n*-hexane or petroleum ether.

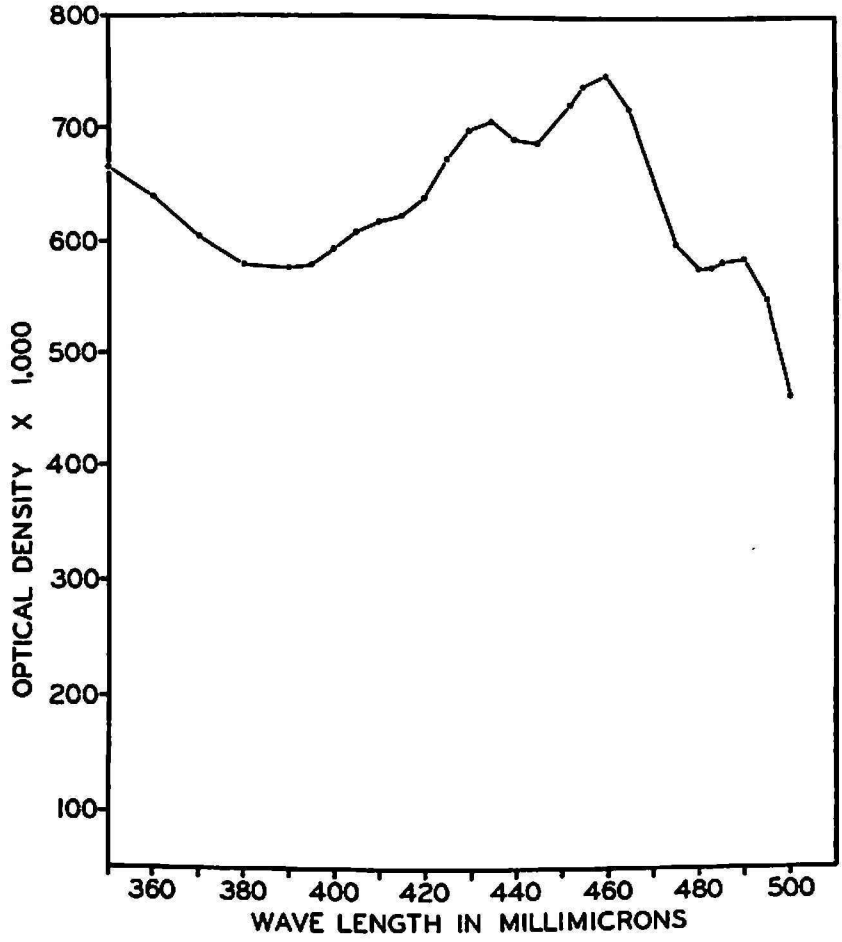

FIG. 2.—Absorption curve of a solution of bixin in ethanol. Absorption maximas are shown at 445, 460, 490 mμ.

BIOLOGICAL ACTIVITY OF THE PETROLEUM ETHER EXTRACT

The petroleum ether extract of annatto was fed to vitamin A-depleted rats at levels of 0.5, 1.0, and 2.0 mg. of extract per day. The 1-mg. level induced a growth response comparable to that of 2 international units of vitamin A and close to that 0.6 μg. of β carotene. It can be concluded, therefore, that it contains in the neighborhood of 1,000 to 2,000 international units of vitamin A per gram of oily extract (table 1). These results confirm the findings of other investigators (*1*,*2*,*3*).

BIOLOGICAL ASSAY OF BIXIN

Bixin was fed to four vitamin A-depleted rats at a level of 4 mg. per day. These animals lost an average of 25 gm. in weight in less than 4 weeks, and did not survive the experimental period.

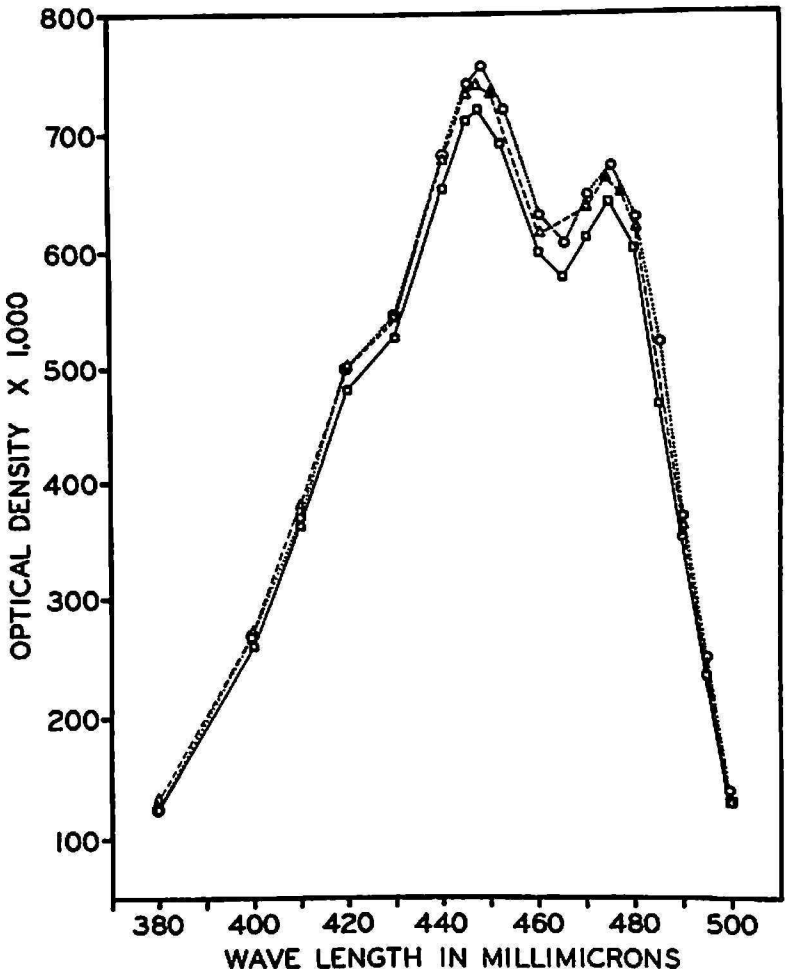

FIG. 3.—Effect of storage and processing in the absorption spectoral curve of B carotene: O ··· O fresh solution of B carotene; □ ··· □ the same solution of B carotene after being stored in the icebox for 5 months; △ - - - △ Solution of B carotene after the aged solution was evaporated to dryness and redissolved in petroleum ether.

EFFECT OF STORAGE AND PROCESSING IN THE ABSORPTION CURVE OF β CAROTONE

A control test was run on a known solution of β carotene in petroleum ether in order to determine whether storage at 8°C. and evaporation of the solvent to dryness in a thin-film rotating evaporator heated to 45°–50°C. altered significantly the characteristic absorption curve of β carotene. In figure 3 appears the curve of the fresh solution before being stored in the icebox, after it had been stored for 5 months, and after the aged solu-

tion was evaporated to dryness and then redissolved in petroleum ether in order to assay it again in the DU spectrophotometer.

The three curves are almost identical, showing a characteristic maxima at 448 and 474 mμ of similar dimension. Therefore, it can be concluded that neither storage at about 8°C. for several months, nor evaporation to dryness under the conditions described altered to any significant extent the absorption curve of β carotone, nor the net amount present in these solutions.

FRACTIONATION OF THE PETROLEUM ETHER EXTRACT

Separation into Two Fractions

As described under Material and Methods, portions of the petroleum ether extract were passed through a magnesium oxide-hyflosupercel column and then eluted with the standard mixture of 3-percent acetone in petroleum ether. The column thus washed was then extracted with methyl alcohol as already described. Both the acetone petroleum ether eluate and the methyl alcohol extract were evaporated and their corresponding residues taken up in sesame oil and each fed to vitamin A-depleted rats at a level of 1 mg. per day (table 2).

The six animals that received the acetone-petroleum ether eluate lost weight and four of them died before the end of the 4th week of the experimental period. Those that received as supplement the residue from the methyl alcohol extract not only survived the experimental period, but also gained an average of 29 gm. in weight. All the negative control animals lost weight and died before the end of the experimental period. The negative control animals received as supplement 4 drops of sesame oil per day.

It can be concluded that the active principle in the petroleum ether extract of annatto is not one of the usual caretenoids. The material that remained strongly attached to the magnesium oxide-hyflo supercel column, was found to be readily removed only by methanol. After evaporating this solvent, the residue left had a vitamin A activity of 1,000 to 2,000 international units per gram.

Separation of the Petroleum Ether Extract into Four Fractions

Using the procedure already described, a portion of the petroleum ether extract was chromatographed again. In this case, however, instead of using a 3-percent acetone petroleum ether mixture for elution, a 10-percent acetone mixture was used. The column was then cut into three parts and each part was extracted at room temperature with methyl alcohol, with the aid of a mechanical shaker.

The absorption curve of each of these fractions was determined in

petroleum ether. The results are shown in figure 4. The acetone-petroleum ether eluate, exhibited peaks similar to those observed when a 3-percent acetone eluate was used. These peaks are at 372, 395 and 420 mμ. The upper third of the column yielded a fraction with a plateau between 389 and 419 mμ. The middle third showed maxima at 425 and 445 mμ and the lower third showed maxima at 446 and 470 mμ. The absorption expectrum

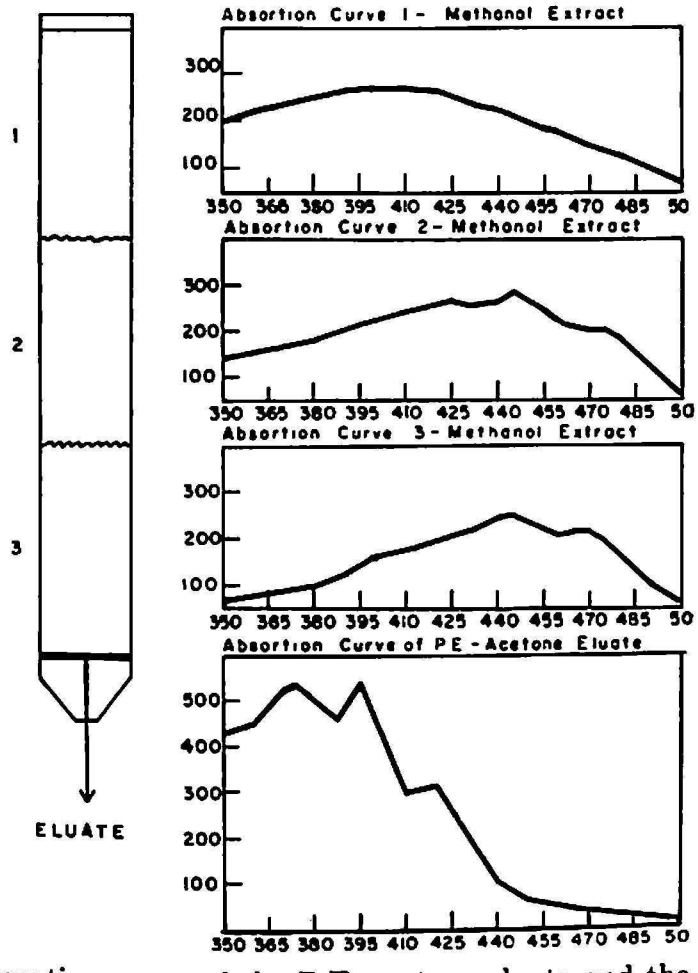

FIG. 4.—Absorption curve of the P.E.-acetone eluate and the 3 zones in which the column was cut and extracted with methanol. Abscissa, wavelength in millimicrons. Ordinate, optical density × 1000.

of the lower zone resembled somewhat that of β carotene which has peaks at 447 and 474 mμ.

We should keep in mind, however, that each one of the three arbitrary zones in which the column was equally divided included a number of subzones. Thus the absorption expectra of the material removed was the resultant of a mixture of several different compounds and not of any particular one. These absorption curves indicate, however, that the pigment or pigments with peaks between 400 and 470 mμ had biological ac-

tivity, while the eluate with peaks below 400 mμ did not have this activity. Further work will be necessary in order to fractionate the active part more thoroughly and at the same time to obtain larger amounts of these fractions for biological assay.

SUMMARY

The high vitamin A activity of the petroleum ether extract of the annatto seed has been confirmed. It ranges from 1,000 to 2,000 international units of vitamin A per gram of extract.

It was found that a standard mixture of 3-percent acetone in petroleum ether did not remove any vitamin A-active material from a magnesium oxide-hyflo supercel column, on which a petroleum ether extract of annatto had been chromatographed. The only solvent among those tried, that effectively removed the material which was left absorbed on the column after elution with acetone-petroleum ether was methanol.

The methanol extract exhibited a biological activity of about the same order of magnitude as the original petroleum ether extract, that is, 1,000 to 2,000 international units of vitamin A.

RESUMEN

Se confirmó que el extracto de la semilla de achiote obtenido con éter de petróleo evidencia una alta actividad de vitamina A, y su contenido de esta vitamina fluctúa entre 1,000 y 2,000 unidades internacionales por gramo del extracto.

Se encontró que una mixtura normal de un 3 por ciento de acetona en éter de petróleo no extrajo material vitamínico alguno de tipo A, de una columna de óxido de magnesio *hyflo super cel*, en la que se cromatografió un extracto de semilla de achiote obtenido con éter de petróleo. El metanol fue el único solvente de los que se probaron, que removía efectivamente el material retenido en la columna después de lavarse con una mixtura de acetona y éter de petróleo. El extracto de metanol evidenció una actividad biológica de una magnitud casi igual que la del extracto original de éter de petróleo, o sea, de 1,000 a 2,000 unidades internacionales de vitamina A.

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Erratum

Vol. 49, No. 1, in the article "Antibody Titers of Dairy Heifers Following Vaccination with a Staphylococcal Toxoid," by José D. Rivera Anaya, Carlos M. Berrocal, and G. Rosado Carbó, pp. 88–98:

p. 89, paragraph 4, line 2 should read: "... incubated at 37°C. for 1 hour."