TABLE III
Comparison Between Fatty-acid Composition of Pasture Lipids and of Depot Fats of Pasture-fed Animals

Source of fat	% Fatty acids										% Trans-acids
	Saturated				Unsaturated						
							C_{18}				
	C_{12}–C_{14}	C_{16}	C_{18}	C_{20}	C_{12}–C_{14}	C_{16}	Monoene	Diene	Triene	C_{20}	
Pasture (ryegrass)	1.8	10.6	1.5	0.4	0.7	4.1	4.6	11.6	62.8	1.9	trace
Wild rabbit	1.6	22.1	6.4	0.8	0.4	4.4	12.7	7.9	42.4	1.3	nil
Horse	2.4	29.7	4.3	0.2	1.4	6.5	32.5	3.8	16.1	3.1	nil
Ox	2.7	27.8	21.6	....	0.3	2.5	42.5	0.5	0.3	1.8	4.5
Sheep	3.8	25.0	22.2	0.7	0.5	1.7	44.2	trace	trace	0.9	11.2
Rumen contents of sheep	1.2	16.9	48.5	5.8	0.2	1.8	19.2	3.5	2.9	....	10.9

ruminants. Mechanisms of the formation of all these constituents based on the activity of rumen bacteria are suggested.

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Identification of Some Marine Oil Constituents by Chromatography[1]

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OF ALL THE LIPIDE MIXTURES of natural origin, marine oils are the most complex and the most difficult to analyze. Among the constituent lipides of marine oils are the fatty alcohols, ether-alcohols, hydrocarbons, waxes, triglycerides, various phospholipides, and vitamins A, D, and E. Present methods for the determination of these lipides require large amounts of material and are laborious, time-consuming, and uncertain. For the most part these procedures are modifications of methods used for the analyses of other fats and oils and are not very efficient even for their original purposes.

A method has been devised by Dieckert and Reiser (1) for the separation and identification of μg. quantities of various lipides on glass-fiber filter paper impregnated with silicic acid. The present study was designed to determine whether that method could be used to advantage in separating and identifying the lipide constituents of marine oils. The paper method is incomparably simpler than other methods where analysis rather than isolation is the objective.

Experimental

Reference compounds. The following compounds, dissolved in A.C.S. chloroform, 1 mg. per ml., were used as reference: winterized cottonseed oil[2] (for triglycerides), vitamin A alcohol, vitamin A palmitate, 7-dehydrocholesterol, cholesterol, vitamin D_3, hexadecanol, cholesteryl palmitate, hexadecyl acetate, hexadecyl palmitate, d-α-tocopherol, d-α-tocopheryl acetate, and squalene.

Solvent systems. The solvent systems used were: cyclohexane; 1% methanol in cyclohexane, v/v; 2% ethyl ether in iso-octane, v/v; 2 g. iodine in 2% ethyl ether in iso-octane, v/v; 1%, 4%, 10%, and 50% ethyl ether in petroleum ether,[3] v/v; 25% and 50% methanol in ethyl ether, v/v. The cyclohexane was technical grade; the methanol and ethyl ether were A.C.S. grade; the iso-octane was rectified by passage through a silica gel column; and the petroleum ether[3] was tested for peroxides before use.

Column. Silicic acid, $SiO_2 \cdot nH_2O$, powder, Baker reagent grade, was used in a column for the preliminary separations.

Paper. Glass-fiber filter paper[4] was impregnated with silicic acid for the paper chromatography by the method of Dieckert and Reiser (1, 3).

Spray reagent. Aqueous sulfuric acid reagent, 50%, v/v, was used as a spray with subsequent heating to char the lipide spots and make them visible on the paper.

Special reagents. Choline containing lipides were identified by the Dragendorff reagent spot test (5).

[1] Supported, in part, by grants from the Fish and Wildlife Service of the United States Department of the Interior, the Robert A. Welch Foundation, and the Office of Naval Research.
[2] Wesson Oil, Wesson Oil and Snowdrift Sales Company, New Orleans, La.
[3] Commercial n-hexane, Skellysolve B, b.p. 60°–70°C., Skelly Oil Company, Kansas City, Mo.
[4] No. X-934-AH, heavy weight, H. Reeve Angel and Company, 52 Duane street, New York, N. Y.

Amino lipides were detected by use of the ninhydrin spot test (5).

Heat source. A glass heating-panel [5] with temperature of 340°C. was used for heating the chromatograms.

Oil samples. The marine oils used were one sample of menhaden oil [6] and two of cod-liver oil.[7,8]

Procedures. The R_f values for the reference compounds were first determined on the silicic acid impregnated glass-fiber filter paper with different solvent systems (1, 3), each value being the average of at least five tests. On the basis of these R_f values a scheme was developed for separating the components of artificial mixtures of the compounds and of marine oils occurring in nature.

Because of the large proportion of triglycerides occurring in marine oils, the mixture was first separated on a column into five groups with five solvent systems, according to the method of Fillerup and Mead (2). Then 10 g. of silicic acid were transferred to a 15-mm. chromatograph tube and packed to a height of approximately 9 cm. by tapping. Three-column volumes of methanol were added and allowed to run at atmospheric pressure. After the column became wet, 4–5 lbs. of nitrogen pressure were applied. This pressure was maintained during all washings and elutions. The column was washed with 3-column volumes each of methanol, acetone, ethyl ether, and petroleum ether.[3] Next 50 mg. of the sample in 5 ml. of petroleum ether [3] were placed on the column, followed by an additional 5 ml. of petroleum ether.[3] After the sample was added, it was eluted with 200 ml. each of 1%, 4%, 10%, and 50% ethyl ether in petroleum ether [3] and 150 ml. of 25% methanol in ethyl ether.

The eluates were collected in 20- to 50-ml. volumes, each concentrated and chromatographed on the silicic acid paper with cyclohexane, 1% methanol in cyclohexane, and 2% ethyl ether in iso-octane. In this manner minor constituents could be detected which might otherwise have been overshadowed or lost. Each eluting solvent was discontinued where no lipide was found in the eluate.

A certain amount of variation, more pronounced with some of the reference compounds than others, made absolute R_f values unreliable. For this reason, in the identification of unknown components, reference compounds were chromatographed simultaneously.

Ascending chromatography was the method employed and, except for the initial determinations of reference R_f values, a great portion of the work was done in one- and two-quart jars with No. 1 Whatman filter paper lining the inside of the jars. With these jars, the rapid movement of the solvents used, and the small strips of paper a period of equilibration appeared to be unnecessary.

The similarity of R_f values for vitamin D_3 and cholesterol in all solvents tried presented a problem which was solved by the addition of iodine to the iso-octane ether solvent system. Vitamin D_3 remained at the point of origin while the R_f value for cholesterol was 0.30.

Attempts were made to make the chromatogram quantitative, as well as qualitative, by measuring the size and density of the charred lipide spots by the use of densitometers. Various substances were used to rectify the glass-fiber filter paper and minimize the irregularity in density. These included a silicone water-repellent spray,[9] a plastic spray,[10] collodion, light cedarwood oil, the plastic spray [9] over light cedarwood oil, and glycerine.

Both reflection and transmission densitometers were used. The transmission densitometer was modified by removing the lens and replacing the aperture discs with black plastic tape to increase the aperture width to 7–8 mm. Density measurements were made of the paper with no spots. Five, 10, and 15 μg. quantities of fat were measured and placed on the paper with a micro-pipette, sprayed, and charred. Density measurements were made of the paper and the spots. Similarly measurements were made of 5 μg. of fat chromatographed with 2% ether in iso-octane.

Results and Discussion

Table I shows the R_f values on silicic acid impregnated glass-fiber filter paper of lipides found in marine oils. The R_f values for d-α-tocopherol and d-α-tocopheryl acetate were so nearly the same that their separation was not effected by this scheme. Table II shows the results of the combined column and paper separation and identification of an artificial mixture of the reference compounds. Table III shows the results of the combined column and paper separation and identification of the components of three samples of marine oil.

TABLE I

R_f Values on Silicic Acid Impregnated Glass-Fiber Filter Paper of Lipides Found in Marine Oils

Compound	Cyclohexane	Cyclohexane-1% methanol	Iso-octane-2%-ether
Winterized cottonseed oil[a]	0.00	0.89	0.65
Vitamin A alcohol	0.00	0.00	0.00
Vitamin A palmitate	0.00	0.87	0.46
7-Dehydrocholesterol	0.04	0.48	0.38[b]
Vitamin D_3	0.07	0.74	0.55
Cholesterol	0.08	0.69	0.44
Hexadecanol	0.16	0.64	0.65
Cholesteryl palmitate	0.63	0.93	0.90
Hexadecyl palmitate	0.61	0.91	0.93
Squalene	0.92	0.90	0.94
d-α-Tocopherol	0.42	0.91	0.79
d-α-Tocopheryl acetate	0.32	0.91	0.83

[a] Wesson Oil, Wesson Oil and Snowdrift Sales Company, New Orleans, La.

[b] In 2% ether in iso-octane, 7-dehydrocholesterol showed two R_f values, 0.00 and 0.38.

Of the five fractions obtained by the column separation, the first three contained all the lipides listed under reference compounds; the fourth, according to Fillerup and Mead (2), contained the fatty acids, and the fifth, the phospholipides. This study is not concerned with the identities of the fatty acid moieties. The first fraction contained vitamin A palmitate, cholesteryl palmitate, squalene, hexadecyl palmitate, and the tocopherols. All triglycerides came through in the second fraction with a part of the aliphatic alcohol. The remainder of the latter appeared in fraction three along with vitamin A alcohol, cholesterol, and vitamin D_3. The 7-dehydrocholesterol, according to preliminary tests, should have appeared in the third fraction. However it was not recovered from the mixture containing all the

[5] Corning heating panel, Webster Bros. Company, 1436 West Fullerton avenue, Chicago, Ill.

[6] Fish and Wildlife Service, Sample No. CPOG1–3A65.

[7] Super D cod-liver oil, Upjohn and Company, Kalamazoo, Mich.

[8] Cod-liver oil, "Cold Process," prepared by enzymatic digestion, Silmo Chemical Corporation, Vineland, N. J.

[9] Fumol, Fumol Corporation, Long Island City, N. Y.

[10] Krylon, the Post Company, 1215 Capitol Avenue, Houston, Tex.

TABLE II

Combined Column and Paper Separation and Identification of Components in an Artificial Mixture

Column fraction	Solvent	Amount of eluate	Mixture[a] (identified on paper)
1a	1% Ethyl ether in "Skelly B"[b]	30 ml.	Vitamin A palmitate, hexadecyl acetate,
1b	1% Ethyl ether in "Skelly B"[b]	20 ml.	hexadecyl palmitate.
1c	1% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A palmitate, cholesteryl acetate,
1d	1% Ethyl ether in "Skelly B"[b]	20 ml.	tocopheryl acetate.
1e	1% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A palmitate, tocopheryl acetate,
1f	1% Ethyl ether in "Skelly B"[b]	20 ml.	tocopherol, cholesteryl acetate, squalene
1g	1% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A palmitate, tocopheryl acetate,
1h	1% Ethyl ether in "Skelly B"[b]	20 ml.	tocopherol.
1i	1% Ethyl ether in "Skelly B"[b]	30 ml.	
2a	4% Ethyl ether in "Skelly B"[b]	30 ml.	Winterized cottonseed oil.[c]
2b	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil.[c]
2c	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil,[c] hexadecanol.
2d	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil,[c] hexadecanol.
2e	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil,[c] hexadecanol.
2f	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil,[c] hexadecanol.
2g	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil,[c] hexadecanol.
2h	4% Ethyl ether in "Skelly B"[b]	20 ml.	Winterized cottonseed oil,[c] hexadecanol.
2i	4% Ethyl ether in "Skelly B"[b]	30 ml.	Winterized cottonseed oil,[c] hexadecanol.
3a	10% Ethyl ether in "Skelly B"[b]	20 ml.	Hexadecanol, Vitamin A alcohol.
3b	10% Ethyl ether in "Skelly B"[b]	20 ml.	Hexadecanol, Vitamin A alcohol.
3c	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A alcohol, cholesterol.
3d	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A alcohol, cholesterol.
3e	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A alcohol, cholesterol.
3f	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A alcohol, cholesterol.
3g	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A alcohol, cholesterol.
3h	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin A alcohol, cholesterol.
3i	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin D_3, cholesterol.
3j	10% Ethyl ether in "Skelly B"[b]	20 ml.	Vitamin D_3, cholesterol.

[a] 7-Dehydrocholesterol was the only component of the mixture impossible to recover and identify.
[b] Commercial n-hexane, Skellysolve B, b.p. 60°–70°C., Skelly Oil Company, Kansas City, Mo.
[c] Wesson oil, Wesson Oil and Snowdrift Sales Company, New Orleans, La.

reference compounds. The fifth fraction contained the phospholipides, reference values for which are reported by Dieckert and Reiser (3). In the oil samples studied, phosphatidylethanolamine was the only phospholipide demonstrable. Ninhydrin tests for phosphatidylethanolamine were much less sensitive than the chromatograms. Tests with Dragendorff's reagent for lecithin were negative.

Vitamin A alcohol and vitamin D_3 seemed to undergo some change during the process of separation and concentration which made them difficult to demonstrate consistently in the natural oils. "Super D" cod-liver oil[7] showed spots corresponding to triglycerides, vitamin D_3, vitamin A alcohol, phosphatidylethanolamine, and an unidentified component. Silmo cod-liver oil[8] and menhaden oil chromatograms indicated the presence of squalene, triglycerides, vitamin A alcohol and vitamin D_3, and phosphatidylethanolamine.

Most of the substances used to rectify the paper for density measurements darkened the paper and were unsatisfactory after drying. Glycerine gave

TABLE III

Combined Column and Paper Separation and Identification of Components of Marine Oil

Column fraction	Solvent	Silmo cod-liver oil[a]	Menhaden oil[b]	Super D cod-liver oil[c]
1a	1% Ethyl ether in "Skelly B"[d]	Negative	Squalene (trace)	Negative
1b	1% Ethyl ether in "Skelly B"[d]	Negative	Squalene (trace)	Negative
1c	1% Ethyl ether in "Skelly B"[d]	Negative	Squalene (trace)	Negative
1d	1% Ethyl ether in "Skelly B"[d]	Negative	Squalene (trace)	Negative
1a, b, c, d combined	.....	Squalene	Squalene	Negative
2a	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Triglycerides
2b	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Triglycerides
2c	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Triglycerides
2d	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Triglycerides
2e	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Negative
2f	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Negative
2g	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Negative
2h	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Negative
2i	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Negative
2j	4% Ethyl ether in "Skelly B"[d]	Triglycerides	Triglycerides	Negative
2e, f, g combined	.....	.....	.....	Triglycerides
3a	10% Ethyl ether in "Skelly B"[d]	Negative	Vitamin D_3	Vitamins D_3 and A alc.
3b	10% Ethyl ether in "Skelly B"[d]	Vitamins D_3, A alc.	Vitamin D_3	Vitamin A alc.; unidentified comp.
3c	10% Ethyl ether in "Skelly B"[d]	Vitamins D_3, A alc.	Vitamins D_3, A alc.	Vitamin A alc.; unidentified comp.
3d	10% Ethyl ether in "Skelly B"[d]	Vitamins D_3, A alc.	Vitamins D_3, A alc.	Vitamin A alc.; unidentified comp.
3e	10% Ethyl ether in "Skelly B"[d]	Vitamins D_3, A alc.	Vitamins D_3, A alc.	Vitamin A alc.
3f	10% Ethyl ether in "Skelly B"[d]	Vitamins D_3, A alc.	Vitamins D_3, A alc.	.....
3g	10% Ethyl ether in "Skelly B"[d]	Vitamins D_3, A alc.	Vitamins D_3, A alc.	.....
4a	50% Ethyl ether in "Skelly B"[d]	Not chromatographed	Not chromatographed	Not chromatographed
5a	25% MeOH in ethyl ether	Phosphatidylethanolamine	Phosphatidylethanolamine	Phosphatidylethanolamine

1% Ethyl ether in petroleum ether[d]—total 200 ml.
4% Ethyl ether in petroleum ether[d]—total 200 ml.
10% Ethyl ether in petroleum ether[d]—total 200 ml.
50% Ethyl ether in petroleum ether[d]—total 150 ml.
25% Methanol in ethyl ether—total 150 ml.

[a] See footnote no. 8. [b] See footnote no. 6. [c] See footnote no. 7. [d] Commercial n-hexane, Skellysolve B, b.p. 60°–70°C., Skelly Oil Company, Kansas City, Mo.

better results than the others, but the wet paper was difficult to handle. The lipide spots were too large to be measured with the reflection densitometer. With the transmission densitometer, differences between portions of the paper were often as great as or greater than the differences between the paper alone and the charred spots. The spots from a chromatogram were irregular, and their variation in size and shape made their densities impossible to measure accurately with this method.

Summary and Conclusion

A scheme for the separation and identification of some constituents of marine oils was developed. A preliminary separation on a silicic acid column with five solvent systems was followed by further separation and identification on silicic acid impregnated glass-fiber filter paper. This method can be used successfully for qualitative determinations, but the irregularities in the density of the glass paper prevent an accurate quantitative assay.

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A Polarographic Investigation of the Kinetics of Epoxidation of Unsaturated Fatty Acid Esters[1]

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IN A PREVIOUS PAPER (3) we reported that the second order specific reaction constants for the perbenzoic acid epoxidation of vinyl oleate and vinyl laurate at 30°C. were, respectively, 1580×10^{-3} and 7.13×10^{-3} l./mole/min. The specific reaction rate constants for the corresponding reactions with peracetic acid would be of greater interest because of the commercial use of peracetic acid for epoxidation. However this kinetic study was precluded by the interference of hydrogen peroxide and diacetyl peroxide in the analysis of commercial peracetic acid. Since our earlier work however the homologous long-chain aliphatic peracids have been synthesized in pure form (2). Specific reaction rate constants obtained, using such an acid, would be expected to be closely related to those from peracetic acid. Furthermore a non-aqueous polarographic technique has been devised (2) which specifically measures peracid content, making valid kinetic data taken even at high conversions. A redetermination of our previously reported results, using perlauric acid, was therefore desirable.

Experimental

Apparatus

A Sargent[4] Model XXI polarograph was used to obtain the current-voltage curves. The capillary had m and t values of 3.13 mg. per second and 1.5 seconds, respectively, yielding a capillary constant of 2.29 $mg.^{2/3}$ $sec.^{-1/2}$ These values were obtained by using an open circuit with the capillary dipping into the electrolytic solution maintained at 25 ± 0.1°C. The electrolytic solution consisted of 0.25 M ammonium acetate in glacial acetic acid. The polarographic behavior of perlauric acid has been described in a previous publication (2).

[1] Presented at the Fall Meeting of the American Oil Chemists' Society, Philadelphia, Pa., Oct. 10, 1955.
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[3] A laboratory of the Eastern Utilization Research Branch, Agricultural Research Service, United States Department of Agriculture.
[4] Mention of a specific trade name does not imply endorsement by the United States Department of Agriculture over similar products not mentioned.

Materials Used

Perlauric acid was prepared by the method of Parker (2) from distilled lauric acid, m.p. 43–44°C.

Methyl laurate, $n_D^{30.4}$ 1.4275, sap. no. 214.5 (theory 214.3), was prepared by the acid-catalyzed esterification of lauric acid with methanol, followed by fractional distillation of the crude methyl laurate.

Vinyl laurate and *vinyl oleate* were prepared by the procedure of Swern and Jordan (4).

Methyl oleate was prepared by the procedure of Knight (1).

Ethyl epoxystearate was prepared by the peracetic acid epoxidation of ethyl oleate and was crystallized from methanol. It had an oxirane oxygen content of 4.93 (theory 4.91), n_D^{30} 1.4460.

Analytical Procedure

The epoxidation reactions were performed by mixing benzene solutions of weighed quantities of perlauric acid and the fatty acid ester and diluting with benzene so that the final concentrations were about 0.1 molar for each reactant. The solution was immediately transferred to the reaction cell maintained at the temperature of the investigation (15°, 25°, or $35° \pm 0.1$°C.). Two-ml. samples were pipetted from the mixture and transferred to the polarographic H-cell containing 50 ml. of the glacial acetic acid-ammonium acetate electrolytic solution. The H-cell was then sealed and degassed for 10 min. with nitrogen to remove dissolved oxygen. At the end of this period a polarogram was recorded. It was found that, because of the extreme dilution of the sample in the H-cell ($10^{-4}M$), there was negligible reaction between the peracid and the unsaturated fatty acid ester during the time required for degassing and recording the polarograms.

Because the polarographic waves of the peracids yield abnormally high maxima, it was necessary to work at low concentrations. Sample sizes were chosen so that the final concentration of the peracid in the H-cell was about $4 \times 10^{-3}M$. At these low concentra-