the diene given by the isomerization method. One, *Penstemon albidus* [105], contains sufficient carbonyl, hydroxyl, and unsaponifiables to explain the anomalous results obtained. The remainder present no obvious reasons for inapplicability of the analyses. In fact, some oils showing from −2% to −5% saturated acids may actually be suitable for the isomerization procedure because such results are not necessarily outside acceptable limits of precision of the method.

Conversely, routine application of the isomerization method to unknown oils may give results that are incorrect but not obviously so. For example, *Picramnia pentandra* [51] appears to have a reasonable, though unusual, composition. However the oil is solid well above room temperature, and the major component has chromatographic characteristics not of the usual monoenes but of stearolic acid. About 85% of the mixed methyl esters is probably from tariric acid, known to occur in other *Picramnia* species (2), but it behaves like stearolic acid in the equipment used.

Summary

Chemical screening of seed oils continues to reveal nature's diversity. This work provides leads to numerous species which warrant further research to investigate their oil and meal in greater detail, to appraise their crop potential, and to assess their practical value for providing new oilseeds.

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Direct Conversion of Lipid Components to Their Fatty Acid Methyl Esters[1]

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THE APPLICATION of gas-liquid chromatography (GLC) for analysis of lipids has created the need for a convenient, quantitative method for conversion of milligram quantities of lipid components to their fatty acid methyl esters. The methyl esters are more amenable to GLC analysis than the fatty acids or their higher-molecular-weight alkyl esters.

Transesterification with methanol and catalytic amounts of sodium methylate has proved to be a rapid and effective method for conversion of glycerides to methyl esters and has been employed for many years. The reaction is substantially complete when a mixture ($1:2^{W/V}$) of glycerides and methanol (containing sodium methylate in amounts equal to about 1% of the weight of the glycerides) are refluxed for 30 min. The same procedure however does not give complete methanolysis of sterol esters and phospholipids.

Little has been published on the methanolysis of sterol esters and phospholipids. Rollet (6) reported some measure of success in the methanolysis of egg lecithin when using tin or zinc to prevent resinification. Shinowara and Brown (7) were able to get good yields of methyl esters from methanolysis of phospholipids with 5–10% of dry hydrogen chloride as catalyst and reaction time of 36 hrs. Stoffel (8) recently described a micromethod for methanolysis of lipid components with methanol containing 5% of dry hydrogen chloride and 2 hrs. of reaction time; the methyl esters were volatilized from the unsaponifiable matter and collected.

This paper describes the results of further investigation of the use of methoxides in transesterification of lipids, particularly sterol esters and phospholipids. Quantitative conversion to methyl esters was obtained with excess methanol, which contained many times the amount of sodium or potassium methoxide normally used for methanolysis of glycerides. A simple chromatographic treatment on a silicic acid column was found effective in removing free sterols and other unsaponifiable matter from the methyl ester product.

Experimental

Sources of Samples. The cholesteryl esters (Tables I and II) were synthesized by the reaction of lard fatty acid chlorides with cholesterol as described (2). The lipid components (Tables III to VI), except for soybean phospholipids, were obtained as fractions in

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TABLE I

Effect of Amount and Concentration of Sodium Methoxide and Reaction Time on Yield of Methyl Esters from Cholesteryl Esters

Expt. No.	Chol. esters[a]	Sodium methoxide solution		Reflux time	Eluted fraction		Yield of methyl esters
		Amount	Normality		Weight[b]	Cholesterol	
	mg.	*mls.*		*hrs.*	*mg.*	*%*	*% theory*
1	25.7	5	0.006	1	26.0	61.1	none
2	25.7	5	0.06	3	10.1	3.7	83
3	25.7	5	0.2	3	10.8	0.6	94
4	25.7	5	0.4	3	10.5	0.0	92
5	34.7	10	0.4	½	14.8	3.4	91
6	27.0	10	0.4	1½	11.6	0.0	97

[a] Synthetic cholesteryl esters of lard fatty acids.
[b] Weight of methyl esters and unchanged cholesteryl esters eluted from silicic acid column with 300 ml. of petroleum ether containing 1% ethyl ether.

TABLE II

Comparison of Effectiveness of Sodium and Potassium Methylate on Methanolysis of Cholesteryl Esters

Expt. No.	Chol. esters[a]	Methoxide soln.		Reflux time	Eluted fraction		Yield of methyl esters
		Amount	Normality		Weight[b]	Cholesterol	
	mg.	*ml.*		*hrs.*	*mg.*	*%*	*% theory*
1	34.7	10	0.06 Na	1	19.8	30.3	64
2	30.0	10	0.06 K	1	12.7	0.7	94
3	34.7	10	0.1 Na	1½	14.0	1.0	89
4	27.0	10	0.1 K	1½	11.4	none	94
5	34.7	10	0.2 Na	½	17.1	17.8	79
6	34.7	10	0.2 K	½	16.8	11.7	88
7	34.7	10	0.4 Na	½	14.8	3.4	91
8	54.0	10	0.4 K	½	23.0	none	96

[a] Synthetic cholesteryl esters of lard fatty acids.
[b] Weight of methyl esters and unchanged cholesteryl esters eluted from silicic acid column with 300 ml. of petroleum ether containing 1% ethyl ether.

the silicic acid chromatographic separation of total lipids of tissues by the method previously described (5). The soybean phospholipid was a commercial sample of "lecithin," which was purified by precipitating three times with acetone from a petroleum ether solution. The lard was a special product rendered from pigs fed a high level of safflower oil. Sesame oil was U.S.P. grade.

Methanolysis. Principal emphasis was given to conditions which would give complete conversion of cholesteryl esters to methyl esters because these are the most difficult to transesterify. The general procedure for methanolysis was as follows. Standard solutions of sodium or potassium methoxide in absolute methanol were prepared so that aliquots added to small samples (10–50 mg.) would provide a suitable working volume as well as a large excess of methanol and methoxide. Metallic sodium or potassium was cut into small bright pieces under petroleum ether and added to a known volume of redistilled absolute methanol in amounts slightly in excess of that required for the desired normality. (Only a small piece of sodium or potassium is added at one time because of vigorous reaction.) After the addition was completed, an aliquot was titrated and the calculated amount of methanol added to adjust the normality. (Precautions should be taken at all times to keep apparatus, reagents, solvents, and sample dry and to avoid exposure of reagents to atmospheric moisture.)

A weighed sample (10–50 mg.) of lipid was added to a small (25–50 ml.) round-bottom flask, provided with a standard taper joint (19/38) and short condenser. The sample was dissolved in 5 ml. of redistilled petroleum ether (B.P. 40–55°C.). A known volume of standard methoxide solution was added by pipette, and the condenser was attached for reflux. Oxygen-free dry nitrogen was bubbled through the mixture slowly by means of a glass capillary introduced through the condenser, and the mixture was refluxed for the periods indicated in Tables I and II. To the reaction mixture while still warm 0.5 N methanol solution of sulfuric acid was added through the condenser in slight excess over the amount calculated to neutralize the methoxide. The contents were then cooled and transferred to a 100-ml. separatory funnel; two 15-ml. portions of petroleum ether were used to make the transfer. About 20 ml. of water were then added, and the mixture was shaken thoroughly. The separated aqueous layer was re-extracted with 20 ml. of petroleum ether in a second small separatory funnel.

The two petroleum ether extracts were combined and washed with several 15-ml. portions of water until the washings were neutral to Congo Red paper. The solution was then transferred to a suitably small, weighed flask, and the solvent was removed by warming on a water bath at about 45° with a gentle stream of nitrogen directed at the surface of the solution. After the solvent was evaporated, the warming under nitrogen was continued at about 70° for 15 to 20 min. in order to evaporate traces of moisture. The product consisted of methyl esters, sterols, and any other petroleum ether-soluble unsaponifiable matter present in the original sample. If the transesterification was incomplete, the product would also, of course, contain some of the original sample. The flask and contents were cooled and weighed, and the product was then ready for silicic acid column treatment for isolating the pure methyl esters, as described below.

Acid-catalyzed transesterifications, according to conditions reported by Stoffel *et al.* (8), were also tried on cholesteryl esters and phospholipids. The products obtained were washed and recovered as described above and subjected to the silicic acid column treatment.

Purification of Methyl Esters by Silicic Acid Chromatography. The methyl ester product obtained from the transesterification was further treated in order to isolate the methyl esters free of unsaponifiable or other impurities. Chromatographic adsorption on silicic acid was found effective. The silicic acid-filter aid mixture (80:20) was standardized in activity by heating 200- to 300-g. batches spread in a half-inch layer in a crystallizing dish at 100°C. for 2 hrs. in an oven and then allowed to cool in a desiccator with vent but without drying agent. Water equal to 4% of weight of the silicic acid-filter aid was added to the bottom of the

TABLE III

Yields and Iodine Values of Methyl Esters from Sterol Esters by Methanolysis and Saponification-Fatty Acid Recovery

Sterol esters source	Method	Sample wt.	Methyl esters		
			Yields[a]		Iodine values[a]
		mg.	*mg.*	*%*	
Plasma N, human	M[b]	50.1	21.4	95	140.7
	S[c]	50.1	21.1	94	143.6
Plasma D, human	M	30.3	12.4	91	142.6
	S	30.3	8.9[d]	66	143.6
Liver, rabbit	M	40.3	18.1	99	84.8
	S	40.3	17.5	96	85.0
Liver A, chick	M	46.2	19.8	95	107.2
	S	49.2	21.2	96	108.2
Liver B, chick	M	37.3	15.8	94	102.5
	S	38.1	16.0	93	103.2

[a] All yields and iodine values on methyl ester basis.
[b] Methanolysis, 10 ml. 0.1 N potassium methoxide, 1½ hrs. reflux, chromatographed.
[c] Saponification, extraction of unsaponifiables, and recovery of fatty acids.
[d] Probable loss in handling.

TABLE IV

Composition of Methyl Esetrs Obtained from Sterol Esters by Methanolysis and by Saponification-Fatty Acid Recovery

Sterol esters source	Method	Fatty acid composition						
		1=	2=	3=	4=	5=	6=	Sat.
		%	%	%	%	%	%	%
Plasma N, human	M[a]	43.3	44.9	1.7	5.5	0.4	0.4	3.7
	S[b]	41.8	46.8	1.5	5.7	0.5	0.6	3.1
Plasma D, human	M	44.6	41.3	2.1	6.9	0.7	0.6	3.7
	S	48.0	40.3	2.1	6.8	0.7	0.5	1.6
Liver, rabbit	M	61.3	12.7	2.0	1.2	0.4	0.0	22.4
	S	62.7	12.3	2.7	1.1	0.5	0.0	20.7
Liver A, chick	M	85.7	9.0	0.3	1.8	0.7	1.9	0.6
	S	87.5	8.8	0.3	1.7	0.7	1.8	(−1.1)
Liver B, chick	M	81.5	9.2	0.1	1.9	0.8	1.6	4.9
	S	79.6	9.4	0.0	2.0	0.8	2.3	5.9

[a] Methanolysis, 10 ml. 0.1 N potassium methoxide, 1½ hrs. reflux, chromatographed.
[b] Saponification, extraction of unsaponifiables, and recovery of fatty acids.

desiccator, and the contents were allowed to equilibrate at least over-night. The mixture was then transferred to a dry bottle with air-tight closure and shaken thoroughly. The column and method of packing and operation of the column were the same as described for separation of lipids (5) except that 10 g. of the adsorbent were employed for 10- to 50-mg. samples of the "crude" methyl esters. The weighed sample of crude methyl esters was transferred quantitatively to the top of the column with several small portions of petroleum ether (total 10 ml.). Then 300 ml. of petroleum ether containing 1%$^{V/V}$ of ethyl ether were added to the separatory funnel, and connections were made for operating the column under a constant pressure of nitrogen. The flow rate through the column was adjusted to about 225 ml. per hour, and the solvent level above the silicic acid was maintained at a fairly constant level until all the solvent had been added. When at least 300 ml. of eluate were obtained, all the methyl esters had been eluted; the sterols or other unsaponifiable matter had been retained on the column. If desired, the latter may be removed with more polar solvents as described (5). The solvent was distilled from the methyl esters on a steam bath to a small volume. This concentrate was then transferred with several small portions of solvent to a small, weighed tube, which was drawn to a sharp cone-shape bottom, and taken to dryness by warming under a gentle stream of oxygen-free nitrogen until constant in weight. An alternative technique would be to transfer the concentrate to a small, weighed flask in similar manner, remove the solvent to constant weight, and make up to known volume with solvent so that aliquots may be taken for analyses. With care in the application of solvent and in solvent evaporation, the small amount of ester can be collected in the small cone, thus permitting convenient use of a microsyringe in removing minute amounts for gas-liquid chromatography. The tube containing methyl ester should be stored at low temperature under nitrogen until all analyses are completed.

Direct Esterification of Fatty Acids. Comparisons were made of yield and analysis of methyl esters obtained by methanolysis with those of fatty acids obtained by the well-known, time-honored method of saponification, extraction of unsaponifiables, and recovery of fatty acids. In some instances the fatty acids so obtained were esterified by one of two procedures as follows.

Acid-Catalyzed Esterification. Ten ml. of absolute methanol containing either 4% dry HCl or 4% concentrated H_2SO_4 (96%) were added to 10- to 100-mg. quantities of fatty acids in a 25-ml. round-bottom flask, fitted with standard taper 19/38 joint and condenser. The mixture was refluxed for 1 hr., cooled, and transferred with three 10-ml. portions of petroleum ether to a small separatory funnel. An equal volume of cold water was added, and the extraction, washing, and removal of solvent from the methyl esters were performed as described for the product obtained by methanolysis.

Reaction with Diazomethane. It was found convenient to carry out the reaction of milligram quantities of fatty acids with diazomethane in a small glass tube, 60 mm. x 15 mm. O.D., the bottom of which was drawn to a point, thus giving a cone. This was advantageous in that the methyl esters settled in the cone and could be taken up, without additional handling, with syringe needle for gas-liquid chromatographic analysis. Procedure for making the methyl esters was as follows. About 10–20 mg. of fatty acids were weighed in the glass tube. An ether solution of diazomethane (usually about 4 ml. containing at least 300% excess over theory) was added, and the solution was allowed to stand in the hood for 1 hr., loosely covered with an inverted beaker. A slow stream of nitrogen was then led into the tube to evaporate any ether or reagent remaining. The methyl esters were then ready for analysis. The ether solution of diazomethane was prepared from nitrosomethylurea as described in "Organic Syntheses" (4).

Methods of Analysis. The Wijs method, as modified for small samples (5), was used to determine iodine values. Polyunsaturated acids were determined by a spectrophotometric method (5), and sterols by the method of Luddy *et al.* (3). Analyses by gas-liquid chromatography were performed with apparatus, columns, and conditions essentially the same as reported by Herb *et al.* (1).

Results and Discussion

The data in Table I show that the amount of methanol or methanol solution of sodium methoxide as well as the concentration of methoxide and time of reaction are important in the transesterification of cholesteryl esters. In Experiments No. 4 and No. 6 no unchanged cholesteryl esters were detected in the product which was eluted from the silicic acid column with 300 ml. of the solvent. In this column treatment methyl esters and cholesteryl esters are removed together; the free sterol and free acids, if any, are retained on the column. The latter can be removed with more polar solvents. Examination of this retained material in Experiment No. 4 showed the presence of 1.5 mg. of free acid calculated as oleic acid whereas none was found in Experiment No. 6. The longer reaction time in Experiment No. 4 probably permitted traces of atmospheric moisture to be absorbed, which hydrolyzed a small amount of ester.

Potassium methoxide, as might be expected, was more active than sodium methoxide and produced greater conversion under comparable conditions, as shown in Table II. These data obtained with synthetic

cholesteryl esters of lard fatty acids suggest that the conditions in Experiments No. 4 and 6 (Table I) and Experiments No. 4 and 8 (Table II) would be satisfactory for the conversion of small amounts of cholesteryl esters of fatty acids to their corresponding methyl esters.

Methanolysis of cholesteryl esters, catalyzed with dry HCL or H_2SO_4 under conditions comparable to those used by Stoffel (8), resulted in yields of methyl esters 92–93% of theory, but the product contained about 4% of unchanged cholesteryl esters.

Further work was carried out on cholesteryl esters isolated from tissue lipids by a chromatographic method (5). Comparison of yields, iodine values, and fatty acid compositions were made on the methyl esters obtained by transesterification under conditions of Experiment No. 4 (Table II) with values on fatty acids (calculated to methyl ester basis) isolated by the more laborious procedure of saponification, removal of unsaponifiables, and extraction of fatty acids. These data are shown in Tables III and IV. It is apparent that the yields of methyl esters by transesterification are fully comparable, if not generally the greater. Likewise no significant differences in composition were detected in the comparative series.

TABLE V

Yields and Iodine Values of Methyl Esters from Phospholipids by Methanolysis and by Saponification-Fatty Acid Recovery

Phospholipid source	Method	Methyl esters	
		Yield[a]	Iodine value[a]
		mg./100 mg. sample	
Plasma M, human	M[b]	51.2	87.2
	S[c]	50.0	88.2
Cells M, human	M	37.9	116.7
	S	33.5	114.0
Liver L, chick	M	57.0	141.5
	S	52.3	138.8
Liver, rabbit	M	50.0	103.4
	S	43.6	100.0
Soybean	M	60.4	108.2
	S	62.8	106.0

[a] All yields and iodine values on methyl ester basis.
[b] Methanolysis, 10 ml. 0.1 N potassium methoxide, 1½ hrs. reflux, chromatographed.
[c] Saponification, extraction of unsaponifiables, and recovery of fatty acids.

Similar studies were conducted on methanolysis of phospholipids, and comparisons were made with yields and analysis of the fatty acids obtained from portions of the same materials which were saponified, the fatty acids recovered and analyzed. These data (Tables V and VI) show that the yields of methyl esters were generally somewhat greater by methanolysis, followed by silicic acid column purification. The analysis of fatty acid compositions of preparations made by either procedure were in good agreement.

The conversion of glycerides to methyl esters by employing excess methanol and catalytic amounts of sodium methoxide is known to be readily accomplished. For completeness of this investigation however several samples of glyceride fats and oils were subjected to the conditions for methanolysis described by Experiment No. 4 (Table II). The yields of methyl esters were 96% of the weight of fat or oil sample. Almost identical yields were obtained by saponification and fatty acid recovery when calculated to the methyl ester basis. No differences in fatty acid composition of the two preparations were found. It was observed that the methanolysis of glyceride fats and oils under the conditions mentioned proceeded quite rapidly, and it seemed probable that for these fats the time of reaction could be shortened considerably and complete conversion to methyl esters still obtained. If so, the method would be more valuable, particularly for the routine analysis of commercial fats and oils by gas-liquid chromatography.

To test this, a much larger sample than used in above experiments was subjected to methanolysis as follows. Two-gram samples of U.S.P. sesame oil and 50 ml. of 0.2 N methanol solution of potassium methoxide were refluxed for 5 min. and 15 min. and the products were isolated by the general procedure already described. The yields of water-white methyl esters after the silicic acid column treatment were 98% of theory for either reaction-time. No free acids were present in these materials as determined by titration. Gas-liquid chromatographic analysis showed no differences in composition of the two preparations or of methyl esters of the same oil prepared by a different procedure (8). For refined glyceride fats and oils of low free fatty acid and unsaponifiable content, the column treatment probably would be unnecessary. Without column treatment but with reagents ready, the time required to prepare methyl esters from sesame oil free of solvent and ready for analysis by gas-liquid chromatography was about 30 min. whereas the silicic acid column treatment including removal of solvent from the eluted fraction requires about 2 hrs. However, since several columns can be operated concurrently for different samples, the time charged per sample would be substantially reduced.

The methyl esters prepared by the 5-min. reflux time were distilled without column at 140° pot temperature and 0.1 mm. of pressure, leaving 16 mg. of undistilled material in the flask. This material was analyzed by gas-liquid chromatography and consisted of methyl esters of the C_{18} acids of sesame oil. No glycerides were detected by infrared absorption spectra. It is concluded that the conversion of glycerides to methyl esters was complete in about 5 min. of reflux time.

Direct esterification of fatty acids with absolute methanol and HCL or H_2SO_4 as catalyst, or methylation with diazomethane as described, gave quantitative yields of methyl esters. No free fatty acids could be detected by titration. Examination for conjugation by ultraviolet spectroscopy showed only slight increases in absorptivity in the diene and triene regions over that found in the original samples of fatty acids. The increase in absorptivity for the acid-catalyzed esterification product amounted to less than 0.1% calculated as linoleic and linolenic acids; for the product made by methylation with diazomethane, about 0.2%.

Summary

Methyl esters were prepared from cholesteryl esters, phospholipids, and glycerides in substantially quantitative yields by methanolysis with large excess of sodium or potassium methoxide in absolute methanol.

A silicic acid chromatographic adsorption column technique was described, which was effective in separating methyl esters from unsaponifiables such as sterols, pigments, etc., and free acids.

Conditions for complete methanolysis of glyceride

TABLE VI

Composition of Methyl Esters Obtained from Phospholipids by Methanolysis and by Saponification-Fatty Acid Recovery

Phospholipids source	Method	Fatty acid composition						
		1=	2=	3=	4=	5=	6=	Sat.
		%	%	%	%	%	%	%
Plasma M, human	M[a]	37.8	11.6	3.1	4.5	1.1	2.0	39.9
	S[b]	40.6	11.0	3.2	4.4	0.9	1.7	39.1
Cells M, human	M	22.6	10.0	2.9	16.3	2.5	2.4	43.3
	S	23.7	9.5	2.8	14.9	2.6	2.8	43.7
Liver L. chick	M	11.0	21.1	2.5	21.5	2.4	2.4	39.1
	S	9.9	21.4	2.6	20.9	2.3	2.4	41.5
Liver, rabbit	M	19.6	29.0	2.1	7.5	1.2	0.8	40.0
	S	22.6	28.9	2.2	6.5	1.0	0.7	38.0
Soybean	M	14.4	42.8	8.5	0.0	0.0	0.0	34.3
	S	19.4	41.5	6.7	0.0	0.0	0.0	32.4

[a] Methanolysis, 10 ml. 0.1 N potassium methoxide, 1½ hrs. reflux, chromatographed.
[b] Saponification, extraction of unsaponifiables, and recovery of fatty acids.

fats and oils requiring only 5 min. of reflux time were described.

Quantitative conversion of fatty acids to methyl esters was accomplished by direct esterification with absolute methanol containing 4% HCL or H_2SO_4 and by methylation with diazomethane.

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Changes in Iodine Value and Refractive Index of Fatty Acids During Alkyd Resin Manufacture and Analysis

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IN MANY specifications for alkyd resins, limits are placed on the iodine value and refractive index of the separated fatty acids. Values obtained by various laboratories are not always in agreement. No work is known in which changes in iodine value and refractive index of fatty acids during alkyd resin manufacture and analysis have been studied and reported. This paper is a report of an investigation undertaken to determine the effects of alkyd resin manufacture and analysis upon the iodine value and the refractive index of fatty acids.

Experimental

Materials. One lot each of commercial linseed fatty acids, soya fatty acids, and low rosin (1%) tall oil fatty acids was selected. All tests and alkyd resins were made with these fatty acids.

Comparison of ASTM Methods and Federal Test Method Standard Number 141. The iodine value and refractive index of each lot of fatty acids were determined according to ASTM method D555-54 (1). A sample of each fatty acid was then passed through a phthalic anhydride determination and a fatty acid separation according to ASTM method D1398-56T (2). The iodine value and refractive index of each separated acid fraction were determined by means of ASTM method D555-54 (1). Each determination was repeated according to Federal Test Method Standard Number 141 (3,4,5,).

Preparation of Alkyd Resins. Alkyd resins were processed in a 2-gal. stainless steel kettle equipped with an agitator. Each resin was prepared with an input of 52.6 parts of fatty acids, 21.7 parts of glycerol, and 33.1 parts of phthalic anhydride. All resins were solvent-processed in xylol. Approximately 4% of xylol, based on resin solids, was present in resins after processing. Two resins were prepared with each lot of fatty acids. One of these resins was processed at 400°F.; the other resin was processed at 450°F. After the processing temperature had been reached, a sample was removed from each resin each hour for a period of 9 hrs.

Analysis of Alkyd Resins. Preliminary analyses revealed that changes in iodine values and refractive indexes of fatty acids during alkyd resin processing were slight. Therefore only the samples removed at intervals of 2 hrs., 5 hrs., and 9 hrs. were analyzed. Each of the samples was saponified, the dipotassium salts were removed by filtration, and the fatty acids were separated according to ASTM method D1398-56T(2). Iodine values and refractive indexes of the separated fatty acids were determined by means of ASTM method D555-54.

Results and Discussion

Effects of Analysis. The results obtained by comparing ASTM and Federal methods of analyses are listed in Table I. No significant changes in either