amount of diene conjugation and in the iodine value in going from the monomer to the polymer is evidence for the participation of the unsaturated side chain in the polymerization. Information on this point is contained in a previous paper (8).

Figures 3, 4, and 5 can be used to determine the number average molecular weight of these vinyl ether polymers once the intrinsic viscosity has been

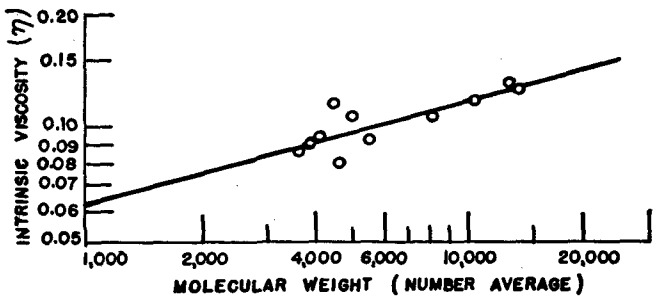

FIG. 5. Intrinsic viscosity-molecular weight relationship of linseed vinyl ether polymers.

determined. A number of polymers obtained during this study had intrinsic viscosities up to a value of 0.27 (Figure 3, curve D). The limitations of the cryoscopic method for determining molecular weight do not permit the measurement of M of a polymer in this range. However, if the relationship of log M *vs.* log $[\eta]$ is linear up to this point, molecular weights of the order of 100,000 have been obtained for soybean vinyl ether polymers. With stearyl vinyl ether polymers the molecular weights obtained thus far do not exceed 25,000. There is evidence in the case of polystyrene that the value of K and a in the Mark-Houwink relation which describe high polymers (mol. wt. of 1 to 2 million) continue to hold for low molecular polystyrenes (mol. wt. of 1 to 10 thousand) (7).

Summary

Molecular weights of the polymers of the vinyl ethers of stearyl, soybean, and linseed fatty alcohols were measured cryoscopically in cyclohexane at three different concentrations. Corrected number-average molecular weights were obtained by extrapolation to zero concentration. For each family of polymers a series of preparations varying in degree of polymerization were studied with number-average molecular weights ranging from 1,500 to 15,000 or higher.

Reduced viscosity measurements at 25°C. were made on benzene solutions of each polymer preparation at three different concentrations. Intrinsic viscosities were obtained by extrapolating to zero concentration. Intrinsic viscosities for the polymers range from 0.05 to 0.20.

Logarithmic plots of molecular weight *vs.* intrinsic viscosity gave linear relationships for stearyl, soybean, and linseed polymers. Values for K' and a in the equation of Mark and Houwink were obtained from these plots.

Acknowledgment

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Analysis of Corn Oil for Total Tocopherols[1, 2]

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ALTHOUGH A NUMBER of the vegetable oils can be analyzed for their tocopherol content satisfactorily by the Parker-McFarlane treatment with sulfuric acid (9) and the subsequent Emmerie-Engel reaction with iron-dipyridyl (3), corn oil is well known to give low and erratic results by the procedure (6, 9). The nature of the interfering substance is unknown, and no suitable method for its elimination from the oil has been reported. This is unfortunate because of the widespread use of corn and its products in the animal diet and the consequent importance of a knowledge of the levels of tocopherol present in the whole grain.

Other procedures which have been applied to corn oil include saponification and adsorption of the unsaponifiable fraction (4); molecular distillation and subsequent hydrogenation (11); and paper chromatography (2). In the chromatographic experiments only *gamma-* and *alpha-*tocopherols were found in corn oils, and these were in an approximate ratio of 90% *gamma* to 10% of the *alpha* form (2, 5). However none of the procedures is suitable for use as a routine method for small samples (one gram or less) with ordinary laboratory equipment.

In the present study efforts were made to learn about the properties of the substance in corn oil which interferes with the Emmerie-Engel procedure and to develop a method for its removal.

Experimental

*Reagents and Apparatus. Gamma-*tocopherol, natural product (Distillation Products Industries), was used for all standardization and recovery experiments.

Alpha, alpha'-Dipyridyl (Matheson, Coleman, and Bell Division, Matheson Company). A solution of 0.25 g. in 100 ml. of ethanol was prepared fresh every two days.

[1] Presented at the fall meeting of the American Oil Chemists' Society, Chicago, Ill., September 24–26, 1956.
[2] Journal Paper No. 1051 of the Purdue University Agricultural Experiment Station, Lafayette, Indiana.

Ferric chloride, hexahydrate, reagent (J. T. Baker Chemical Company). A solution of 0.05 g. in 50 ml. of ethanol was prepared fresh daily.

Benzene, A. R. (Merck and Company), was redistilled before use.

Ethanol, 99.5% (Commercial Solvent Corporation), was distilled after overnight contact with 20 g. of soda lime, reagent (Merck and Company), and 5 g. of zinc dust, A. R. (Mallinkrodt Chemical Works), per liter of ethanol. Solutions containing ethanol were stored in black-painted bottles.

Ethyl ether, A. R. (Mallinkrodt Chemical Works).

Hexane, commercial (Skellysolve B, Skelly Oil Company), was purified by passing it through a column of activated silica gel desiccant (Davison Chemical Company), then washing, drying, and distilling as proposed by Emmerie and Engel (4).

Iso-octane (Phillips Petroleum Company) was redistilled for use as the solvent for spectrophotometric measurements.

Diatomaceous and activated bleaching earths, Official, Am. Oil Chemists' Society (A. S. LaPine and Company).

Nitrogen, high purity, dry (Linde Air Products Company).

Potassium hydroxide, reagent, pellets (J. T. Baker Chemical Company). A 50-g. quantity was made to 1 liter with boiled, deionized water and deaerated with nitrogen just before use.

A Beckman Model DU spectrophotometer with matched, 1-cm. silica cells was used for absorption spectra.

A Beckman Model B spectrophotometer, fitted with a covered test-tube holder, was used to measure absorbance at 520 mμ in the tocopherol assay. The spectrophotometer was used in a dimly lighted room. Selected round cuvettes, 19 x 150 mm. (Wilkens-Anderson Companl), were used.

The molecular still (pot-type) was operated with an oil diffusion pump.

Crude corn oil. The ether extract of a uniform lot of single cross corn (WF 9 x M 14) was used for comparison of different treatments in the method development.

Nature of the Interference. When 1 ml. of a 2% solution of crude corn oil in hexane was mixed with ferric chloride and the dipyridyl, added according to the Emmerie-Engel procedure, color development was rapid within the first 2½ min., then continued at a slower rate. Absorbance of the solution after 2½ min. showed an apparent tocopherol content of 0.239%. The addition of 50 micrograms of *gamma*-tocopherol prior to the reaction resulted in quantitative recovery (99%) when absorbance was read also at 2½ min. However the continuous drift to higher absorbance readings showed clearly that a substance which reacts more slowly than the tocopherols was contributing to give high results. Similar observations on vegetable oils have been reported by Baxter (1), Tošić and Moore (12), and others. Preliminary treatment of the hexane solution of corn oil by the Parker-McFarlane procedure sharply reduce the apparent tocopherol content; only 62% of the *gamma*-tocopherol added before treatment was recovered. However the drift in absorbance values was practically eliminated. *Gamma*-tocopherol added after the sulfuric acid treatment was recovered quantitatively; likewise, in the absence of oil, it was unaffected by the treatment. Modifications in the amount and concentration (8) of the sulfuric acid used in the pretreatment did not produce satisfactory results.

Molecular Distillation of the Crude Oil. One gram of the crude oil, after extraction with 5% of $NaHCO_3$, was placed in a small pot-type of molecular still and allowed to distill at different temperatures (Table I). On reacting the distillate fractions with the iron-dipyridyl reagents, the preponderance of color development was found in the 170° fraction. This is in accord with previous studies, in which the tocopherols of soybean oil showed a distillation maximum at 145°C. in a cyclic still (10). The 220° fraction and the residue also showed some color development however, and absorbance drift was confined largely to these fractions. The 170° fraction showed no substantial drift.

Spectral Absorption of Oil and Distillate. Since the 90° fraction showed general absorption over the range 260 to 300 mμ, it evidently contained a substantial amount of non-tocopherol substance. However the absorption curve of the 170° fraction (Curve B of Figure 1) resembled that of pure γ-tocopherol (Curve C) in its contour. The pure tocopherol showed a third less total absorbance at 295 mμ when compared on the basis of equal Emmerie-Engel values (absorbance at 520 mμ). However the crude oil (Curve A) showed nearly 10 times as much ultraviolet absorbance as its equivalent of 170° distillate.

This strong absorption in the range from 270 to 330 mμ is not shown by crude soybean oil (Figure 2). When compared at equal concentrations, absorbance

TABLE I

Analysis of the Molecular Distillates of Corn Oil by the Emmerie-Engel Procedure

Distillation		Apparent Tocopherol
Temp.	Time	
°C.	*hrs.*	*g./100 g. initial oil*
90	24	0.012
170	4[a]	0.113
170	4[b]	0.001
220	4	0.017
Residue		0.020

[a] First period.
[b] Second period.

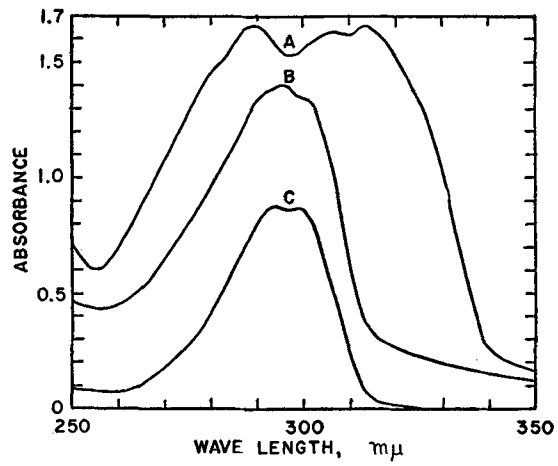

FIG. 1. Absorbance of corn oil and its molecular distillate.
A. Crude oil, 0.0106 g./ml.
B. The 170°C. distillate from an 8-fold amount of the oil.
C. Absorbance of the amount of *gamma*-tocopherol which gives, at 520 mμ, the same absorbance as (B) after the Emmerie-Engel reaction.

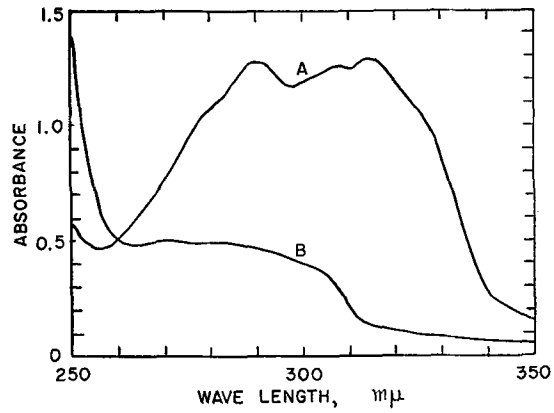

FIG. 2. Absorption spectra of (A) crude corn oil, 0.0084 g. per ml.; and (B) crude soybean oil, 0.0168 g. per ml.

of corn oil at 295 mμ was 5-fold that of soybean oil and at 315 mμ 20-fold that of soybean oil. Various samples of crude oil from both white and yellow corn showed, in general, the same type of absorption curves.

The data obtained in distillation and spectral studies therefore indicated that the interfering substance was separable from the tocopherols by molecular distillation and suggested that the substance has absorption in the region of 270–315 mμ.

Removal of Interference with Dilute KOH. Extraction of a hexane solution of corn oil (1%) with a one-half volume of 5% aqueous KOH was found to be an effective procedure for removing the interference (Table 2). The extracted solution, reacted directly

TABLE II

Comparison of Methods for Preparing Corn Oil for Analysis[a]

Method of Purification	Apparent tocopherol	Recovery of added γ-tocopherol	Rate of increase in apparent tocopherol with time
	%	%	*slope*[b] × 10^{-3}
None	0.239	99.0	13.4
Parker-McFarlane	0.079	62.6	1.2
Molecular distillation (170°C., 1st period)	0.113	87.6	0.5
5% aq. KOH: hexane	0.161	97.8	2.3
Activated bleaching earth: benzene	0.123	87.5	12.4
5% aq. KOH: hexane; act. bl. earth: benzene	0.084	91.6	1.6
Diatomaceous earth: benzene	0.189	95.4	12.6
5% KOH: hexane; diatomaceous earth: benzene	0.132	96.5	1.8

[a] Emmerie-Engel procedure: reaction of 0.02 g. oil.
[b] Method of least squares.

with the iron-dipyridyl mixture, showed an apparent tocopherol content which compared favorably with the apparent tocopherol content obtained by molecular distillation. The comparison is even more favorable when a correction is made for the recovery of only 87.6% of added tocopherol in the latter. Colored substances were still present in the alkali-treated oil. There was a good recovery of *gamma*-tocopherol added before treatment, and the absorbance drift was small as indicated by the slope of line after 2½ min. of reaction time. More concentrated KOH solutions did not show advantages over the 5% solution, and none of the KOH solutions was effective in extracting the interference from corn oil in benzene solution. Aqueous sodium bicarbonate, aqueous acetic acid, concentrated H_3PO_4, and concentrated HCl were all unable to remove the principal interference from hexane solution.

Other types of treatments to remove the interference were also not successful. Solvent extraction of the oil with various percentage, aqueous solutions of methanol, ethanol, acetone, and diacetone-alcohol was ineffective. Adsorption with diatomaceous earth by shaking a suspension in a benzene solution of corn oil was effective in removing colored substances but failed to reduce substantially the ultraviolet absorption of the solution. Column adsorption with this combination also resulted in some loss of added tocopherol. Fuller's earths of the types commonly used for bleaching oils affected more severe losses of added tocopherol.

Recovery of Interfering Substance from the KOH Extract. After treatment of a 1% solution of corn oil in hexane with 5% aqueous KOH, the hexane solution was washed by shaking with water, the phases were separated, and all aqueous extracts were combined (Figure 3). To this alkaline extract a one-

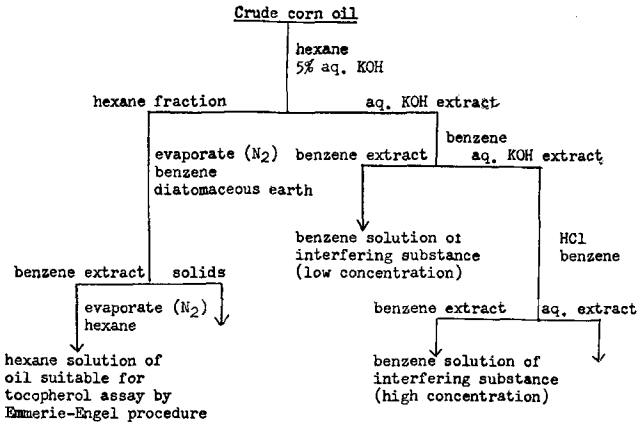

FIG. 3. Scheme for pretreatment of corn oil for tocopherol assay and for concentration of the interfering substance.

fourth volume of benzene was then added, and the mixture was shaken. After separating the two phases, the procedure was repeated twice with the alkaline extract. The solvent extracts were combined and washed with water. The aqueous phase was then acidified with HCl and shaken with benzene, as before. Each of the two benzene extracts was then evaporated to dryness under nitrogen, and hexane was added up to the original volume of the oil-solvent solution. Portions were then examined spectroscopically, and other portions were added to comparable portions of the treated corn oil.

The spectral curves (Figure 4) revealed that the crude corn oil (Curve A) had lost most of its absorptive power after treatment with 5% KOH (Curve B). Benzene extracted a small amount of the absorptive substance from the KOH solution (Curve C) and more than 10 times as much after acidification (Curve D). The sum of absorbances of the fractions (Curves B, C, and D) shows almost complete recovery of the absorbance of the original oil (Curve A). Comparative absorbance of the amount of *gamma*-tocopherol (Curve E), which would give an iron-dipyridyl reaction equal to that of the KOH-treated oil, indicated that some residual non-tocopherol ultraviolet absorbance was still present in the oil.

An indication of the extent to which this residual substance enters into the iron-dipyridyl reaction is shown in the slopes of lines which depict the rate of

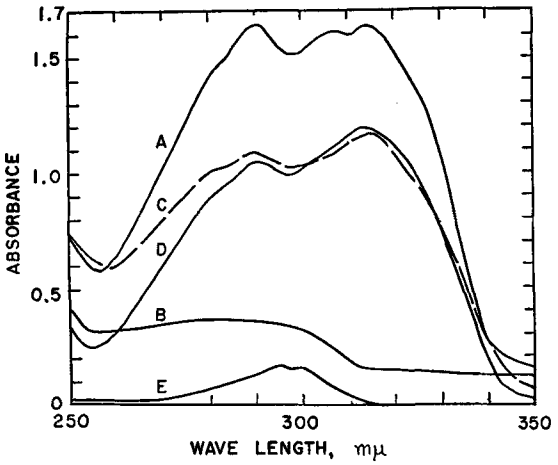

FIG. 4. Absorption spectra of (A) crude corn oil, 0.0100 g/ml.; (B) oil after extraction with 5% aq. KOH; (C) benzene-soluble substance extracted from KOH (all readings multiplied by 14); (D) benzene-soluble substance extracted from 5% KOH after acidification; and (E) the absorbance of the amount of *gamma*-tocopherol which gives, at 520 mμ, the same absorbance as (B) after the Emmerie-Engel reaction.

absorbance drift after 2½ min. of reaction time (Figure 5). The rate of drift for the crude oil (Curve A) was cut sharply after KOH-treatment (Curve B). The benzene-extractable substance recovered from the aqueous KOH phase after acidification (Curve C) produced about the same rate of drift as the crude oil. When a proportional amount of the extract was added back to the treated oil (Curve D), the recombined mixture showed absorbance drift similar to that of the original oil. The slopes of straight lines fitted to the data were: A, 0.0134; B, 0.0023; C, 0.0125; and D, 0.0134. Baxter (1) has suggested that the absorbance drift during the 8-min. period following the initial (2-min.) reading should not be more than 10% of the apparent tocopherol present at the initial reading. The slope of the B curve indicates that this criterion will be met by KOH-treated oils under almost all circumstances.

Method Adopted. Corn, ground on a burr mill to approximately 20-mesh size, was extracted with cold ethyl ether for 20 hours in the dark. The extract was

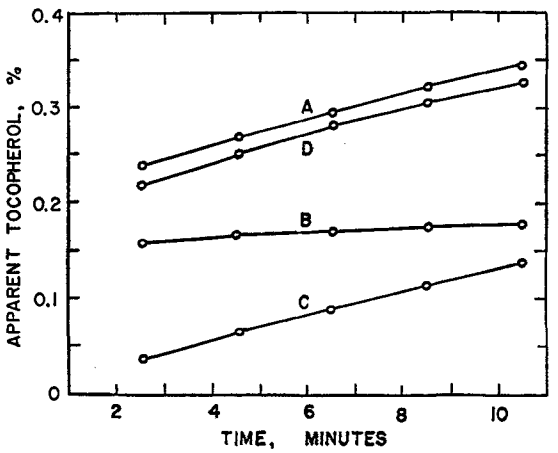

FIG. 5. Results of pretratment of corn oil as measured by Emmerie-Engel values.
A. Crude corn oil.
B. Oil after extraction of a hexane solution with 5% aq. KOH.
C. Benzene-soluble substance extracted by 5% aq. KOH.
D. Oil with 5% aq. KOH extract re-added (benzene-soluble portion).

TABLE III

Tocopherol Content of Oil from Corn Inbreds

Inbred	Total tocopherol		Rate of increase in apparent tocopherols with time [a]
	Direct analysis	Internal standard	
	%	%	*slope* [b] × 10^{-3}
Oh 43E	0.283	0.323	1.9
Hy	0.226	0.236	1.5
Pa	0.057	0.062	1.7
Mo G	0.050	0.052	1.9
Average of 10 inbreds [c]	0.145	0.154	18 [d]

[a] Emmerie-Engel procedure: reaction of 0.02 g. oil.
[b] Method of least squares.
[c] Sixty analyses of different ears.
[d] Calculated from 10% increase in apparent tocopherol, 8-min. period after initial reading (Ref. 1).

filtered through fluted, coarse filter paper (Whatman, No. 12) and evaporated almost to dryness on a steam bath; and the resultant oil was dried for 1 hr. in a vacuum oven at 50°C. The oil (2 g. or less) was transferred to a small centrifuge tube, which was then stoppered and held at —20°C. for no longer than three days before analysis.

On the day of analysis the oil was brought to room temperature, stirred thoroughly, and centrifuged. A weighed amount was dissolved in hexane. Aliquots of the oil-hexane solution were layered on 5% aqueous potassium hydroxide in 2 glass-stoppered centrifuge tubes. To one tube was added hexane, and to the other a known amount of *gamma*-tocopherol in hexane. The final concentration of the oil and *gamma*-tocopherol in the hexane were 1% and 0.0025%, respectively. The volume ratio of oil solvent to aqueous alkali was 2 to 1.

The tubes were stoppered and shaken for 10 min. and centrifuged, and an aliquot was pipetted from the solvent layer into a clean, glass-stoppered centrifuge tube. The hexane was evaporated under nitrogen while the tubes were held in a 45°C. water bath. To the residue was added a volume of benzene equal to the volume of the original aliquot, and 0.5 g. of diatomaceous earth per 10 ml. oil-benzene solution to remove the remaining pigments. The mixture was stoppered and shaken for 30 seconds and centrifuged. The solvent fraction was decanted. Suitable aliquots were then pipetted into amber, glass-stoppered bottles, and the solvent was evaporated under nitrogen while the bottles were held in a 45°C. water bath. The residue was then dissolved in 1 ml. of hexane.

Total tocopherol (as *gamma*-tocopherol) was determined by a modified Emmerie and Engel procedure. A setting for the reagent blank was obtained in the spectrophotometer by making use of adjacent sensitivity settings. To the sample residue dissolved in 1 ml. hexane was added 1 ml. of ferric chloride solution. Within 30 seconds 1 ml. *alpha, alpha'*-dipyridyl solution was added, and an interval timer was started. The bottle was rotated to mix the contents, and at 30 seconds 7 ml. of ethanol were added from a serological pipette (with bulb). The solution was mixed, allowed to stand until 2 min. had elapsed on the timer, and poured into a cuvette in the spectrophotometer. The slit width had already been adjusted for the reagent blank. Absorbance was determined at 2½ min. and compared with a calibration curve prepared with *gamma*-tocopherol under similar conditions.

The tube to which *gamma*-tocopherol had been added prior to the purification procedure was used as an internal standard for the entire procedure. Adjust-

ments were made for any deviations introduced by the volume of oil or oil residue, and calculations were made in the following manner: μg *gamma*- tocopherol originally present in sample aliquot $= (A_1)$ (μg *gamma*-tocopherol added per aliquot)$/(A_2 - A_1)$; where A_1 is the absorbance of corn oil and A_2 is the absorbance of corn oil plus added *gamma*-tocopherol. Kaunitz and Beaver (7) used similar calculations.

Application of the Method. The method has been applied successfully to the analysis of a number of corn inbreds and crosses and has served to show that a several-fold range exists in tocopherol content. Some examples are shown in Table III. It has been observed that the absorbance drift usually falls close to the arbitrary value of 10% suggested by Baxter.

Summary

A method has been developed to overcome difficulties in the analysis of corn oil for tocopherol. The poor recovery of tocopherol added to corn oil prior to the Parker-McFarlane treatment does not occur with the present method, and the absorbance drift which occurs when crude corn oil reacts with iron and dipyridyl is largely eliminated.

The interfering substance responsible for the drift did not distill appreciably in a molecular still at 170°C., and attempts to remove it from the oil by selective adsorption or solvent extraction were unsuccessful. It was extractable with dilute potassium hydroxide from a hexane solution of the oil and could be transferred from the alkaline extract to benzene. If the alkaline extract were acidified and then shaken with benzene, a much larger amount of the substance could be transferred. Extracts containing the substance showed strong absorption at wave-lengths between 290 and 315 millimicrons.

Alkali treatment of the oil and subsequent adsorption on diatomaceous earth reduced the content of interfering constituents to permit analyses for total tocopherol by the Emmerie-Engel method. By using this method, it was found that the oil from different corn inbreds and crosses varied over a six-fold range in tocopherol content.

Acknowledgment

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The Use of Coloring Ingredients in Fatty Food Products. Their Physiology, Chemistry, and Stability

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THERE IS RELATIVELY little structural similarity in the chemical structures of the four coloring materials, ethyl bixin, *beta*-carotene, Yellow AB, and Yellow OB, to account for the similarity of visible and ultraviolet spectra (except for double-bond conjugation and the similarity of Yellows AB and OB).

Table I shows that other physical properties of the compounds are equally dissimilar and presents the relative solubilities of these compounds.

On turning to the coloring values of these compounds, there is a rather wide variation in amounts of the pure substance necessary to color a ton of margarine. Based on extinction coefficients, Espoy and Barnett (6) reported the following equivalence:

1 g. *beta*-carotene ≡ 4 g. FD&C Yellows 3 and 4 (mixed) ≡ 0.7 g. ethyl bixin.

On the basis of actual visual examination they reported the following ratios:

1 g. *beta*-carotene ≡ 3.3 g. FD&C Yellows 3 and 4 (mixed) ≡ 0.5 g. ethyl bixin.

Analyses of many samples have shown there was up to 30% variation in the amount of color present; this has been confirmed by our experience with the quantity of color specified by various manufacturers (Table I).

Figure 1 shows the ultraviolet spectra of the commercial colors as determined in our laboratories. These data agree with curves reported by Espoy and Barnett (6) in 1955.

Stability Studies

Until a short time ago fat-soluble annatto colors were prepared by extracting the fat-soluble color

TABLE I

	Ethyl Bixin	β Carotene	Yellow AB	Yellow OB
Empirical formula	$C_{27}H_{34}O_4$	$C_{40}H_{56}$	$C_{16}H_{13}N_3$	$C_{17}H_{15}N_3$
Molecular weight	422.54	536.85	247.29	261.31
Melting point	149°C.	181–182°C.	102–104°C.	125–126°C.
Form	Red rhombic crystals	Red rhombic leaflets, deep purple prisms	Orange powder, orange-red platelets	Orange powder, orange-red platelets
Solubility				
Water	I	I	I	I
Ethanol	Sl.S.	I	S	S
Ethyl ether	Sl.S.	S	S	S
Hexane		S		
Acetone	S		S	S
C_6H_6	S	S	S	S
CCl_4	S	S		
Vegetable oils	Sl.S.	V. Sl. S.	S	S
Experience use level, g./ton	2.7–3.5	7.2	10–12[a]	10–12[a]

[a]Assuming equal parts of each are used according to usual practice.