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tion curve for isomerized pure linolenic acid is intermediate between those of F_1 and F_2 , which is to be expected for a mixture containing both types of trienes. The presence of the alpha-eleostearic acid type of triene raises the possibility that the isomerization of linolenic acid might have attained completion earlier than 2 hrs. when the extinction coefficient was lower. The extinction coefficient, k_{268} , for pure linolenic acid obtained by the sealed tube method (Table II) is the highest so far reported for this acid. This together with a smaller value in the diene region will render the estimation of linoleic and linolenic acids in mixtures more accurate.

The absorption curve for methyl arachidonate II has peaks in the diene, tetraene, and pentaene regions. The absence of a peak in the triene region shows that complete conjugation of arachidonic acid has also been attained under these conditions. Thus the peaks in the diene and pentaene regions reveal the presence of nonconjugated diene and pentaene in the original sample. The extinction coefficients at 315 $m\mu$ and 346 $m\mu$, respectively (Table II, last column), are about the highest as compared to other methods.

Analysis of Samples of Oils. Analysis of the oils for the component polyunsaturated acids by the sealed-tube method compares well with those of the other methods. It should be noted that because of a higher extinction coefficient at all characteristic peaks the estimation of the minor component by this method is likely to be the more reliable. The presence of a tetraene in the linolenic acid concentrate, as noted earlier, prompted a re-examination of linseed oil by all the methods with special reference to this acid. A peak was observed in the tetraene region by all the methods, and no peak was seen in the pentaene region. The contents of tetraenoic acids, calculated as arachidonic acid by the 6.6% KOH-glycol and 21.0% KOH-

glycol methods, Table IV, do not agree and only the k₃₁₅ values could be recorded for the potassium t-butoxide methods. Until the nature of this tetraenoic acid (length of the carbon chain and position and geometry of the double bonds) is settled, nothing more than its presence can be shown.

Summary

A temperature and time study revealed that the best conditions for the isomerization and estimation of polyunsaturated acids are heating in a sealed tube for 2 hrs. at 140° with potassium-t-butoxide in t-butanol. Under these conditions conjugation of linolenic and arachidonic acids appears to have attained completion. With linoleic acid a higher degree of conjugation than by other methods and a shift of peak to lower wavelength $(231-232 \text{ m}\mu)$ are observed. Extinction coefficients obtained by this method for isomerized pure linoleic (k232, 98.0) and linolenic (k₂₆₈, 138.4) acids are the highest so far reported. Ultraviolet and infrared examination of the conjugated trienes isolated from isomerized linolenic acid concentrate showed that, under these conditions, trienes similar to both alpha- and beta-eleostearic acids are produced. Analyses of samples of oils for component polyunsaturated acids, by this method, compare well with those by other methods. The presence of small amounts of a tetraenoic acid in linseed oil is noted.

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3-Amino-1-Propanol as a Complexing Agent in the **Determination of Total Gossypol**

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THE ESTIMATION of total gossypol has proven to be useful for evaluating the influence of processing conditions on the distribution of the gossypol in cottonseed between the meal and oil (10, 14). Recent reports (3, 5), indicating an apparent relationship between total gossypol content and the nutritive value of cottonseed meal for nonruminants, suggest that this constituent may receive increasing attention as a quality factor of cottonseed meal. Although analytical methods currently employed for the estimation of total gossypol (1, 9) are adequate from the view-point of accuracy and precision, they require a minimum 6-hr. hydrolysis period for cleavage of bound gossypol. In the method proposed here total gossypol is completely removed from cottonseed meal in a 30-minute extraction, during which the gossypol is complexed with neutralized 3-amino-1propanol in dimethylformamide. The difference in absorption of aliquot portions of the extract before and after reaction with aniline serves as a measure of the total gossypol content and allows proper correction for background absorption of the extracts.

The time required for analysis is 2 hrs. as compared to about 7 hrs. for the present methods. For control purposes the time required could be further reduced to 1 hr. by minor modification of the procedure. In addition to meals, the procedure is also applicable to cottonseed meats, crude oils, and soapstocks, offering the advantage of a single total gossypol method for all cottonseed products.

Analytical Method

Reagents

1. Isopropyl alcohol-hexane mixture. Mix 60 volumes of reagent grade isopropyl alcohol and 40 volumes of commercial hexane [A.O.C.S. Specification H 16-56 (1)].

2. Complexing reagent. Pipet 2 ml. of 3-amino-1propanol (practical grade) and 10 ml. of glacial acetic acid (reagent grade) into a 100-ml. volumetric

¹One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

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flask, cool to room temperature, and dilute to volume with dimethylformamide (N,N-dimethylformamide, reagent grade or commercial grade redistilled between 152-153°C.). The reagent is stable for one week after preparation.

3. Aniline. Reagent grade redistilled over zinc dust. Store in refrigerator and redistill when the absorbance of the reagent blank exceeds 0.022.

4. Standard gossypol solution. Weigh 25 mg. of pure gossypol (7, 8), dissolve in and make to 50-ml. volume with the complexing reagent. If exactly 25 mg. of gossypol are used, the solution will contain 0.5 mg. per ml.

Calibration Curve

Pipet 2, 4, 6, 8, and 10 ml. of the standard gossypol solution into 50-ml. volumetric flasks. To each standard add sufficient complexing reagent to make the total volume 10 ml. Use 10 ml. of the complexing reagent as a blank. Heat the flasks in a boiling water bath (100°C.) for 30 min., cool and dilute to volume with the isopropyl alcohol-hexane mixture. Pipet duplicate, 2-ml. aliquots of each standard and of the blank into 25-ml. volumetric flasks. Dilute one set of aliquots to volume with the isopropyl alcoholhexane mixture and reserve as reference solutions. To the other set of aliquots add 2 ml. of aniline, heat in a boiling-water bath (100°C.) for 30 min., cool to room temperature, and dilute to volume with isopropyl alcohol-hexane mixture. Allow the flasks to stand at room temperature for 1 hr. after dilution and mixing.

With a spectrophotometer determine the absorbance of the reagent blank at 440 m μ using the diluted blank aliquot without aniline as a reference solution. Determine the absorbance of each gossypol standard reacted with aniline, using the appropriate diluted standard as a reference solution. Subtract the absorbance of the reagent blank from that of each standard to obtain the corrected absorbance.

Calculate the calibration factor by dividing the mg. of gossypol in the 2-ml. aliquot of each standard by the appropriate corrected absorbance. Average the factors for all the gossypol standards. The amount of gossypol in the sample aliquots is then found by multiplying the corrected absorbance of the sample aliquot by the calibration factor. Once obtained, the calibration factor need not be redetermined except for periodic checks of instrument response. In the event that a colorimeter is used rather than a spectrophotometer, the calibration factors will probably vary with the concentration of gossypol. In this case it is necessary to plot the concentration of gossypol in the standards against the corresponding absorbance and use the calibration curve for the calculation of the gossypol content of the sample aliquots.

Sample Preparation

1. Cottonseed. Grind about 50 g. of sample through a Bauer mill² with the plates separated so that the seed are broken but not pulverized. Separate the kernels from the hulls and lint by screening, and grind the hull-free kernels to pass a 2-mm. screen in a Wiley mill.²

2. Cake, pellets, and meal. Grind about 50 g. of a representative sample to pass a 1-mm. screen in a Wiley mill.

3. Crude oils. Heat the sample to about 50°C., mix well, and filter through paper suitable for the filtration of oil. Should it be necessary to keep the sample for an extended period, it should be stored at about 0° F.(-18° C.) to minimize change in gossypol.

4. Soapstock. Heat the sample to about 50°C. and mix well immediately before sampling. Store samples under the same conditions as crude oil.

Sample Size

The analytical sample should contain from 1 to 5 mg. of gossypol. For maximum precision the aliquot used should contain about 0.1 mg. of gossypol. Suggested sample weights and aliquots are as follows:

Expected total gossypol content,	Sample weight,	Aliquot for analysis,
%	g.	ml.
Below 0.1	2.0 - 4.0	5 - 10
0.1 - 0.2	2.0	2
0.2 - 0.4	1.0	2
0.4 - 0.6	0.75	2
0.6 - 0.8	0.50	2
0.8 - 1.0	0.30	2
1.0 - 2.0	0.20	2

Procedure

Weigh sufficient sample material to contain 1 to 5 mg. of total gossypol into a 50-ml. volumetric flask, and add 10 ml. of the complexing reagent (a rapid delivery pipet may be used). Use 10 ml. of the complexing reagent as a reagent blank. Heat both the sample and the blank in a boiling-water bath (100° C.) for 30 min., cool to room temperature, dilute to volume with isopropyl alcohol-hexane mixture, and mix. Filter through paper of medium retentivity, collecting the filtrate in a small glass-stoppered flask.

Pipet duplicate aliquots of the filtered extract and of the reagent blank into 25-ml. volumetric flasks. Dilute one of the aliquots to volume with the isopropyl alcohol-hexane mixture and reserve as reference solution. To the other aliquot add 2 ml. of aniline, develop the color, and determine the corrected absorbance as outlined above for the standards. Determine the mg. of gossypol in the sample aliquot by means of the calibration factor or by reference to the calibration curve as described for the gossypol standards. Calculate total gossypol in the sample as follows:

Total gossypol,
$$\% = \frac{5 \times G}{WV}$$

G = Mg. gossypol in sample aliquot W = Weight of sample in g.

V = Volume of aliquot used for analysis

Experimental and Discussion

The use of complexing agents was investigated as means of reducing the time required for cleavage of bound gossypol. Aromatic amines, which are known to extract bound gossypol from cottonseed meals (4), were not investigated since their use would preclude the evaluation of gossypol by an aniline colorimetric reaction. Preliminary experiments showed that amino alcohols, such as 2-amino-1-butanol, 1 amino-2propanol, 2-amino-2-methyl-1,3-propanediol, tris(hydroxymethyl) aminomethane, and 3-amino-1-propanol, all form complexes with gossypol similar to those reported (12) for long chain aliphatic amines. Of these 3-amino-1-propanol was selected since the re-

²Mention of the names of firms or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

agent is stable and readily available, and the absorption characteristics of the complex formed with gossypol are such that proper correction can be made for background absorption in applying the aniline colorimetric reaction as a measure of extracted gossypol.

Dimethylformamide, a stable high-boiling solvent in which both neutralized aminopropanol and the gossypol complex are readily soluble, was chosen as the reaction medium since it permits the extraction to be conducted at 100°C. and minimizes the time required for cleavage of bound gossypol. The isopropyl alcohol-hexane mixture used for dilutions allows application of the procedure to cottonseed meats, meals, crude oils, and soapstocks. Ketone solvents must be avoided as they react with aminopropanol to form colored solutions.

Gossypol-Aminopropanol Complex

Under the conditions employed in the analytical procedure, reaction of gossypol with 3-amino-1-propanol results in the formation of a complex in which the absorption maximum is shifted from 370 m μ to 376-398 m μ (Figure 1). When the complex is re-



acted with an excess of aniline, the spectra exhibit the typical dianilinogossypol maximum at 440 m μ (Figure 1), identical with that of pure gossypol reacted with aniline under comparable conditions, and exhibit no evidence of the maximum at 376–398 m μ characteristic of the gossypol-aminopropanol com-

	T.	ABLE I	
	Effect of Aminopropanol an Reaction of Go	nd Acetic Acid Conce ossypol with Aniline	ntration on
_	Concentration in 10 ml. ²	Treatment	Absorbance

Concentration in 10 mi."		hoforo	after		
3-Amino-1- propanol	Acetic acid	aniline reaction	aniline reaction ^b		
ml.	ml.				
None	None	None	0.347		
0.2	None	30 min, at 100°C.	0.131		
0.1	0.2	None	0.298		
0.1	0.2	30 min. at 100°C.	0.323		
0.2	0.2	30 min. at 100°C.	0.292		
0.2	1.0	30 min. at 100°C.	0.333		
0.2	2.0	30 min. at 100°C.	0.333		
0.2	5.0	30 min. at 100°C.	0.342		
0.04	1.0	30 min. at 100°C.	0.333		
0.10	1.0	30 min. at 100°C.	0.333		
0.20	1.0	30 min. at 100°C.	0.333		
0.30	1.0	30 min. at 100°C.	0.330		
0.50	1 1 0	1 Summa at $100^{\circ}0$	0314		

 2.50 mg. of gossypol and specified amounts of 3-amino-1-propanol and acetic acid made to 10-ml. volume with dimethylformamide.
 2/50 aliquots reacted with 2 ml. aniline, for 30 min. at 100°C., absorbance readings with Bausch and Lomb Spectronic-20 at 440 mµ. plex. This indicates that, with an excess of aniline, the equilibrium is almost entirely displaced in favor of the formation of dianilinogossypol. The data in Table I also confirm the conversion of the complex to dianilinogossypol.

Optimum Composition of Complexing Reagent

Since an equilibrium exists when the gossypolaminopropanol complex is reacted with an excess of aniline, the effect of variation in the concentration of aminopropanol and acetic acid on the reaction was investigated. The data shown in Table I, where the concentration of gossypol was maintained constant, indicate that an excess of acetic acid over that required for neutralization of the aminopropanol leads to optimum color development. Heating the gossypol with neutralized aminopropanol before the subsequent aniline reaction results in a higher absorbance than is obtained when the preliminary heating period is omitted. In terms of permissible variation, aminopropanol concentration can vary from 0.1 to 0.3 ml. and acetic acid from 1.0 to 5.0 ml. with no effect on the color developed with aniline. Based on these results, 10 ml. of a complexing reagent containing 0.2 ml. of aminopropanol and 1.0 ml. of acetic acid were selected for the extraction of total gossypol in the analytical procedure.

The ratio of gossypol to aminopropanol in the extraction step is another variable which limits the range over which the procedure is applicable. To investigate this factor, concentrations of gossypol ranging from 0.5 to 5.0 mg. were treated with 10 ml. of the complexing reagent and reacted with aniline as outlined in the procedure. Calibration factors calculated from absorbance measurements (Table II)

TABLE II Effect of Aminopropanol : Gossypol Ratio on Color Developed in Aniline Reaction

Concentration	Ratio	After aniline reaction		
Gossynol	aminopropanol : gossypol	Absorbance b	Factor ^c	
0.50	400:1	0.053	0.377	
1.00	200:1 133:1	$0.108 \\ 0.168$	$0.370 \\ 0.357$	
$2.00 \\ 2.50$	$100:1 \\ 80:1$	$\begin{array}{c} 0.222\\ 0.280\end{array}$	$0.360 \\ 0.359$	
$3.00 \\ 3.50$	$66:1 \\ 57:1$	$0.337 \\ 0.398$	$0.356 \\ 0.352$	
4.00	50:1 45·1	0.450	$0.356 \\ 0.354$	
5.00	$\frac{1}{40:1}$	0.569	0.353	

^a Specified amount of gossypol treated with 10 ml. of complexing reagent containing 0.2 ml. of 3-amino-1-propanol and 1.0 ml. of acetic acid and heated for 30 min. at 100° C. ^b 2/50 aliquots reacted with 2 ml. of aniline for 30 min. at 100° C. Absorbance readings with Bausch and Lomb Spectronic-20 at 440 mµ.

e Factor = $\frac{mg.\ gossypol\ in\ aliquot}{absorbance}$

indicate that gossypol concentration in the analytical sample can vary from 1.0 to 5.0 mg. with no effect on the results obtained. This range of concentration is adequate for the analysis of all cottonseed products.

Stability of Extracts and Reagents

Extracts of cottonseed meal and pure gossypol obtained by treatment with the complexing reagent are exceptionally stable. Unchanged values for total gossypol were obtained for extracts stored for as long as six days prior to colorimetric analysis. The complexing reagent itself is stable up to one week at room temperature. Reagent blanks are negligible and have been found to range from 99 to 100 transmittance. After reaction with aniline, extracts of cottonseed meals and gossypol standards exhibit a slight increase in absorbance up to 1 hr. after color development and dilution. Readings taken after periods of 1 to 5 hrs. show no significant change in absorbance. The initial change in absorbance is probably caused by a shift in equilibrium on dilution as the use of increased aniline concentration or time of reaction had no effect.

For purposes such as plant control, absorbance values of samples could be determined immediately after mixing and dilution to volume, provided that calibration factors were determined under the same conditions. This would allow a determination to be completed in 1 hr. If this technique should be used, a variation of 10 min. in reading the absorbance would result in only a 2% error in total gossypol content, almost within the precision of colorimetric measurement (2).

Extraction and Color Development

Total gossypol is completely extracted from cottonseed meal in 30 min. with the complexing reagent (Table III). Since variation in extraction time from

Effect of Variation on the Determin	s in Extraction ation of Total G	Time and Color ossypol in Cotton	Development seed Meal
Extraction		Color development	
Time of heating with complexing reagent	Total gossypol found ^a	Reaction time with aniline	Total gossypo found ^b
min.	%	min.	%
10	0.863	10	0.960
$\tilde{2}\tilde{0}$	0.945	20	0.961
30	0.956	30	0.956
45	0.964	45	0.960
60	0.960	60	0.961

^a Color developed by heating with aniline for 30 min. at 100°C. ^b 30-minute extraction period with complexing reagent.

30 to 60 min. has no effect on the results obtained, minor variation in extraction time is not critical. The reaction of complexed gossypol with aniline is complete in about 10 min., and increase of reaction time to 60 min. produces no further change. To provide a suitable safety factor and to allow adequate time for evaporation of excess solvent, when larger sample aliquots are employed, a 30-minute period is specified in the procedure.

Specificity

Extracts of typical cottonseed meals obtained by treatment with the complexing reagent all exhibit absorption maxima at 376–398 m μ , typical of the gossypol-aminopropanol complex (Figure 2). Gossypol and any derived or gossypol-like pigments in meals are apparently all converted to a single complex on treatment with 3-amino-1-propanol as specified. As previously shown in the case of pure gossypol, reaction with aniline converts the gossypol-aminopropanol complex in meal extracts to dianilinogossypol with absorption maxima at 440 m μ (Figure 2).

Application of Procedure

Application of the proposed procedure to the analysis of total gossypol in cottonseed meals gives results in substantial agreement with those obtained by use of the current A.O.C.S. method (1) and with a p-anisidine method (9) modified to include a reducing agent



during color development (6) (Table IV). Both of these procedures utilize a 6-hr. hydrolysis with oxalic acid in methyl ethyl ketone for cleavage of bound gossypol prior to colorimetric analysis. The slightly lower values obtained by the A.O.C.S. procedure are attributed to the fact that a portion of the gossypol pigments are probably oxidized during the 6-hr. hydrolysis and do not react with aniline during the color development. Addition of a reducing agent to the p-anisidine reagent (6) apparently reverses this oxidation, and the pigments are capable of reaction with the color reagent. The combination of a rapid extraction and formation of a stable complex in the proposed procedure prevents oxidation or loss of extracted gossypol.

When applied to crude oils and soapstocks, the proposed procedure yields values essentially equivalent to those obtained by use of a method (11) recommended for the analysis of total gossypol in crude cottonseed oils (Table V). Although no procedure has been specifically proposed for soapstocks, the p-anisidine method for oils has been reported to be a valid measure of total gossypol in these materials (13).

Precision

The reproducibility attainable by the procedure was evaluated by 15 analyses of a prepress-solvent meal

TABLE IV Comparison of Total Gossypol Values in Cottonseed Meals by Several Methods			
	Total gossypol		
Type of cottonseed meal	A.O.C.S. method a	p-Anisidine method ^b	Proposed method
Screw-pressed Screw-pressed Screw-pressed Hydraulic-pressed Prepress-solvent Prepress-solvent Prepress-solvent Prepress-solvent Hexane-extracted, tempered meats Hexane-extracted, tempered meats Hexane-extracted, alkali-cooked Butanone-extracted, raw meats Mean supe	% 0.69 0.82 1.24 0.90 1.21 0.71 0.80 0.85 0.90 0.85 1.03 1.16 0.35 0.88	$\% \\ 0.74 \\ 0.84 \\ 1.27 \\ 0.93 \\ 1.23 \\ 0.76 \\ 0.84 \\ 0.92 \\ 0.96 \\ 0.86 \\ 1.07 \\ 1.18 \\ 0.39 \\ 0.92 \\ 0.9$	% 0.74 0.86 1.30 0.93 1.26 0.75 0.85 0.91 0.96 0.92 1.10 1.19 0.38 0.93

^a Oxalic acid — methyl ethyl ketone hydrolysis; aniline reaction; A.O.C.S. Ba 8-55 (1). ^b Oxalic acid — methyl ethyl ketone hydrolysis; p-anisidine reagent modified to include reducing agent (6, 9).

	Total gossypol pigments		
Type of sample	p-Anisidine method ^b	Proposed method	
	%	%	
Crude oils			
Hydraulic-pressed	0.087	0.089	
Screw-pressed	0.198	0.204	
Prepress-solvent	0.314	0.311	
Pure gossypol in refined and bleached oil	0.095	0.094	
Acidulated soapstocks			
Blended-centrifugal-refined a	0.033	0.030	
Hydraulic—batch-refined	0.083	0.080	
Prepress-centrifugal-refined	0.421	0.451	
Blended-centrifugal-refined a	0.495	0.532	
Hydraulic-centrifugal-refined	0.788	0.794	
Screw-press-centrifugal-refined	1.56	1.58	

conducted over a period of several months; single analyses were performed in each instance. The average value was 0.958%, and the standard deviation ± 0.0084 . The coefficient of variation was 0.88% and compares favorably with the maximum precision attainable in conventional photometric analysis, generally considered to be about 0.5 to 1.0% of the amount present (2).

Summary

A method is proposed for the determination of total gossypol in cottonseed meals, crude oils, and soapstocks based on a rapid extraction of gossypol by neutralized 3-amino-1-propanol in dimethylformamide to form a stable complex, followed by colorimetric analysis of an aliquot of the extract by means of an aniline reaction. A determination can be completed in about 2 hrs. and with minor modification in 1 hr. compared to about 7 hrs. for current methods. Results obtained by the proposed procedure on meals, oils, and soapstocks are in essential agreement with those found by use of other accepted methods. Desirable features, such as stability of reagents and extracts and a high degree of reproducibility, suggest that the procedure will satisfy the requirements for a rapid and simplified method for the analysis of all cottonseed products for total gossypol.

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Pilot-Plant Preparation of Edible Safflower Oil

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RODUCTION of safflower seed in the United States States reached a record high in 1956 when, according to recent estimates, about 100,000 acres of safflower were planted. Increasing interest in this oilseed and its potential for adaptation in portions of the Western Great Plains and Pacific Coast States as a replacement for wheat has revived efforts to find broader fields of use for the oil.

Currently much of the safflower oil produced in this country is used by the coatings industry, principally in the manufacture of non-yellowing alkyd resin and drying oils. In recent months at least two pharmaceutical preparations containing safflower oil have appeared on the market and also some food manufacturers apparently are becoming interested in the oil. In some sections of the world safflower oil reportedly is used primarily for food purposes (1, 3). There is conflicting evidence regarding its oxidative and flavor stabilities since some investigators have found the oil to be poor in these respects (5, 6). In the tests reported here however safflower oil was found to possess extremely good edible characteristics, and its oxidative stability was considerably improved when the oil was treated with a metal scavenger and an antioxidant. Whether the differences in findings between previous reports and this report result from seed variental factors. from variations in

processing treatments, or from differences in methods of evaluation is not known.

The safflower oil used in the present tests was obtained from a commercial processor in two lots, one in early 1956 and the second about eight months later. Both lots were alkali-refined to a low free fatty acid content by the processor and were characterized as "nonbreak" oils. Several 100-pound batches from each lot were bleached in pilot-plant equipment with 1% of Super Filtrol² at 105°–110°C.(221°–230°F.) for 15 min. and filtered. The bleached oils were deodorized 4 hrs. at 218°C.(425°F.) and at 4-6 mm. of mercury absolute pressure, with about 3% of stripping steam per hour. The oils were removed from the deodorizer at 48° C.(120° F.) and stored at -18° C.(0° F.) in glass bottles. The deodorized oils were evaluated for flavor stability, using taste-panel techniques developed at this laboratory (4). Oxidative stability (A.O.M.) was evaluated for each sample by determining peroxide development after an 8-hour oxidation in the Swift stability apparatus (2).

Results

Characteristics of the two lots of safflower oil used to prepare edible oil for evaluation are given in Table I. Although the fatty acid composition of both lots

¹ One of the divisions of the Agricultural Research Service, U. S. Department of Agriculture.

² The mention in this article of commercial products or equipment or the names of their manufacturers does not constitute endorsement by the U. S. Department of Agriculture of such firms or products.