the meal and pigment glands for possible industrial or other uses (1, 2, 3, 4).

This discussion has been based on the present stage of development of the fractionation process and the present possible utilization of the products. Pilot-plant work under way indicates improvements in the process, such as a higher recovery of the meal as purified fine meal. The development of additional and more profitable uses for the products would increase their value and provide a broader and more stable market for cottonseed. Such developments would make fractionation, already technologically interesting, a field economically advantageous.

Summary

A preliminary cost study was made of a combination screw-press extraction-fractionation plant. The economic advantage of the combination plant over the screw-press operation as shown here is small in comparison with the large additional investment required for the combination plant. The present value of the combination process lies in the production of two new products, a purified high-protein meal and a concentrated pigment gland fraction, and in the possibility of producing a high-grade oil as the removal of the whole pigment gland prevents the pigment material from coming in contact with the oil. Pilot-plant work under way indicates improvements in the process such as a higher recovery of the meal as purified meal. The development of additional and more profitable uses for the products would increase their value and provide a broader and more stable market for cottonseed.

Acknowledgment

The authors wish to express their appreciation to Joseph L. Hecker and to Jack E. Hawkins for making the drawings for the charts.

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[Received October 10, 1949]

Countercurrent Distribution Studies on Fat Soluble Plant Pigments[1]

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PARTITION of immiscible solvents, the procedure used by Willstätter in his classical work on the separation of plant pigments (8), has been almost entirely replaced within the past two decades by chromatographic adsorption methods (6). With the development of systematic extraction procedures such as are made possible by the countercurrent distribution apparatus of Craig (2), the merits of solvent partition procedures again need to be re-examined. In the present paper the countercurrent distribution of a model system composed of chlorophyll-a, chlorophyll-b, and carotene is described. Separations obtained experimentally are discussed in relation to those predicted by the theory of Martin and Synge (4) and to their equations as modified herein. A simple nomograph has been constructed.

Materials and Methods

Chlorophyll-a and chlorophyll-b were isolated from frozen spinach by chromatographic adsorption on powdered sugar as described by Zscheile and Comar (9). Each pigment was further purified by two successive adsorptions on powdered sugar columns. The effectiveness of the separation was then established by chromatographic and spectrophotometric methods. Carotene used in this work consisted of a mixture of alpha and beta (Eastman Kodak Co. 2702) and of crystalline α-carotene and crystalline β-carotene (General Biochemicals inc., No. 15849 and 16447).[3]

Countercurrent distributions were carried out in the 25-tube Craig analytical apparatus, using equal volumes of aqueous ethanol and hexane (90% ethanol and hexane, mutually saturated). On completion of the countercurrent distribution operation, both layers of each tube (8 ml. hexane and 8 ml. aqueous ethanol) were withdrawn into 25, 50-ml. volumetric flasks and diluted to volume with absolute ethanol. This dilution procedure resulted in a single phase solution for subsequent spectrophotometric determinations of chlorophyll-a, chlorophyll-b, and carotene. It was necessary to determine therefore the absorption coefficients of the pigments in that solvent mixture. The resulting equations for determining the concentrations of chlorophyll-a, chlorophyll-b, and carotene are similar in form to those of Comar and Zscheile (1):

[1] Presented at the fall meeting of American Oil Chemists' Society, November 2, 1949, in Chicago, Ill.

[2] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

[3] The mention of these products does not imply that they are endorsed or recommended by the Department of Agriculture over others of similar nature not mentioned.

$$\text{Chlorophyll-a (mg./L)} = 12.72\ D^{6650\ \text{Å}} - 3.907\ D^{6475\ \text{Å}}$$

$$\text{Chlorophyll-b (mg./L)} = 26.87\ D^{6475\ \text{Å}} - 7.18^{6650\ \text{Å}}$$

$$\text{Carotene (mg./L)} = 3.58\ D^{4500\ \text{Å}} - 0.900\ D^{6650\ \text{Å}} - 1.76\ D^{6475\ \text{Å}}$$

The amounts of chlorophyll-a, chlorophyll-b, and carotene in any given tube of the apparatus were determined by substituting the experimentally determined optical densities (D) at the appropriate wave lengths in the above equations.

Results

The countercurrent distribution pattern for a mixture consisting of 14.8 mg. chlorophyll-a, 12.4 mg. chlorophyll-b, and 1.04 mg. carotene is shown in Figure 1. The concentration in mg. per liter of each chlorophyll pigment contained in each tube is plotted against the tube number. The concentrations of carotene have been multiplied by 10. Also included are the theoretical curves calculated from the partition coefficients as described by Williamson and Craig (7). Partition coefficients used for this purpose were themselves averages calculated from the experimental points near the peak of each curve and are: $K_{chl\ a} = 1.061$, $K_{chl\ b} = 0.592$, and $K_{carotene} = 4.093$.

First, it will be observed that excellent agreement is obtained between theoretically calculated and experimentally observed distribution curves. Second, it will be noted that pure chlorophyll-a and pure chlorophyll-b can be isolated only from opposite sides of their respective curves and that a mixture exists in the middle. In contrast, carotene is well separated. For reasons to be discussed later, it is desirable to measure the degree of separation by dividing fractionations at the point of "cross over" of the curves. The overlap of the chlorophyll-b curve upon the chlorophyll-a curve, or, more specifically, the ratio of area under the tail of the "a" curve from the point of intersection to the total area of the "b" curve may be used as a convenient measure of impurity. The overlap in this case is 23.7%.

Pigment fractionations obtained by countercurrent distribution will quite naturally be considered in relation to those obtainable by chromatographic adsorption on sugar. For comparative purposes a mixture of pure chlorophyll-a and pure chlorophyll-b was adsorbed on a column of powdered sugar and developed with 25% diethyl ether in petroleum ether. After passage through 260 mm. of the column, the chlorophyll-a band contained less than 1% of chlorophyll-b as impurity.

The separation of α- and β-carotene was attempted by using the same solvents, Craig apparatus, and procedure described above. No fractionation of α- and β-carotene could be detected by spectrophotometric methods. This pair of compounds however is also separated quite satisfactorily by chromatographic adsorption methods. These results are reported because they serve to define the limitations of the technique.

Discussion

It is apparent that application of more plates should improve the fractionation of the chlorophyll pigments. Since a 53-plate model of distribution equipment is available (3), a calculation of distribution curves for 53 plates was made employing the partition coefficients given above. It can be seen by comparing the curves of Figures 1 and 2 that the degree of separation would in fact be greatly improved since the area of overlap of chlorophyll-a in the chlorophyll-b curve has been reduced from 23.7% to 14.7% in this instance.

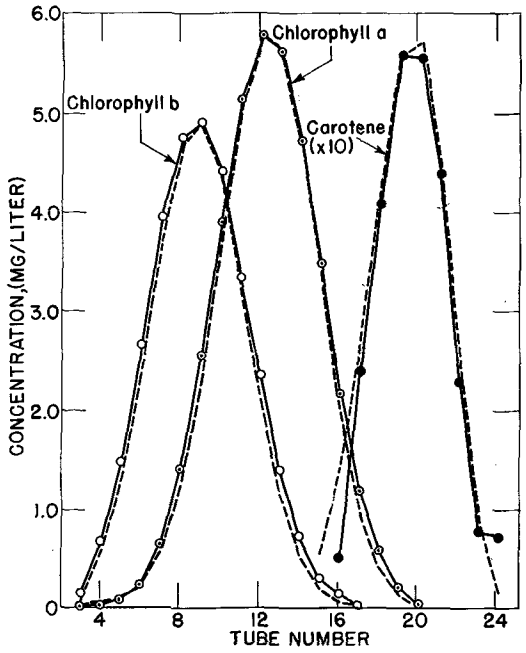

FIG. 1. Countercurrent distribution of chlorophyll-a, chlorophyll-b, and carotene in the 25-tube Craig distribution apparatus. Solid lines represent experimental data; broken lines show the calculated theoretical distribution.

These results point to the need for a more general treatment of the problem of degree of separation in terms of partition coefficients and number of plates. Such an algebraic description was, in fact, developed by Martin and Synge for partition chromatography in 1941 (4). Based on similar considerations, Mayer and Tompkins (5) have recently given an analysis of degree of separation for ion exchange columns which again is applicable only in the case of large numbers of plates and of transfers. There remains a need for analysis where relatively small numbers of plates and transfers are involved as in the Craig counter-current

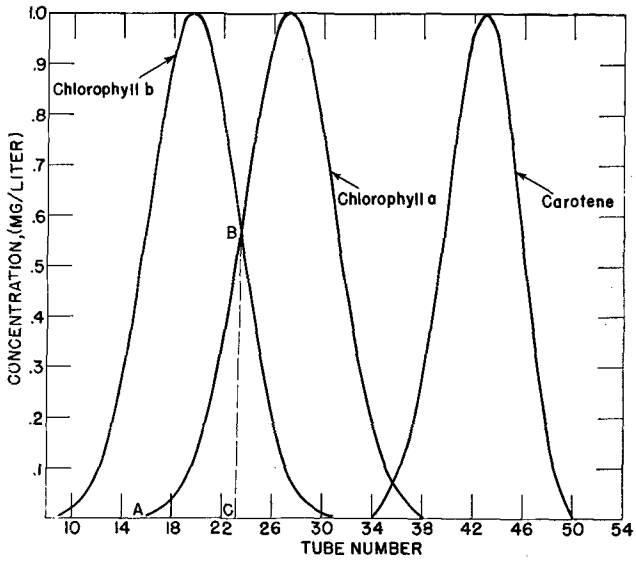

FIG. 2. Theoretical distribution of chlorophyll-a, chlorophyll-b, and carotene for a 54-tube Craig countercurrent distribution.

distribution apparatus. Such a method is proposed in the following paragraphs.

The degree of separation of equal amounts of two compounds is defined as the amount of one compound expressed as impurity in a mixture of the two when measured at the point of crossover of their distribution curves (4). As shown in Figure 2, the percentage impurity of chlorophyll-a in chlorophyll-b is the ratio of the area, ABC, to the total area under the chlorophyll-b curve.

Martin and Synge derived an equation to describe separation of a binary system by partition chromatography. Where equal volumes of immiscible solvents are used, their equation may be written in general form as follows:

$$(I) \qquad \frac{1+\alpha}{1+\beta} = \frac{r - \sqrt{r}\,t + \frac{t^2}{4}}{r + \sqrt{r}\,t + \frac{t^2}{4}} = R$$

where

α = partition coefficient of substance A, according to Martin and Synge,

i.e., $\frac{\text{gm. A in non-mobile (hypo) phase}}{\text{gm. A in mobile (hyper) phase.}}$

When equal volumes of solvent are used, $\alpha = \frac{1}{K_a}$, K_a being the partition coefficient in the terminology of Craig (7).

β = partition coefficient of substance B, according to Martin and Synge. $\beta = \frac{1}{K_b}$.

r = serial number of the plate at the point of intersection.

t = abscissa of the normal curve of error, from which degree of separation can be found. (The degree of separation in this case is the area under the normal curve from t to ∞ as given in tables of areas of the normal curve of error).

R = the ratio, $\frac{1+\alpha}{1+\beta} = \frac{1+\frac{1}{K_a}}{1+\frac{1}{K_b}} = \frac{1+K_a}{1+K_b} \cdot \frac{K_b}{K_a}$

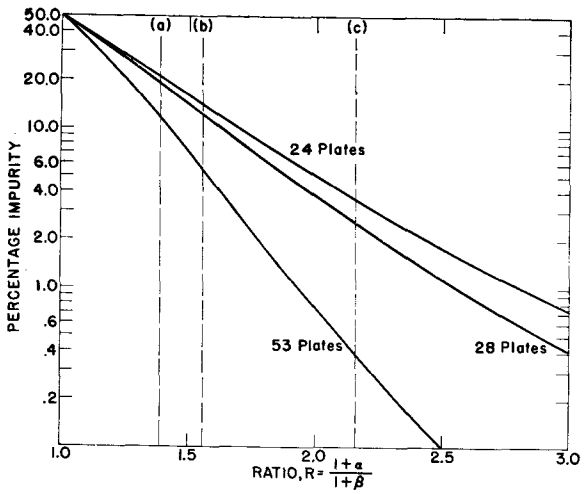

FIG. 3. The relationship of degree of separation (per cent impurity) to "R" for 24-, 28-, and 53-plate countercurrent distributions.

Since $\frac{t^2}{4}$ is small under practical conditions, it has been dropped from the equation. The equation can then be solved for t to give:

$$(II) \qquad t = \sqrt{r}\,\frac{R-1}{R+1} = \sqrt{r}\,\frac{K_b - K_a}{K_a + K_b + 2K_a K_b}$$

For small numbers of transfers such as generally employed in the Craig apparatus, the use of the plate number of the point of intersection, "r," in the above formula leads to results which are not consistent with theoretical results as computed by the use of binomial expansions. A closer approximation to binomial expansion results can be obtained by using "n," the number of transfers, instead of "r" in equation II. Agreement between separations calculated by this more or less empirical relation and those calculated from the binomial expansion is shown in the table.

COMPARISON OF PERCENTAGE OVERLAPS FOR 24 TRANSFERS AS COMPUTED FROM BINOMIAL EXPANSIONS AND FROM EQUATION III, $t = \sqrt{n}\left[\frac{R-1}{R+1}\right]$

R	K_a	K_b	Overlap—%	
			Binomial	Eq. No. III
1.07	3.0	4.0	61.6	43.6
1.12	2.0	3.0	31.3	38.6
1.20	1.0	1.5	29.5	32.8
1.26	0.6	0.9	29.3	28.4
1.27	0.9	1.5	25.3	28.1
1.31	0.4	0.6	37.5	25.5
1.35	0.8	1.5	21.3	23.3
1.50	0.5	1.0	21.2	16.4
1.58	0.9	3.0	8.2	13.4
1.73	0.7	2.5	6.2	9.3
1.82	0.7	3.0	5.3	7.6
2.00	0.5	2.0	4.7	5.2
2.06	0.25	0.7	13.4	4.5
2.14	0.5	2.5	2.5	3.8
2.25	0.5	3.0	1.4	3.0
3.00	0.25	1.5	1.6	0.71
3.33	0.25	2.0	0.72	0.41
3.75	0.25	3.0	0.11	0.23
4.00	0.25	4.0	0.00	0.16
Pigments				
[1]1.384	0.5918	1.0608	23.7	21.5
[2]1.561	1.0608	4.0930	15.1	14.2
[3]2.161	0.5918	4.0930	5.5	3.6

[1] Chlorophyll-a in chlorophyll-b.
[2] Carotene in chlorophyll-a.
[3] Carotene in chlorophyll-b.

Practical conditions of countercurrent extraction place limits on the magnitude of the partition coefficients K_a and K_b. On the basis of experience, these limits can be set as $K_b < 4$ and $K_a > 0.25$, K_b always being the larger of the two. Practical R values thus range from >1 to <4 and include most cases where the Craig technique would be used.

Within these limits, then, equation (II) may be rewritten

$$(III) \qquad t = \sqrt{n}\,\frac{R-1}{R+1} = \sqrt{n}\,\frac{K_b - K_a}{K_a + K_b + 2K_a K_b}$$

where t, R, K_a, and K_b are defined as above and n is the total number of transfers (ordinarily one less than the total number of tubes).

This equation may also be written in the following forms:

$$(IV)[4] \qquad n = t^2\left[\frac{R+1}{R-1}\right]^2 = t^2\left[\frac{K_a + K_b + 2K_a K_b}{K_b - K_a}\right]^2$$

[4] Since preparation of this paper, a manuscript by Peter L. Nichols has been sent us in which he has independently derived the equation: $n = t^2 S$ from somewhat different considerations. However his value for S differs from our $\left[\frac{R+1}{R-1}\right]^2$ above.

and

$$(V) \qquad R = \frac{\sqrt{n} + t}{\sqrt{n} - t} = \frac{1 + K_a}{1 + K_b} \cdot \frac{K_b}{K_a}$$

Equations (III) and (IV) will be found the most important in practice. Equation (III) gives the degree of separation when a given number of plates is applied to a given pair of substances. The problem of how many plates need be applied to attain a desired degree of separation is one of frequent occurrence. Since countercurrent apparatus is available, providing 24, 28, and 53 plates (3), calculations of degree of separation have been made by means of equation (III) corresponding to these numbers of plates. A useful plot of this calculation is shown in Figure 3 where the ratio R is plotted against percentage of impurity. The percentage of impurity was determined from the calculated "t" by reading the corresponding areas from tables of the normal curve of error.

The applicability of this graph to the separation of plant pigments can be illustrated by the broken lines of Figure 3. The line labeled "a" refers to the separation of chlorophyll-a and chlorophyll-b (R = 1.384); "b" to chlorophyll-a and carotene (R = 1.561); and line "c" to chlorophyll-b and carotene (R = 2.161). Thus it is predicted that in the 24-plate apparatus, chlorophyll-b will contain 21.5% of chlorophyll-a as impurity; that chlorophyll-a will have 14.2% carotene as impurity; and that chlorophyll-b will contain 3.6% carotene as impurity. (The corresponding ratios of overlap as calculated from the binomial expansion are 23.7%, 15.1%, and 5.5%, respectively). Use of the 53-plate apparatus should reduce the amount of chlorophyll-a in chlorophyll-b to 12.1%, the carotene in chlorophyll-a should now be 5.6%, and the carotene content of chlorophyll-b should be reduced to 0.38%. (The corresponding ratios of overlap as calculated from the binomial expansion are 14.7%, 11.5%, and 0.0%, respectively.) The number of plates needed to achieve a given degree of separation for a given pair of compounds can be calculated from equation (IV). Similar data to that in Figure 4 was presented graphically by Martin and Synge, calculated from their equation. Reading from Figure 4, it is apparent that 37 plates would be required to reduce the chlorophyll-a impurity in chlorophyll-b to 15.9%, 150 plates to reduce it to 2.27%, and 340 plates to reduce it to 0.13%.

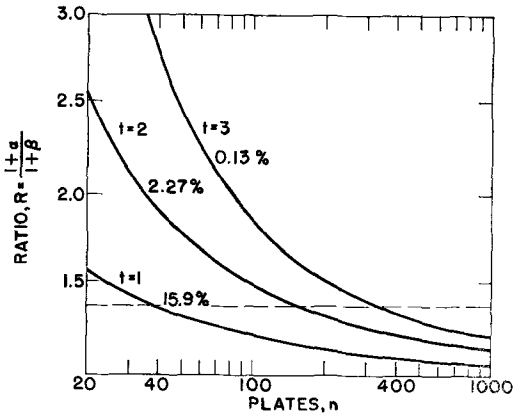

FIG. 4. Graph similar to that given by Martin and Synge showing the number of theoretical plates necessary to achieve a specified degree of separation, as calculated from equation (IV).

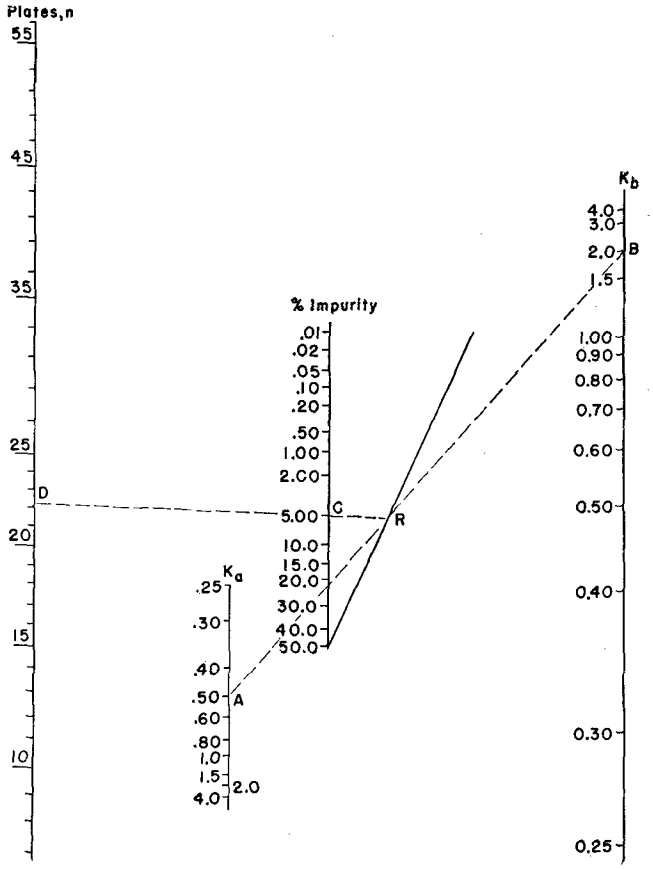

FIG. 5. Nomograph relating plates, n, per cent impurity, R, K_a, K_b as defined in the equations.

While the following comparison between chromatographic adsorption and countercurrent distribution is admittedly unwarranted, it is interesting to consider the equivalent height of a theoretical plate on an adsorption column calculated by using the above theory. Chlorophyll-a containing less than 1% of chlorophyll-b was obtained from a chromatographic separation on a 260-mm. column described earlier. According to Figure 4, at least 340 solvent partition plates would be required to give this degree of separation. The height of each plate would therefore have been $\frac{260}{340}$ or 0.76 mm. While such a comparison cannot be defended rigorously, it does place adsorption analysis and countercurrent distribution in their proper perspective as applied to chlorophyll separations.

A nomograph, giving the relationship between partition coefficients, number of transfers, and degree of separation has been constructed. The nomograph or alignment chart may be used to find any one of the 4 variables in the equations if the other three are known. For example, suppose one wishes to achieve a separation in which 5% of substance A is present as impurity in substance B, where $K_a = 0.5$, and $K_b = 2.0$. To find the number of plates required for this separation:

a) Draw a line AB between the values of $K_a = 0.5$ and $K_b = 2.0$ on Figure 5 (K_b is chosen larger than K_a).

b) From the point R on the diagonal line, draw another line CR through the 5% point on the impurity scale, extending it to cross the n axis. The value of the n-scale at this point estimates the number of plates needed for the desired 5% impurity separation.

The reverse of the above process may be used to determine the percentage of impurity of A in B, using a given number of plates, that is, the second line can be drawn from R to the number of plates used and the percentage of impurity read from the percentage scale. The error of the nomographic solution is usually less than the approximations introduced in the derivation of the equations and the use of the nomograph should greatly facilitate the application of the equations.

Acknowledgment

The authors are indebted to J. C. Cowan for his advice and encouragement throughout the course of this work and to Vera Turner for her technical assistance.

Summary

Chlorophyll-a, chlorophyll-b, and carotene have been used to study the operation of countercurrent distribution apparatus. An incomplete separation of the chlorophylls was obtained by the use of the 25-tube Craig apparatus, but nearly complete separation of the chlorophyll and carotene pigments resulted. Degree of separation can be estimated by the modification of the equation of Martin and Synge which follows:

$$t = \sqrt{n}\ \frac{R - 1}{R + 1} = \sqrt{n}\ \frac{K_b - K_a}{K_a + K_b + 2K_a K_b}$$

Comparisons were made between the degree of separation predicted by these formulae and that calculated by the use of the binomial expansion. The utility of such predictions is illustrated by the problem of separating plant pigments. A nomographic solution of these equations is presented to facilitate their application.

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[Received March 28, 1950]

Determination of Total Gossypol Pigments in Cottonseed Materials

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THERE has been a recognized need for a reliable routine method for the determination of total gossypol pigments in cottonseed materials. Such a method must be fairly rapid and precise. Several methods have been suggested (4, 5). They are gravimetric methods, requiring hot aniline extractions, time-consuming precipitations, and individual attention. A method is proposed which involves an acid hydrolysis of the "bound" gossypol pigments in aqueous methyl ethyl ketone, and the colorimetric determination of the gossypol pigments by the method described recently (3) for free gossypol pigments.

The term "total gossypol pigments," as used in this paper, designates gossypol and closely related pigments, which after hydrolysis and reaction with *p*-anisidine give reaction products identical spectrophotometrically with that of pure gossypol. In this connection it must be pointed out that gossypol pigments such as gossyfulvin are convertible with ease to gossypol (1) and the gossypurpurin reacts with *p*-anisidine to give a product spectrophotometrically identical with that obtained from pure gossypol and the same reagent (3).

Reagents

a) Aqueous acetone: 700 ml. A.C.S.-grade acetone plus 300 ml. of distilled water.

b) Methyl ethyl ketone — water azeotrope: Mix 1106 ml. of reagent grade methyl ethyl ketone with 110 ml. of water and distill through a column such as a Vigreaux column. The azeotrope boils at 73.5°C. and contains 11.0% water by weight (2). Store in a brown bottle.

c) Oxalic acid solution: Dissolve 12.60 g. of A.C.S. grade oxalic acid dihydrate ($H_2C_2O_4 \cdot 2H_2O$) in the methyl ethyl ketone azeotrope and make up to 1 liter with the azeotrope. This solution is 0.1 molar in oxalic acid.

d) Barium acetate solution: Dissolve 136.73 g. of A.C.S.-grade barium acetate hydrate [$Ba(C_2H_3O_2)_2 \cdot H_2O$] in distilled water and dilute to 1 liter with water. This solution is 0.5 molar in barium acetate.

e) Isopropanol: Reagent-grade diluted to 80% by volume with distilled water.

f) Glacial acetic acid: A.C.S. reagent-grade.

TABLE I

Influence of Time of Hydrolysis on Total Gossypol Pigments Found

Time of Heating at 75°C.	Total Gossypol[1]			
	Raw Meats	Cooker Meats	Hydraulic-Pressed Meal	Screw-Pressed Meal
Hours	%	%	%	%
2	1.24	1.35	1.31-1.41	0.53
3	1.24	1.44	1.48	0.55
4	1.22	1.46	1.54	0.58
5	1.24	1.50	1.55	0.60
6	1.24	1.51	1.56	0.60
7	1.23	1.50	1.58	0.61
8	1.23	1.51	1.56	0.60
16	1.23	1.51	1.55	0.60

[1] Air-dry basis.

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.