STUDIES OF THE UNSAPONIFIABLE FRACTION OF LIPINS DERIVED FROM MARINE SOURCES

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M.L. KARNOVSKY, M.Sc.(Rand.), A.R.I.C.

Presented in the University of Cape Town
for the Degree of Doctor of Philosophy

Department of Chemistry
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INTRODUCTION

Unsaponifiable matter

Cocks[1], in a report on the Determination of Unsaponifiable Matter in Oils and Fats, has defined this fraction as follows :-

> "Unsaponifiable matter consists of that material present in oils and fats which, after saponification of the oil or fat by caustic alkali and extraction by the solvent specified ..... remains non-volatile on drying at 80° C."

The saponification of fats has in general, for laboratory purposes, been carried out with potassium hydroxide, since potassium soaps are suitably soluble. Ethyl alcohol has been the usual saponification medium, in order to obtain homogeneous reaction mixtures, and to avoid emulsions as far as possible during the extraction.

The extraction has then been carried out, usually on the diluted saponification mixture, using petroleum ether, ethyl ether or some similar solvent, and the unsaponifiable fraction has been obtained, after suitable washing of the solutions, by evaporating off the solvent. Such extractions, when carried out on bulk samples of fat, may prove troublesome, due both to the formation of emulsions mentioned above, and the necessity of using very large volumes of materials for the extraction of the usually rather small amount of unsaponifiable matter present in most oils. Continuous extractors, such as that described by Hilditch[2a] have been devised to obviate these difficulties.

The estimation of the unsaponifiable matter content of fats and oils is a process involving the quantitative isolation of that fraction from a weighed amount of fat, and has been carried out according to several defined techniques. Such techniques specify the weights of material to be used, the nature of the solvent (usually petroleum ether, or ethyl ether) and other experimental details of the estimation. The principal method using petroleum ether is that of the American Chemical Society, or F.A.C. method[3], in which an extended number of extractions are necessary in order to obtain complete removal of the unsaponifiable fraction from the saponification mixture. Several methods using ethyl ether have been proposed, of which the main ones are those of Kerr and Sorber[4] and of the Society of Public Analysts[1].

Experience has shown that the use of petroleum ether often leads to incomplete recovery of unsaponifiable matter[5], and in fact this solvent has been used in cases where preferential removal of certain constituents of the unsaponifiable fraction was desired[5f]. Though ethyl ether is a more suitable extraction solvent than petroleum ether, its use was found to lead to complications both in the extraction and in subsequent washing procedures, except within a limited range of volume relationships[5e]. Comparisons have been made between the use of petroleum ether and ethyl ether[6], and the changes of policy of the Association of Official Agricultural Chemists with regard to the selection of an official standard method for the estimation of unsaponifiable matter during the years around 1925, reflect the difficulties that have been encountered in the

establishment of a quantitative technique for the isolation and estimation of that fraction.

In 1935, the Society of Public Analysts described a method[1] using ethyl ether as the solvent, that has proved the most efficient technique available. Comparison of this method with others by Kirsten[7] and the experience of other workers[7c] have shown it to be most reliable and rapid in the majority of cases, minimising as it does the risk of emulsions during extraction, and the presence of soaps or free fatty acids in the unsaponifiable matter isolated. The general trend both in England and America is towards the adoption of this method as routine laboratory practice. (See Appendix A)

With regard to the efficiency of the S.P.A. method as applied to marine oils, Kirsten[7] has reported more complete extraction of unsaponifiable fractions from the saponification mixtures of these fats than is obtained by other methods, but both in the original description[1] and in later papers[8] reference has been made to difficulties experienced with those materials.

The Unsaponifiable Matter of Marine Fats

Marine animal fats, especially the liver oils of fish, have been found to differ from land animal and vegetable fats in that they contain larger proportions of unsaponifiable matter. The following types of compound have been isolated from unsaponifiable fractions of these fats :

(a) Carotenoid pigments.

(b) Vitamins A.

(c) Sterols, notably

(d) Vitamins D.

(e) Hydrocarbons — notably squalene.

(f) α-glyceryl ethers.

(g) Fatty alcohols.

Of these constituents, a, b, c, d, f and g may occur in the unsaponified oil as esters of fatty acids.

Isolation of Constituents

The carotenoids have usually been separated and isolated chromatographically[9] from the oil or from the unsaponifiable fraction. Sterols have been obtained by crystallisation methods, and by precipitation as their digitonides[10], Vitamins A by chromatographic means[11], by vacuum distillation[12], molecular distillation[13], and fractional crystallisation[14]. Vitamin D has been isolated, after removal of other substances[15], by chromatography[16], fractional crystallisation[17] and molecular distillation[18]. The α-glyceryl ethers have been obtained by crystallisation methods, and distillation of their acetates[19], and squalene by vacuum distillation from the oil itself, or as the solid hexahydrochloride[20]. The fatty alcohols have been obtained by crystallisation and distillation[21].

Estimation of the components of unsaponifiable fractions

Although the fatty acid components of oils and fats have been the subject of close analytical investigation (e.g. by the Hilditch fractionation technique[2b]), the

literature reveals that the treatment of the unsaponifiable fraction has in general not been quantitative and analytical. Studies have usually aimed at the isolation and identification of the various individual components, but little attention has been paid to the development of techniques for the accurate estimation of those components -- except in the case of the commercially important Vitamins A and D.

These substances may be estimated by biological assays[22], and, in the case of the Vitamins A by a well tried chemical method -- the antimony trichloride colorimetric method[23]. Chemical methods for the determination of Vitamins D present difficulties due to interference of similar substances. Both Vitamins A and D may be estimated by a physical method i.e. the spectrographic method, but again, estimation of Vitamins D is more subject to interference from other materials than is that of Vitamins A[24,25].

The sterols have long been determined by the method of Windaus as their addition complex with digitonin, and although this method does not possess the accuracy of an inorganic gravimetric determination[26], providing sufficient excess of digitonin and a long enough time of precipitation are allowed, reasonable results may be expected. A limitation of this method is that insoluble digitonides form only with sterols conforming, in configuration of the C_3 carbon, with cholesterol, i.e. compounds of the β type are precipitated, but the epi-compounds are not[27]. The sterols may also be estimated by colorimetric means, as a result of several well-known reactions[28], but owing to the complex nature of unsaponifiable matter, direct application of such colorimetric methods is not feasible.

Carotenoid pigments constitute usually only a very small fraction of the unsaponifiable matter — the main member of this group that is encountered in marine oils, i.e. astacin, being usually removed in the alkaline soap solutions. These materials have been colorimetrically and spectrographically determined. Squalene has previously been estimated, not entirely successfully, as will be reported later (Section 4), but no truly quantitative analytical technique has been employed. No analytical methods for the determination of α -glyceryl ethers have been described, although Swain and Morton[29] announced a future report on the development of an approximate method and André and Bloch[30] have recorded rough results obtained by a technique of "fractional diffusion". The fatty alcohols have also not been treated analytically.

Aim of the Present Research

Due to the lack of a standard technique for the extraction of unsaponifiable matter, and the lack of analytical techniques for several unsaponifiable constituents, unsaponifiable fractions of fats have been examined in general for only one or two components at a time. Consequently, no "balance sheets" of unsaponifiable fractions, showing the relative amounts of the known components, have been available. It was felt that the development of techniques particularly for the estimation of α -glyceryl ethers and of hydrocarbons would constitute a contribution to existing knowledge on the subject, and would provide a valuable means of collecting preliminary data in the detailed studies of particular unsaponifiable fractions.

Thus, new and interesting constituents of unsaponifiable matter might be revealed. Further, it was considered that the development and application of these analytical techniques would provide valuable aids in physiological studies. It was felt, too, that in the case of marine oils high in unsaponifiable matter content, analytical examination of these fractions should supplement fatty acid data, and the oil be discussed as a whole. (See Section 5)

The following programme was therefore drawn up :-

(1) A study of the efficiency of recovery of the unsaponifiable matter from marine oils

Previous reports of the composition of various unsaponifiable fractions have referred to material extracted in a variety of ways. It was necessary, before embarking on analytical studies of unsaponifiable components, to extract the unsaponifiable fractions as completely as possible from the saponification mixtures by a standardised technique.

To this end, and since data on the efficiency of extraction of unsaponifiable matter from saponification mixtures — especially those of marine oils — are diffuse and indefinite, it was decided to subject the method of the Society of Public Analysts, which has proved the most successful method, to a critical appraisal. As reported above, reference has been made in the literature to some shortcomings of this method when applied to marine fats[1,7a,8], and experience in this laboratory has indicated that such an examination of the S.P.A. method with reference to marine fats would be of value.

(2) Investigation of a possible method of estimating α-glyceryl ethers

The α-glycol nature of these substances[31] made their estimation with lead tetra-acetate, or periodic acid, an attractive possibility.

(3) Survey of the occurrence of α-glyceryl ethers

It was planned to use any method devised in (2) for a survey of the extent of occurrence of α-glyceryl ethers in fats generally. This would provide the first co-ordinated view of the distribution of these compounds, and would give basic data for more detailed studies in individual cases.

(4) Development of a method for estimating hydrocarbons, particularly squalene, in marine oils

The modification of existing procedures necessary in the case of marine oils, because of the greater quantity and variety of unsaponifiable constituents was considered. The Fitelson[32] technique (Appendix B) for the detection of olive oil in edible oil mixtures was regarded as most promising, and examination of its application to marine oils was desirable.

(5 and 6) The application of existing and new methods to detailed studies of the unsaponifiable fractions of individual species

It was decided to examine the unsaponifiable fraction of the liver oil of a specimen of Heptranchias pectorosus

-- the Seven-gilled shark, and to examine the unsaponifiable fractions of the fats, generally, of Cetorhinus maximus -- the Basking shark.

(7) A short survey of the Provitamin D contents of some local marine invertebrate oils

This was to be carried out at the request of, and with the object of assisting, local industry.

THE RECOVERY OF UNSAPONIFIABLE MATTER FROM MARINE OILS BY THE SOCIETY OF PUBLIC ANALYSTS' METHOD

In survey studies carried out in this laboratory, the Society of Public Analysts' procedure[1]* for the estimation of unsaponifiable matter has been applied extensively to marine fats of widely varying types. While satisfied with this technique as a conventional routine method, it has been felt — and our experience has been confirmed in another laboratory[8] — that the technique as described does not lead to quantitative recovery of unsaponifiable matter in all cases. In particular, the ease of recovery of unsaponifiable matter has been found very dependent on the nature of the unsaponifiable substances, and on their amounts in so far as this affects the concentration of soaps in the saponification mixture[7a,7c]. Before embarking upon an extended study of the unsaponifiable components of marine fats, these points have been investigated in greater detail, and the results are here placed on record.

The variations which can occur in the ease of recovery of unsaponifiable fractions from different marine oils by the S.P.A. method are exemplified by the data of Fig. I. From these it is clear that, even after six extractions with ether, recovery may in some cases be incomplete. The varia-

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* See Appendix A.

tions in soap content of the saponification mixtures due to the widely varying percentages of unsaponifiable matter in the oils are one obvious cause of this effect, since the recovery of unsaponifiable matter was rendered more difficult (see Figs. II and III) as the unsaponifiable contents of individual oils were reduced by dilution with arachis oil. This phenomenon was particularly marked in the case of Heptranchias pectorosus, the unsaponifiable matter of which is rich in α-glyceryl ethers (89.2% calculated as selachyl alcohol).* The data are represented in a different way in Fig. IV, where it is made clear that the efficiencies of successive extractions vary with the total unsaponifiable matter content, and therefore with soap concentration. As the unsaponifiable matter content increased, the efficiency of the first extraction increased, whilst that of all subsequent extractions decreased.

That the precise nature of the soaps of the saponification mixture is of minor importance in ordinary cases is shown by the fact that when the unsaponifiable matter of the liver oil of Heptranchias pectorosus was dissolved at a fixed concentration in a range of very different oils, the efficiencies of extraction of unsaponifiable matter were virtually unaltered (see Fig. V). Castor oil, although exhibiting a definitely higher efficiency of extraction at first, showed an overall decrease in efficiency. The recovery curve for this oil fell ultimately below those for the other fats, indicating that the soaps of ricinoleic acid retain this type of unsaponifiable matter more effectively. This is perhaps understandable in view of the hydroxylic character of both soaps and unsaponifiable matter in this case.

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* Section 2.

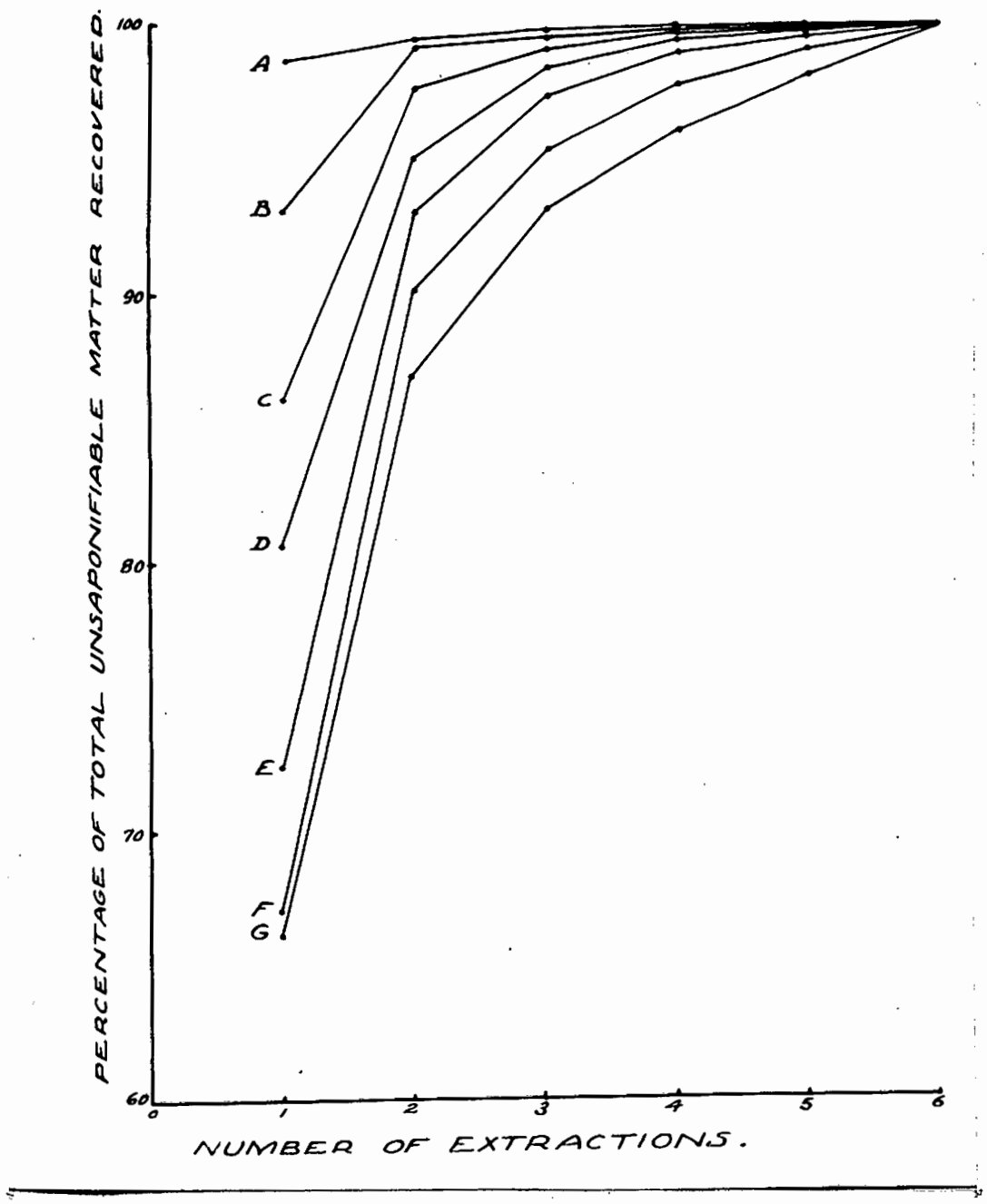

Fig. I

Course of the extraction of unsaponifiable matter from several marine oils, by the S.P.A. Method (six extractions regarded as effecting complete recovery)

A.	Liver oil of	Centroscymnus fuscus	(90.82%	unsaponifiable matter)
B.	Head " "	Physeter macrocephalus	(47.50%	" ")
C.	Total " "	Mytilus meridionalis	(24.80%	" ")
D.	Liver " "	Hexanchus griseus	(20.00%	" ")
E.	" " "	Heptranchias pectorosus	(19.36%	" ")
F.	" " "	Squalus species	(8.20%	" ")
G.	" " "	Merluccius capensis	(3.80%	" ")

Note. The curve for Dentex rupestris (51.20% unsaponifiable matter) is indistinguishable from B. above. See Fig. VIII.

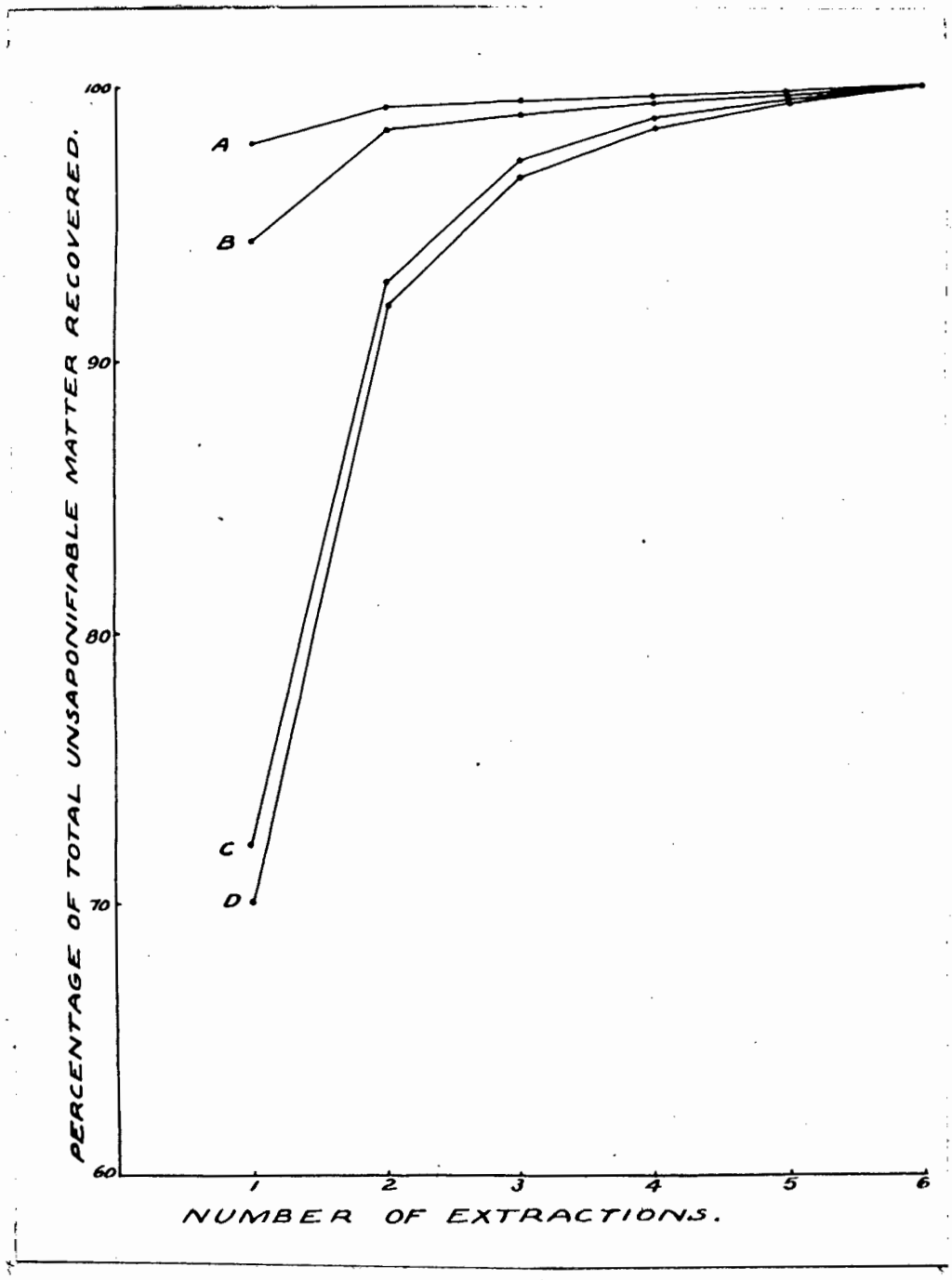

Fig. II

Course of the extraction of the unsaponifiable matter of the liver oil of Heptranchias pectorosus at various levels of unsaponifiable matter content

(Dilutions with arachis oil)

A. 85.1% unsaponifiable matter in oil
B. 65.1% " " " "
C. 19.3% " " " "
D. 10.6% " " " "

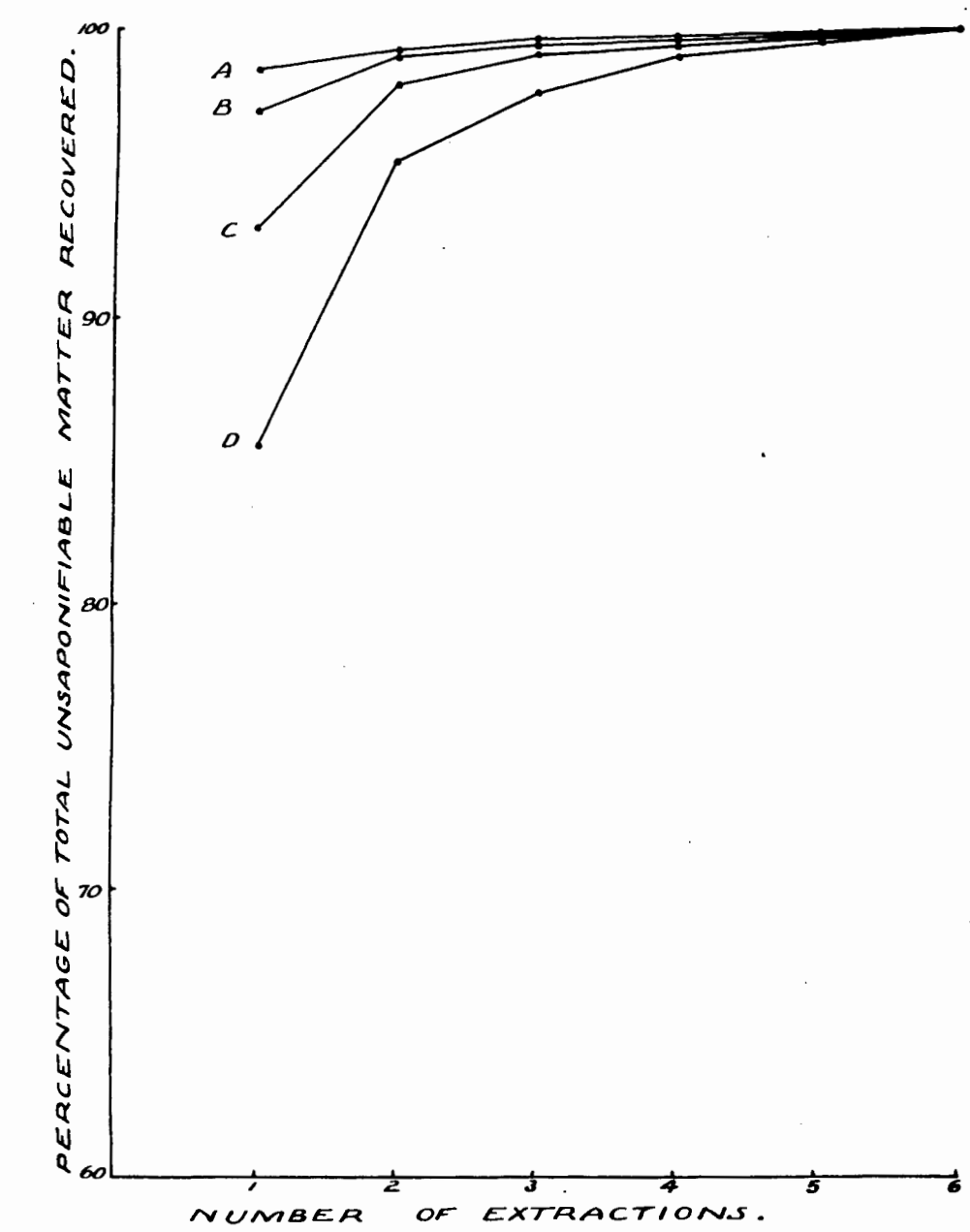

Fig. III

Course of the extraction of the unsaponifiable matter of the liver oil of Centroscymnus fuscus at various levels of unsaponifiable matter content

(Dilutions with arachis oil)

A. 90.8% unsaponifiable matter in oil
B. 51.5% " " " "
C. 20.1% " " " "
D. 9.3% " " " "

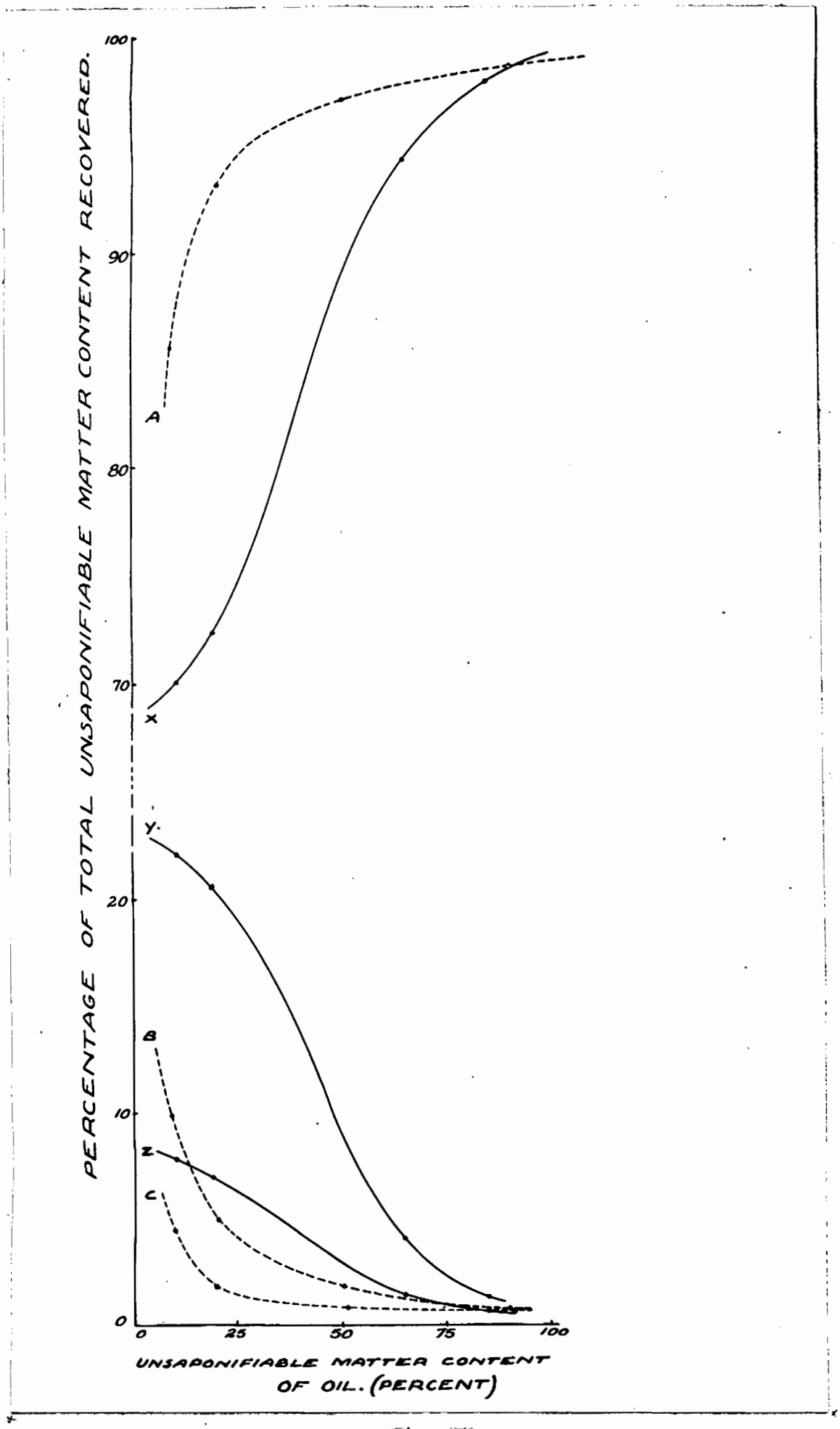

Fig. IV

Variation of efficiency of separate extractions
with unsaponifiable matter content of oil

Liver oil of Centroscymnus fuscus

A. 1st extraction
B. 2nd "
C. 3rd -- 6th extractions combined

Liver oil of Heptranchias pectorosus

X. 1st extraction
Y. 2nd "
Z. 3rd -- 6th extractions combined

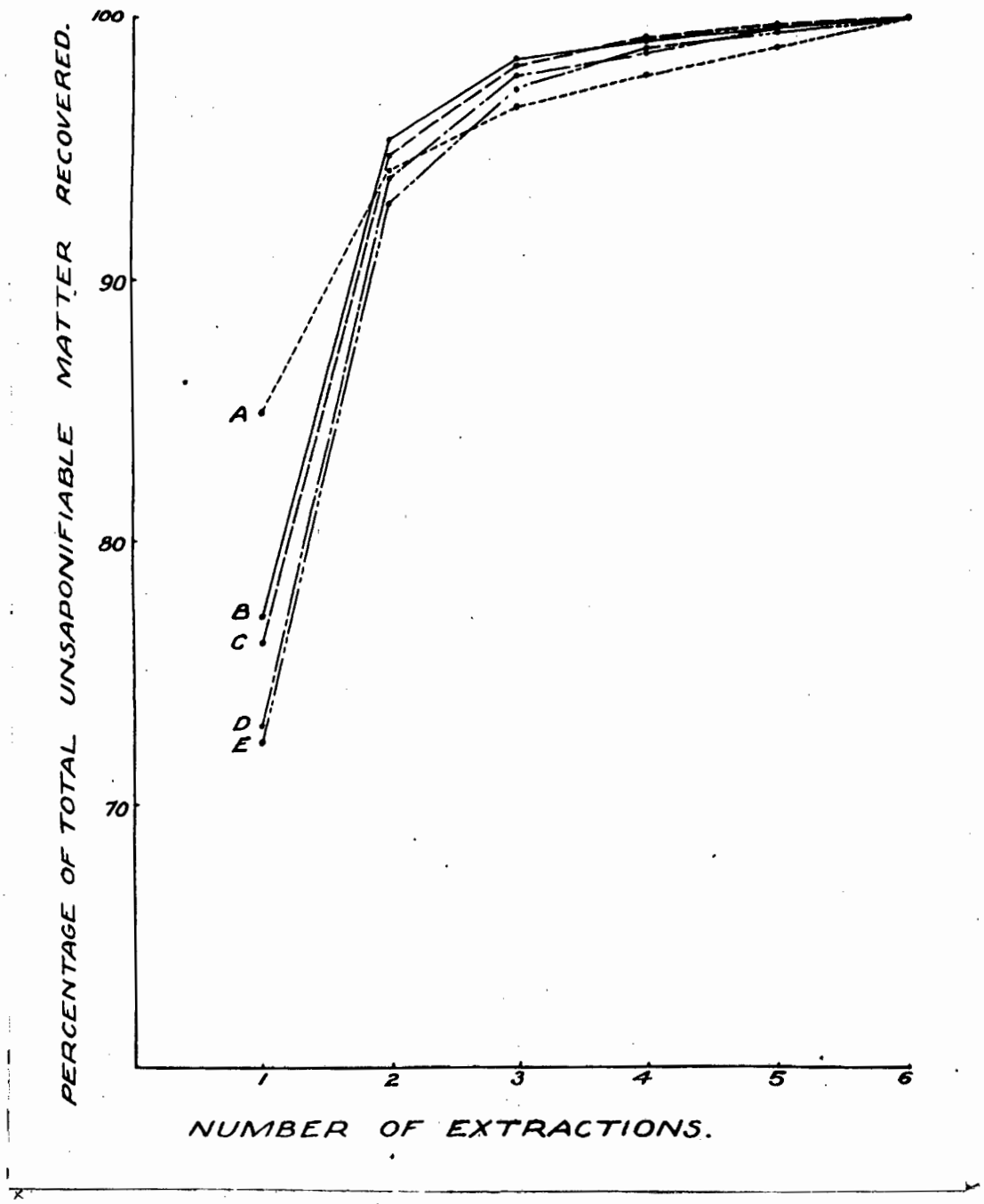

Fig. V

Course of the extraction of the unsaponifiable matter of the liver oil of Heptranchias pectorosus from the saponification mixtures of

A. Castor oil
B. Coconut oil
C. Butter fat
D. Arachis oil
E. Liver oil of Heptranchias pectorosus

The independence of unsaponifiable matter recovery in respect of normal soap types has made possible a study of the effects of the nature of unsaponifiable substances on the efficiency of their recovery. This feature of the S.P.A. method has so far not been adequately discussed. In Fig. VI are recorded the results obtained when oils with unsaponifiable fractions high in content of squalene, vitamin A, fatty alcohols, sterols, and α-glyceryl ethers respectively, were diluted to the same unsaponifiable matter content with arachis oil. The curves show up marked variations in ease of extraction — squalene, vitamin A and sterols being less tenaciously retained by the saponification mixtures than fatty alcohols and α-glyceryl ethers. The difficulty experienced by Swain[8] in determining the unsaponifiable matter content of Pacific dogfish liver oils is almost certainly to be associated with a high α-glyceryl ether content.

Since the present author has been carrying out detailed studies of oils high in Glyceryl ether content*, the course of the extraction of such ethers, and the effects of their presence on the extraction of other unsaponifiable components have also been examined, and it is considered relevant to insert the results obtained at this stage. Thus, Fig. VII shows the course of the recovery of α-glyceryl ethers under a variety of conditions**. The α-glyceryl ether content of the second extract shows a tendency to be greater than that of the first extract — indicating that other substances tend to be extracted before glyceryl ethers.

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* Sections 2 and 3.

** Experiments have indicated that the more saturated glyceryl ethers (e.g. Batyl alcohol) are more easily extracted than unsaturated ones (e.g. Selachyl alcohol).

Fig. VI

Fig. VI

Course of the extraction of unsaponifiable matter from five marine oils of standard unsaponifiable matter content (20%)

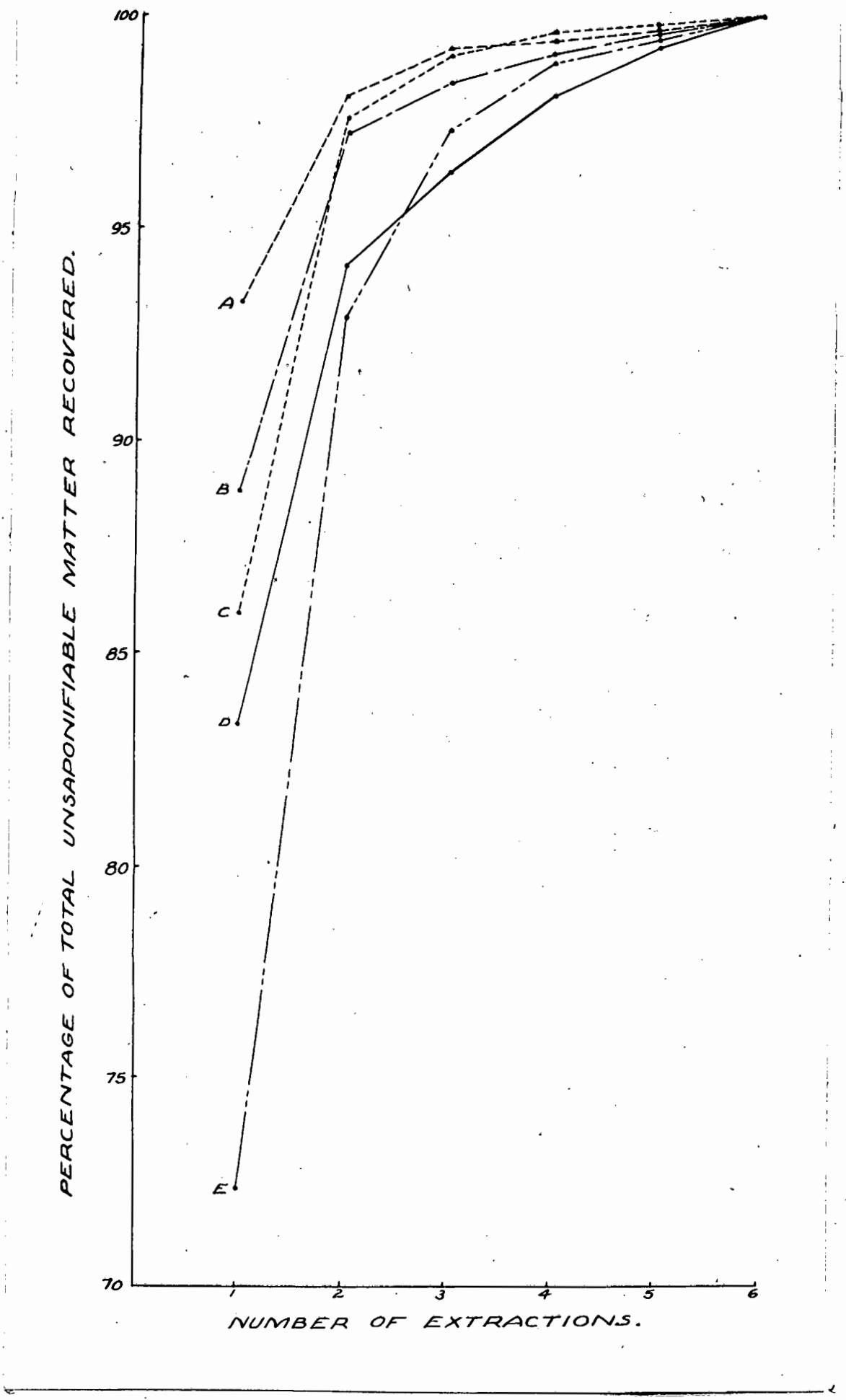
A. Centroscymnus fuscus liver oil
(unsaponifiable fraction mainly squalene)

B. Dentex rupestris liver oil
(unsaponifiable fraction 90% vitamin A)

C. Mytilus meridionalis oil
(unsaponifiable fraction mainly steroid)

D. Physeter macrocephalus head oil
(unsaponifiable fraction mainly fatty alcohols)

E. Heptranchias pectorosus liver oil
(unsaponifiable fraction mainly glyceryl ethers)


PERCENTAGE OF TOTAL UNSAPONIFIABLE MATTER RECOVERED.
100
95
90
85
80
75
70
A
B
C
D
E
1
2
3
4
5
6
NUMBER OF EXTRACTIONS.

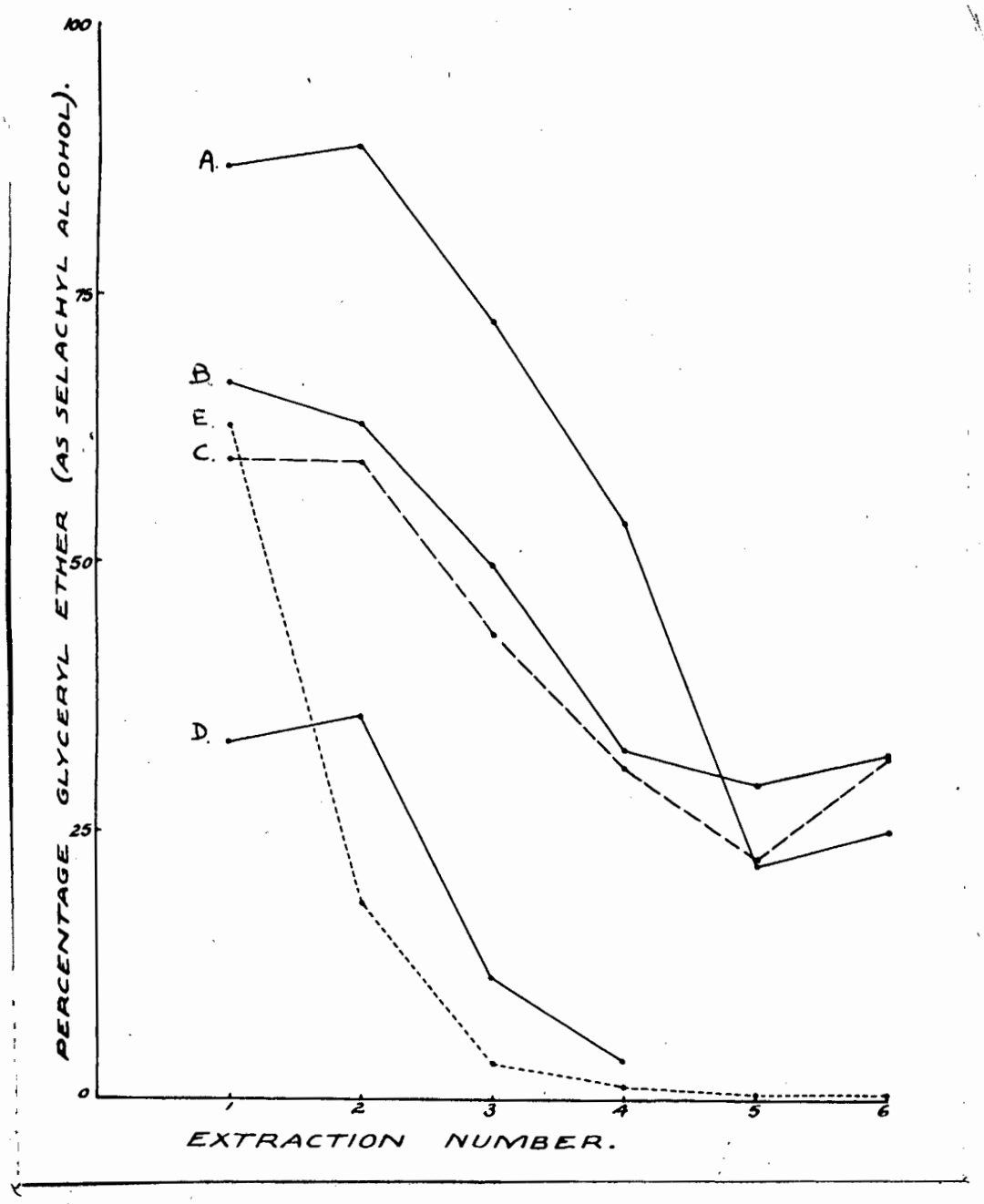


Fig. VII

Glyceryl ether contents of the separate extracts of unsaponifiable matter

A. Liver oil of Heptranchias pectorosus (20% unsaponifiable matter)
B. Diluted liver oil of " " (8% " ")
C. " " " " " " (4% " ")
D. 7% batyl alcohol + 14% cholesterol in arachis oil

(A, B, C, D, calculated on weight of each extract)

E. Results as in "A" calculated on weight of oil saponified

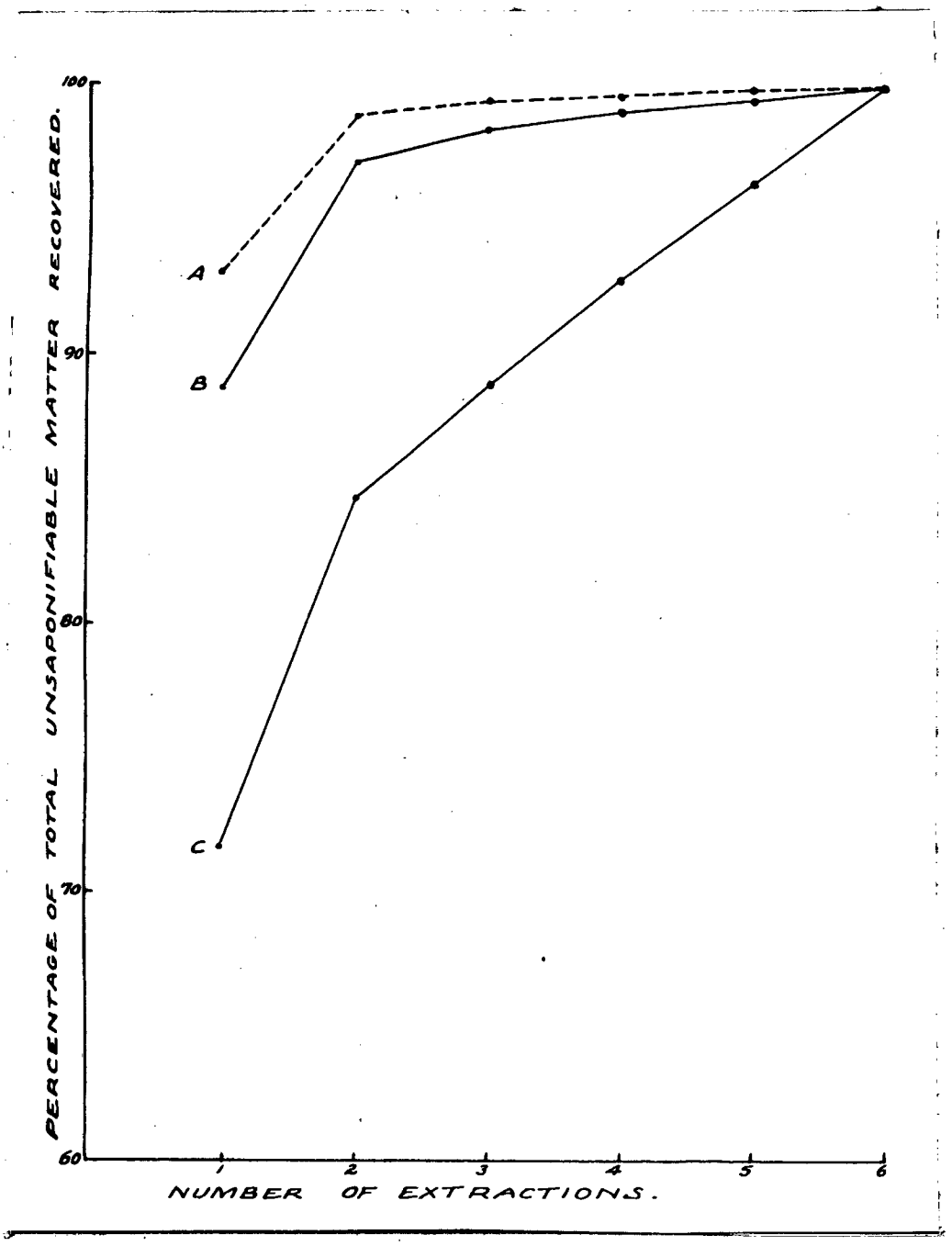


Fig. VIII

Comparison of the course of extraction of the unsaponifiable matter from two oils rich in vitamin A

A. Dentex rupestris liver oil
(unsaponifiable matter = 51.2% of which 90% is vitamin A; 1.6% selachyl alcohol)

B. "A" diluted with arachis oil to unsaponifiable matter content = 21.4%

C. Polyprion americanus liver oil
(unsaponifiable matter = 28.3%, of which 56% is vitamin A; 13% selachyl alcohol)

In subsequent extractions the percentage of α-glyceryl ethers drops away rapidly, however, indicating that at this stage other substances are being extracted in increasing quantity along with the glyceryl ethers. This trend ceases only after the fifth extraction when in every case an increase in glyceryl ether content of the extract was observed. The extended number of extractions necessary to effect the recovery of unsaponifiable matter from oils high in glyceryl ether content is further evidenced by Fig. VIII, in which two liver oils of high vitamin A content are compared. In the case of Dentex rupestris the α-glyceryl ether content of the unsaponifiable matter is very small (1.6%) whereas that of Polyprion americanus is considerable (13.0%). The extraction of the unsaponifiable matter of the Polyprion oil proceeded with greater difficulty than that of Dentex rupestris, even when the unsaponifiable matter content of the latter had been considerably decreased by dilution with arachis oil.

CONCLUSION

The extent of the error involved in restricting the number of ether extractions to three as in the S.P.A. method of analysis can be readily assessed from the above data. Table I indicates the number of milligrams of unsaponifiable matter extracted in a fourth, fifth and sixth extraction of the saponification mixture derived from 2 gm. of each of the oils treated in Fig. I. The percentage unsaponifiable matter content calculated after each stage is given in brackets; that determined for three extractions appears after the specific name.

Table I

	Number of mg. of unsaponifiable matter		
	4th Extraction	5th Extraction	6th Extraction
Centroscymnus fuscus (90.68%)	1.6 (90.69)	1.1 (90.78)	1.1 (90.82)
Physeter macrocephalus (47.33%)	1.9 (47.42)	2.4 (47.52)	0.5 (47.54)
Mytilus meridionalis (24.65%)	2.5 (24.77)	1.0 (24.82)	1.0 (24.87)
Hexanchus griseus (19.65%)	4.6 (19.91)	1.2 (19.93)	1.2 (20.02)
Heptranchias pectorosus (18.84%)	6.2 (19.15)	2.4 (19.26)	1.9 (19.36)
Squalus species (7.83%)	3.9 (8.03)	2.2 (8.14)	1.6 (8.22)
Merluccius capensis (3.5[illegible]%)	2.3 (3.66)	1.5 (3.73)	1.5 (3.81)

It is clear that, from the analytical point of view, serious discrepancies arise in the case of marine oils high in glyceryl ether content (e.g. Hexanchus, Heptranchias) and particularly where the unsaponifiable matter content is low (e.g. Squalus and Merluccius -- 63.3% and 15.5% glyceryl ether respectively). In view of the wide occurrence of α-glyceryl ethers in marine oils*, the results obtained by the S.P.A. method on such oils must therefore be regarded with due reserve. Using an adequate number of extractions, however, the method has proved the most effective one available, and in routine analysis four extractions have been regarded as giving quantitative recovery.

* Section 3.

EXPERIMENTAL

Extraction of unsaponifiable fractions

The shortened S.P.A.[1]* method was used, but since successive extracts were worked up separately, some modifications were introduced :

(1) More than the specified 2 gm. of oil were employed in most cases, but the quantities of all reagents were adjusted in proportion. In this way reasonable weights of material were obtained in each extract.

(2) All oils were saponified for 1.5 hours instead of the 1 hr. recommended.

(3) Extractions were effected in all cases by shaking with ether for exactly one minute.

(4) All extracts except the first were washed with amounts of water and alkali calculated on the basis of their bulk and the S.P.A. recommendations. This procedure was not adopted in the case of the first extract, however, because of its high alcohol content. Such extracts were always diluted with ether (50 ml. per 2 gm. of oil taken) before washing as above was commenced.

(5) Extracts, after evaporation of the ether, were dried to constant weight in suitably tared small distilling flasks under vacuum at 100°C.[7a,b]

Adjustment of Unsaponifiable Matter Content

This was effected either by dilution of the oil or of the unsaponifiable matter derived from it with arachis oil (Figs. II, III, IV, VI, VII, VIII) or with other oils (Fig. V). The diluting oils all contained less than 1% of unsaponifiable matter. Their contributionsto the unsaponifiable fractions of the mixtures were therefore small.

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* See Appendix A.

Precision of the Method

All experiments were carried out in duplicate at least, and the total recoveries at successive stages of extraction rarely differed by more than 1%. Total recoveries of unsaponifiable matter fell consistently well within the recommended tolerance range of ±0.2%, except in a few cases of exceptionally high unsaponifiable matter content.

Determination of α-glyceryl ethers

Glyceryl ethers were determined by oxidation with periodic acid*; the Heptranchias pectorosus liver oil was established before use to give corresponding results by both volumetric and gravimetric methods, and all estimations involving unsaponifiable matter from this oil were therefore carried out volumetrically. Accuracy in the analysis of final extracts always presented difficulty as the weights of material (usually 3-8 mg.) were so small.

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* Section 2.

[illegible] OF α-GLYCERYL ETHERS FROM UNSAPONIFIABLE [illegible]

The α-glyceryl ethers (e. g. oleoyl, chimyl, selachyl and batyl alcohols) were first isolated from the unsaponifiable fractions of elasmobranch liver oils by Japanese workers[19]. Tsujimoto[20], in his examination of many liver oils of this class for squalene, had already reported the presence of non hydroxylic components of these unsaponifiable fractions. These workers obtained data on the molecular formulae of the components, but the final determination of their constitution was accomplished by Heilbron and his co-workers, who established the fact that they were α-glyceryl ethers of fatty alcohols[21,23]. Their conclusions were supported by physical measurements[24] and by later synthetic experiments[25]. André and Bloch[20] had shown that these components occur in the original oils as fatty acid esters, which on saponification would result in the formation of the free glycols.

$$\begin{array}{l} CH_2OCOR_1 \\ | \\ CHOCOR_2 \\ | \\ CH_2OCH_2CH_3 \end{array} \xrightarrow[\text{alcohol}]{\text{KOH in}} \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OCH_2CH_3 \end{array}$$

[where CH_2CH_3 :- we have

= $C_{16}H_{29}$ = oleoyl alcohol = α-glyceryl ether of myristyl alcohol

= $C_{18}H_{33}$ chimyl alcohol = α-glyceryl ether of cetyl alcohol

= $C_{18}H_{35}$ selachyl alcohol = α-glyceryl ether of oleyl alcohol

= $C_{18}H_{37}$ batyl alcohol = α-glyceryl ether of octodecyl alcohol]

The extent of occurrence and distribution of glyceryl ethers in natural fats has never been studied in detail. In almost all investigations to date the presence of these compounds has been demonstrated by their actual isolation from unsaponifiable matter[cf.29]. No method for their estimation has been available, although André and Bloch[30] recorded roughly quantitative data for the glyceryl ether content of the liver oil of Scymnorhinus lichia. This data was obtained by a "fractional diffusion" technique -- a method impracticable for the routine rapid examination of natural fats for their glyceryl ether content. More recently, Owain and Morton[29] have reported an approximate method for the determination of selachyl and related alcohols by a chromatographic method, but details of this are not yet available.

Selective oxidation of α-glyceryl ether constituents of unsaponifiable matter

Since the α-glyceryl ethers are the only 1:2-glycols which have been found recorded as components of unsaponifiable fractions of natural fats, their estimation by the use of reagents such as lead tetra-acetate[31] or periodic acid, which act specifically on 1:2-glycols, has been explored.

Both these reagents effect the oxidation of α-glyceryl ethers according to the equation :

$$CH_2OR.CHOH.CH_2OH \longrightarrow CH_2OR.CHO + CH_2O$$

and the course of the reaction has been followed by estimation both of reagent consumed and of formaldehyde generated.

The use of lead tetra-acetate was soon abandoned when it was found to attack substances other than α-glyceryl ethers, in particular squalene[36], present in the unsaponifiable fractions of many fats. Periodic acid, applied first in acetic acid[37] and later in ethyl alcohol[38] and ethyl acetate[39] solutions, however, proved a more selective reagent.

It was considered desirable for the method of estimating α-glyceryl ethers to be a direct sequel to the ordinary Society of Public Analysts' method[1] for the estimation of unsaponifiable matter, using the usual 2 gm. of fat, or more where necessary, and to be applicable to the often very small quantities of material obtained from that operation. Consequently, conditions for the oxidation were worked out using 0.0325 N. periodic acid.

Under these conditions the oxidation of batyl alcohol was found to be complete (see Fig. IX) in four hours. Acetic acid was not employed in the final procedure because of the difficulty of adjusting the pH of the reaction mixture in the ensuing arsenite method[40] for estimating the excess of oxidising agent when this solvent was employed. Under the conditions finally decided upon, the sample of batyl alcohol available assayed at 98.1(3)% pure on the basis of reagent consumed, and 99.9% pure on the basis of formaldehyde generated in the reaction.

Samples of unsaponifiable matter were prepared for oxidation by dissolving them in either ethyl alcohol or ethyl acetate, or in mixtures of the two. As the results of Table I indicate, there is no interference by either of these two solvents. It was a matter of experience (cf.

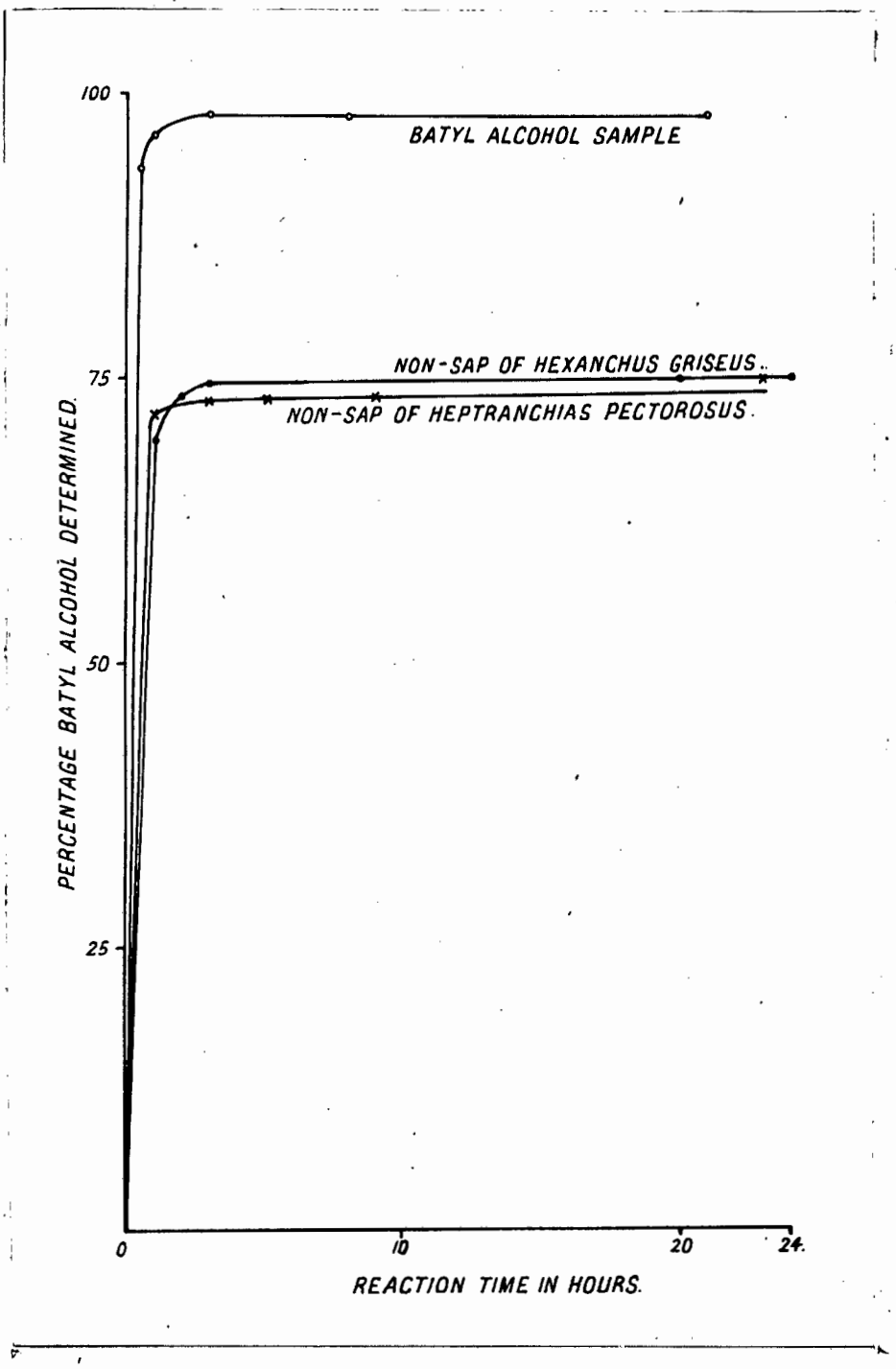

Fig. IX

Course of the oxidation of batyl alcohol and of two unsaponifiable fractions with periodic acid, as followed by the volumetric procedure

Tables I and II) that duplicate analyses of separately prepared samples of unsaponifiable matter from a given fat differed more than duplicate analyses carried out on a single sample of unsaponifiable matter.

The consumption of reagent in all cases is of course determined by the total reducing power of the unsaponifiable matter to periodic acid, whereas the formaldehyde generated can be taken as almost certainly derived only from the oxidation of α-glyceryl ethers present. Nevertheless, there has been close correspondence (see Table IIIA) between the periodic acid consumed and the formaldehyde produced in the case of very many fats, and this can be taken as strong evidence that α-glyceryl ethers are the only 1:2-glycols present in these materials. In survey studies which are in progress, however, many cases (examples in Table IIIB) have arisen in which the consumption of reagent was in excess of that calculated from the amount of formaldehyde produced. In samples of this type, the α-glyceryl ether content of the unsaponifiable matter has been calculated on the basis of the formaldehyde produced, and the excess consumption of oxidising agent is attributed to the oxidation of other substances, some possibly of a 1:2-glycol nature, which are now under investigation.

TABLE I

Comparison of ethyl alcohol and ethyl acetate as media in the periodic acid oxidation of an unsaponifiable fraction of the liver oil of Heptranchias pectorosus

(Volumetric procedure)

Solvent	Volume of aliquot	Weight of unsaponifiable matter in aliquot	Volume of reagent	Result : % glyceryl ether, as selachyl alcohol
Ethyl acetate	5 ml.	0.0581(0)gm.	8 ml.	71.6(8)
	"	0.0544(6)gm.	"	71.6(9)
Ethyl alcohol	"	0.0566(0)gm.	"	71.68)
	"	0.0544(4)gm.	"	71.89)

TABLE II

Percentage α-glyceryl ether as determined on duplicate samples of unsaponifiable matter from the liver oils of some marine fishes

(Volumetric procedure)

Species[41]	Percentage glyceryl ether, as selachyl alcohol, in	
	Sample 1	Sample 2
Galeorhinus canis	32.0(9)	30.9(8)
Isurus glaucs	23.4(0)	22.8(0)
Callorhynchus capensis	63.6(3)	64.5(9)
Carcharias taurus	23.6(0)	23.0(0)
Lophius piscatorius	5.8(3)	5.8(4)
Merluccius capensis 1	34.3(0)	34.1(0)

TABLE III

Comparison of volumetric and gravimetric data in the determination of ∝-glyceryl ethers in the oils of some marine fishes

A

Species[41]	Percentage ∝-glyceryl ether in the unsaponifiable matter, as selachyl alcohol	
	Volumetric	Gravimetric
* Galeorhinus canis (liver oil)	30.9(8); 32.0(9)	35.3 ; 33.5
Echinorhinus spinosus (")	9.6(5)	9.5
* Callorhynchus capensis (")	64.5(3); 65.6(6)	66.6
Centroscymnus fuscus (")	3.0(2)	2.6
Heptranchias pectorosus 1. (")	71.2(3)	70.8
" " 2. (")	94.8(4)	94.3
Chimaera monstrosa (")	14.6(3)	14.5
Hexanchus griseus (")	77.1(0)	77.7
Galeocerdo arcticus (")	81.3(3)	79.6
Cetorhinus maximus (flesh oil)	99.0(2)	96.2

B

Scylliorhinus pantherinus (liver oil)	24.2(0)	13.3
Carcharias melanopterus (")	32.2(6)	6.1
Merlucoius capensis 2(")	22.2(4)	15.5
Sarda sarda (")	64.4(0)	4.8
" " (head oil)	32.1(4)	6.5
Sciaena hololepidota (head oil)	24.7(8)	11.3
Scomber colias (body oil)	4.3(9)	1.4
Sardina sagax (whole fish)	14.2(9)	2.1

* denotes duplicate results carried out on separately prepared samples of unsaponifiable matter.

EXPERIMENTAL

Preparation of a sample of Batyl alcohol (cf. Davies, Heilbron and Jones[31])

The unsaponifiable matter (5.4 gm.) from a sample (25 gm.) of the flesh oil of Cetorhinus maximus was taken up in methyl alcohol (30 ml.) and the solution refrigerated for 16 hours. Solid material which had separated (0.80 gm.; m.p. 58.2 - 60.0° C.) was then filtered off and washed with cold methyl alcohol. The filtrate and washings (40 ml.) were left at 0° C. for a further 16 hours, and a second deposit of solid material (0.25 gm.; m.p. 59.0 - 59.5° C.) was removed. The methyl alcoholic filtrate was then shaken with hydrogen at 1.5 - 2 atmospheres pressure in the presence of a palladium-strontium carbonate catalyst, until the absorption of hydrogen ceased.

The solution containing the suspended catalyst, and some white crystalline material which had separated, was brought to the boil to dissolve the latter, and the catalyst filtered off and washed. The warm filtrate (60 ml) was cooled at 0 - 5° C. for 15 hours, and a crystalline deposit (2.90 gm.; m.p. 61.5 - 65.5° C.) filtered off. Further quantities of solid material (0.35 gm.; m.p. 51 - 58° C.; 0.15 gm.; m.p. 48 - 49° C. and 0.10 gm.; m.p. 52 - 55° C.) were obtained from successive evaporations and coolings of the solution.

Batyl alcohol (m.p. 70.5 - 71.0° C.)[31] was obtained by recrystallisation of the first crop above (2.9 gm.) of crystals, until no further rise in melting point occurred. For oxidation experiments, a sample of this

material (0.3883 gm.) was dissolved in a mixture (50 ml.) of equal volumes of ethyl acetate and ethyl alcohol, and 5 ml. aliquots were withdrawn for each experiment.

The results of a full examination of the unsaponifiable fraction of this oil will be reported later.

Oxidation Experiments

(a) Reagents

Periodic acid. Sodium paraperiodate[42] (2.40 gm.) was dissolved in water (15 ml.) containing sulphuric acid (0.80 ml.). To this was added 235 ml. of 96% ethyl alcohol, which was purified where necessary by distillation from potassium hydroxide in the presence of aluminium turnings. A white precipitate formed. This was filtered off and the reagent allowed to stand overnight before use.

Sodium arsenite. This was about 0.02 N., containing 10 gm. $NaHCO_3$ per litre, and was adjusted to give a back-titration figure of 3 - 5 ml. iodine (0.02 N.) for 5 ml. periodic acid reagent in the blank determination. (See under (c) Oxidation below.)

It was also found useful to know in each batch of solutions the direct titre of the standard iodine solution used (0.02 N.) against the arsenite solution, so that it was immediately evident in any experiment whether an excess of periodic acid had indeed been added.

(b) Preparation of Unsaponifiable Matter

The unsaponifiable matter was isolated by the method of the Society of Public Analysts[1] but with

four ether extractions* instead of the three recommended. The oxidation was carried out on solutions of unsaponifiable matter in ethyl alcohol or ethyl acetate, containing from 35 - 50 mg. material per 5 ml. solution.

(c) The Oxidation

An aliquot (5 ml.) of the solution of unsaponifiable matter, containing 35 - 50 mg. of material (50 mg. selachyl alcohol ≡ 0.146 millimol.) was pipetted into a 300 ml. conical flask. To this was added 5 ml. (≡ 0.163 millimol.) of periodic acid reagent. The solutions were well mixed, and swirled at 10 minute intervals for the first half-hour, and subsequently at half-hour intervals. If the α-glyceryl ether content was high, a white precipitate (iodic acid) separated within about 10 minutes. At the end of four hours, saturated sodium bicarbonate solution (25 ml.) was added from a measuring cylinder, and sodium arsenite solution[40] (25 ml.; see under reagents above) was pipetted in. Organic matter was precipitated (less evident where ethyl acetate was used) and the iodic acid dissolved. Potassium iodide solution (3 ml. of 10%) was then added and the mixture swirled thoroughly and allowed to stand 15 minutes. At the end of this time, the flask was washed down with water (ca. 15 ml.) and the excess sodium arsenite titrated with standard (0.02 N.) iodine solution to starch indicator. Blank determinations (5 ml. solvent replacing the solution of unsaponifiable matter) were carried out simultaneously. From the titre of periodic acid consumed, the percentage glyceryl ether, as selachyl alcohol, was calculated.

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* See Section 1.

Determination of the Formaldehyde generated in the Reaction

To the oxidation mixture, after standing for four hours as above, water (30 ml.) was added, followed by N. hydrochloric acid (9 ml. to give a normality of HCl of 0.1 - 0.2 N.) and N. sodium arsenite solution (3 ml.)[43]. The solution was swirled thoroughly. When the colour generated had disappeared, 60 ml. of a buffer solution (made by mixing 1 volume of 1.7 N. hydrochloric acid with 2 volumes of 1.7 N. sodium acetate[44]) were added, and the whole filtered through a pad of filter-aid with gentle suction. The flask and pad were washed with a further 20 ml. of buffer solution, and then with about 10 ml. of water from a wash-bottle. To the clear filtrate (pH 4.6) 5% alcoholic dimedone solution (2 ml.) was added, and the solution thoroughly shaken, and allowed to stand for 18 hours. The precipitate was then filtered off, in a tared sintered glass crucible, and washed with distilled water. Drying was carried out at 85° C. for 20 minutes, and the crucible then cooled in a desiccator and weighed. A blank was usually carried out on the reagents, but was never found positive. The method is not regarded as giving results to greater accuracy than about 1.5%.

Note :- Where the α-glyceryl ether content of an unsaponifiable fraction was low, it was always advantageous to use 5 ml. aliquots containing more than 50 mg. of unsaponifiable matter. In these cases ethyl acetate was the more useful solvent, owing to the greater solubility of unsaponifiable matter in this solvent than in ethyl alcohol. The use of aliquots greater than 5 ml. necessitated adjustments in all operations subsequent to the oxidation, and in the case of ethyl acetate in particular caused unnecessary difficulties.

THE OCCURRENCE OF α-GLYCERYL ETHERS IN THE UNSAPONIFIABLE FRACTIONS OF NATURAL FATS

The data at present available on the occurrence of α-glyceryl ethers in the unsaponifiable matters of natural fats are the result, in large measure, of studies of elasmobranch liver oils by Japanese workers[19], who first achieved the isolation of selachyl, batyl and chimyl alcohols from a number of such fats. Later workers also based constitutional studies on material derived from elasmobranch oils[31,33]. Isolated observations indicate, however, that α-glyceryl ethers are by no means restricted to elasmobranch fats. Thus they have been isolated from a crustacean (the Japanese crab, Paralithodes camtchatica)[45], from a cephalopod mollusc (Ommastrephes sloani pacificus)[46], from a coelenterate (the gorgonia, Plexaura flexuosa)[47], from an echinoderm (the starfish, Asterias rubens)[48], from whale and herring oils[49], and from the bone marrow of cattle[50]. Further, Swiss workers have recently isolated batyl alcohol in small amounts from the unsaponifiable lipins of arteriosclerotic human aortas[51], and of hogspleen[52]. They have also succeeded in isolating chimyl alcohol from extracts of bull and swine testes[53]. In all cases the data as to actual α-glyceryl ether contents must be regarded as only roughly quantitative, both because a standardised procedure for the isolation of unsaponifiable matter was not used, and because

of the lack of an analytical method for the estimation of α-glyceryl ethers.

In this section, therefore, the amounts of formaldehyde generated in the oxidation with periodic acid — according to the analytical method described previously — of a wide variety of fats from marine and other sources, have been determined. The results are recorded in terms of percentage α-glyceryl ethers, calculated as selachyl alcohol, in the Table.

Discussion of Results

(a) Elasmobranch Fishes[54]. Fats from some 25 species have been examined, and the liver oils of all but two species were found to contain appreciable amounts of glyceryl ethers in their unsaponifiable fractions.

The Heptranchidae liver oils studied — namely those from the seven- and six-gilled sharks, showed the greatest content of glyceryl ethers — the unsaponifiable fractions (about 20%) consisting almost entirely of these substances. In the case of the seven-gilled shark, body and intestinal oils were also examined, and the former, in particular, was rich in glyceryl ethers.

Among the Carcharinidae, the tiger shark studied is to be noted for the high glyceryl ether content of its liver oil. The case of the commercially important Soupfin shark is also of interest — the unsaponifiable matters from the liver oils of 8 adult specimens studied contained from 17.7 - 34.4% of glyceryl ethers, whereas those from yolksac and

embryo liver oils contained 2.1% and 0.4% respectively. These data suggest the accumulation of glyceryl ethers in the liver oils of this species with increasing age. In this connection it may be noted that the hammerhead shark liver oil which was found devoid of glyceryl ethers was derived from a very young specimen[55].

Of the Isuridae, the basking shark has proved of considerable interest, and a more detailed report on this species will be made later. Whereas the liver oils of two of the specimens examined were rich in squalene (38.8% and 28.8% respectively*) their content of glyceryl ether was very low. In contrast, the flesh oils (10.1% and 5.4% in the flesh) contained 96.2% and 60.0% glyceryl ethers respectively in their unsaponifiable fractions (21.4% and 19.3%) and extracts from other tissues also showed appreciable quantities of these compounds. The examination of flesh oils from other species whose liver fats are rich in squalene is contemplated. (Cf. Centroscymnus : 94.1% squalene in the liver oil.)

Of the Squalidae, a Squalus species (unidentified) yielded a liver oil containing very large amounts of glyceryl ethers — an observation which tallies with the experience of other workers ([29], [19c](1924), [35a]). The spiny shark was also of interest, as it yielded a liver oil rich both in squalene and Δ-glyceryl ethers (cf. Scymnorhinus lichia[30]). The Chimaera studied yielded an oil of similar type (cf. [19c], [35a], [56]).

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* See Section 4.

(b) Teleost fishes. Although previous workers[56] have implied the probable presence of α-glyceryl ethers in fats from Teleost fishes, only one report of their actual isolation from these sources has been found[49]. The present investigation of fats from different organs of 20 species has demonstrated the widespread occurrence of glyceryl ethers in small amounts in this sub-class. In one or two species, e.g. the Cape eel, oils were obtained containing an appreciable amount of α-glyceryl ethers in their unsaponifiable fractions. In the cases of the stonebass and the yellow-tail, considerable amounts of the highly unsaturated vitamin A were associated with the α-glyceryl ethers. Head and body, as well as liver oils, showed appreciable contents, the body oil of the supersole being particularly worthy of note.

(c) Molluscs. Of the seven mollusc oils examined, those of the limpet and of the Top shell were lowest in glyceryl ether content. The most interesting oil of this group was that of the hepato-pancreas of the Octopus -- a bright red oil, obtained in a yield of 13.3% -- whose unsaponifiable fraction was almost one-third glyceryl ether (cf.[46]). The oil from the rest of the viscera was also strongly positive for these compounds.

(d) Crustaceans. The Cape spiny lobster, the only arthropod examined, was treated on a carefully differentiated anatomical basis, and all the oils analysed proved to contain very appreciable amounts of α-glyceryl ethers -- the unsaponifiable fraction obtained from the stomach oil having the highest content of these substances. It is to be noted

that here, as in the Octopus, the main fat depot was the hepato-pancreas.

(e) Amphibians, Reptiles, Birds. The Cape Clawed Toad (Xenopus laevis) which has been the object of much experimental work in South Africa and elsewhere, and has formed the basis of a test for pregnancy[57], was examined as representative of the Amphibians, and the Mole snake, as a reptile. The visceral and body oils of both proved positive, and it is of interest to note that the liver oil of the former gave a negative result. Among the birds the domestic fowl was chosen, but the three oils examined showed only small α-glyceryl ether contents.

(f) Mammals. Of the two whale liver oils tested, that of the Humpback whale gave a small positive result. The head oil of a sperm whale (spermaceti) which might have been expected, from its high content of saturated fatty alcohols, to contain considerable quantities of glyceryl ethers, was found to contain only a very small amount. The white rat proved to contain α-glyceryl ethers in the liver, body cavity, intestinal and faecal fats, but the subcutaneous fat was devoid of these compounds. A sample of human liver oil in contrast to that of the rat, gave a negative result. In view of the isolation of batyl alcohol from ox-marrow fat by Holmes et al[50], the present technique was applied to a sample of this material. The glyceryl ether content was of the order reported by these workers.

(g) Vegetable fats. The vegetable oils examined, with the exception of a sample of Tung oil, gave negative or negligible results.

RESULTS

Specific name		Common name	Oil derived from***	% unsaponifiable matter in oil	Glyceryl ether content of unsaponifiable matter, as %age selachyl alcohol	Glyceryl ether content of oil, calculated as selachyl dioleate
PISCES[41]						
(a) **Elasmobranchii**						
Heptranchidae						
Heptranchias pectorosus	(1)	Seven-gilled, or bulldog shark	Liver	22.3(9)	94.3*	55.7
	(2)		"	19.5(3)	89.2**	44.4
			intestine	59.1(2)	3.0	4.5
			flesh (0.4%)	12.4(3)	20.6	6.5
	(3)		liver (40.4%)	26.1(1)	88.7*	58.9
Hexanchus griseus	(1)	Six-gilled shark	" (60%)	22.8(2)	77.7*	45.1
	(2)		"	32.1(0)	92.7*	77.5
Carcharinidae						
Carcharinus walbeehmi		Walbeehm's sharp-nosed shark	"	3.6(8)	20.5*	1.9
Carcharinus melanopterus		Lazy grey shark	"	35.6(0)	6.1	5.5
" obscurus	(1)	Dusky shark	"	5.4(3)	9.4*	1.3
" "	(2)		" (58%)	7.9(5)	0.8	0.2
" glaucus		Blue shark	"	8.7(2)	52.5*	7.2
" limbatus		Blackfin shark	"	3.1(0)	21.2*	1.7
Galeocerdo arcticus		Tiger shark	"	13.1(0)	79.6*	27.1

Galeorhinus canis	(1)	Soupfin shark	liver	1.8(5)	17.7	0.8
	(2)		"	3.0(0)	34.4*	2.6
	(3)		"	1.8(7)	24.2	1.2
	(4)		embryo liver (44.3%)	8.8(2)	0.4	0.1
			yolk sacs (14.0%)	10.3(6)	2.1	0.6
Mustelus laevis	(1)	Smooth hound	liver (46%)	5.1(2)	22.8$	3.0
	(2)		" (46%)	4.2(3)	22.5	2.8
" canis		Common hound	"	2.7(1)	28.5	2.0
Sphyrna zygaena		Hammerhead shark	"	4.1(0)	0.0	0.0
Isuridae						
Isurus glauca		Blue pointer shark	" (6.5%)	4.3(2)	18.7	2.1
Carcharodon carcharias		Man-eating shark	"	36.1(5)	0.0	0.0
Cetorhinus maximus	(1)	Basking shark	" (70%)	47.7(5)	0.9*	1.1
			flesh (10.1%)	21.3(9)	96.2	52.4
			kidney (0.8%)	45.9(0)	2.8	3.3
			spleen (0.52%)	59.5(3)	8.5	12.9
			epididymis (0.52%)	53.3(3)	5.5	7.6
	(2)		liver	32.7(5)	1.6	1.3
	(3)		" (78%)	35.3(2)	2.3	2.0
			flesh (5.4%)	19.2(5)	60.0*	28.9
Odontaspidae						
Carcharias taurus		Slender toothed shark	liver	2.4(1)	16.9*	1.0
Scylliorhinidae						
Scyliorhinus capensis		Cape dogfish	" (23.2%)	2.5(6)	14.9	1.0
" africanus		Striped dogfish	" (42.3%)	2.9(2)	9.5	0.7
" pantherinus	(1)	Variegated dogfish	" (46%)	2.9(5)	13.3	1.0
	(2)		" (43.8%)	2.4(3)	16.6	1.0

Squalidae						
Echinorhinus spinosus	(1)	Spiny shark	liver (70%)	45.9(5)	10.7**	12.5
	(2)		"	49.7(0)	9.5*	12.0
Squalus spp.		Dogfish	"	27.4(0)	77.3*	53.9
Centroscymnus fuscus	(1)	Deepsea dogfish	" (71.5%)	91.5(0)	1.5**	3.6
	(2)		" (60.0%)	86.7(0)	2.6*	5.7
" species			" (90%)	94.1(0)	1.2**	2.8
			flesh (0.4%)	22.3(3)	2.2	1.2
Batoidei						
Rhinobatus spp.		Sand shark	liver	3.5(8)	24.7%*	2.3
Chimaeridae						
Chimaera monstrosa		Common chimaera	" (58%)	74.8(0)	14.5*	27.6
Callorhynchidae						
Callorhynchus capensis		Cape Joseph	" (50.0%)	2.4(4)	66.6*	4.1
(b) **Teleostomi**						
Sardina sagax	(1)	Sardine	whole fish	4.0(9)	2.1	0.3
	(2)		"	2.9(7)	6.5	0.5
Congermuraena australis		Cape eel	liver (5.6%)	11.8(0)	8.7	2.6
			body (2.9%)	6.9(6)	4.2	0.8
Mugil cephalus		Haarder or Bullet	liver (7.2%)	5.7(9)	1.3	0.2
			intestine (11.6%)	9.3(0)	1.5	0.4
			Head (19.3%)	2.8(5)	0.4	<0.1
Merluccius capensis		Stockfish (Hake)	liver	3.5(5)	15.5	1.4

Austroglossus microlepsis	Super sole	liver (9.9%)	9.5(2)	9.3	2.3
		body (4.9%)	5.3(1)	36.7	5.0
Polyprion americanus	Stone bass	liver (4.2%)	22.3(5)	12.8	7.3
Seriola lalandii	Yellowtail (albacore)	"	8.4(3)	17.5	3.8
Sciaena hololepidota	Kabeljaauw	"	8.7(3)	2.7	0.6
"		head	3.2(6)	11.3	0.9
Pagrus gibbiceps	Red stumpnose	liver	14.4(3)	5.9	2.2
Pagellus erythrinus	Rosefish	" (7.2%)	5.9(1)	10.3	1.6
		body (4.1%)	2.9(5)	12.5	0.9
		head (10.0%)	3.4(3)	14.4	1.3
Boopsidea inornata	"Fransch Madam"	total viscera (2.6%)	17.5(0)	4.8	2.1
		body (3.8%)	3.7(6)	10.8*	1.0
		head (7.2%)	4.0(2)	6.0	0.6
Dentex rupestris	Red steenbras	liver	51.2(0)	1.6	2.0
Dentex undulosus	Seventy-four	"	6.4(3)	4.1	0.7
Caranthus aeneus	Blue hottentot	entire fish (4.0%)	3.8(1)	0.0	0.0
Box salpa	Bamboo fish	entire fish (5.3%)	3.0(7)	2.3	0.2
Gymnocrotaphus curvidens	John Brown	liver	(oil saponified in tissue)	1.7	-
"		body (1.3%)	9.1(5)	7.4	1.7
"		head (9.1%)	3.2(0)	21.0	1.7
Scomber colias	Mackerel	head	7.2(0)	3.5	0.6
		body	9.4(0)	1.4	0.3

Thunnus thynnus	Tunny	liver	40.5(4)	3.5	3.6
Sarda sarda	Bonito	"	7.6(1)	4.8	0.9
"		head	1.2(3)	6.5	0.2
Tetrodon species	Toby or "Blaasop"	liver (23.6%)	3.2(1)	13.0*	1.1
Lophius piscatorius	Angler-, or monk-fish	"	2.6(2)	0.6	<0.1
MOLLUSCA					
Mytilus meridionalis (Krauss)	Blue mussel	whole animal minus shell (1.2%)	24.8(3)	4.1	2.6
Oxystele tigrina (Dillwyn)	Periwinkle	whole animal (1.3%)	16.1(1)	3.6	1.5
Patella granularis (Linn.)	Limpet	whole animal minus shell (1.85%)	22.1(5)	1.2	0.7
Haliotis midae (Linn.)	Abalone or Venus ear	total viscera (1.9%)	13.5(0)	7.7	2.6
Turbo sarmaticus (Linn.)	Top shell	whole animal minus shell (1.3%)	26.7(4)	1.43	0.8
Octopus rugosus (Bosch)	Rock octopus	hepato-pancreas (13.3%)	7.4(3)	30.1	5.7
		rest of viscera (1.0%)	27.5(5)	11.4	8.0
ARTHROPODA					
Jasus lalandii (M.Edw.)	Cape spiny lobster (Crayfish)	flesh (0.5%)	23.1(2)	10.2*	6.0
		stomach (1.3%)	11.9(1)	19.9	6.0
		intestine (0.3%)	38.3(4)	0.9	0.9
		gonads (6.5%)	8.4(7)	9.3%	2.0
		hepato-pancreas (21.4%)	5.3(0)	17.6	2.4

VEGETABLE OILS

peanut (groundnut)	---	0.5(C)	0.0	0.0
cotton-seed	---	1.0(G)	0.0	0.0
sesame seed	---	2.3(6)	0.2	0.0
tung nut	---	0.6(4)	1.9	<0.1
castor-seed	---	0.7(2)	0.2	0.0

* Indicates close agreement between reagent consumed and formaldehyde generated
** Volumetric determination.
*** Where available, yield of oil is indicated in brackets.

EXPERIMENTAL

The experimental methods used were those described in the previous section. . In the case of the vegetable fats, as much as 20 gm. of oil was taken for each test. Throughout the work, many cases occurred in which there was discrepancy between the amount of reagent consumed and the amount of formaldehyde generated. One interfering factor was found to be vitamin A, which reduced both periodic and iodic acids, with the liberation of iodine. That this is not the only source of interference has been shown by the occurrence of discrepancies in the case of many oils known to be devoid of Vitamin A. The existence of α-glycols other than α-glyceryl ethers in the unsaponifiable fractions of such oils cannot be excluded completely. These points are the subject of further studies.

APPLICATION OF THE FITELSON METHOD OF "SQUALENE" DETERMINATION TO SOME MARINE OILS

Since the initial detailed reports by Tsujimoto[50] of the occurrence of an unsaturated hydrocarbon — for which he proposed the name "squalene" — in the liver oils of several sharks, much attention has been directed to this compound. In addition to extensive survey studies on marine animals by Japanese[58] and French[59] workers, constitutional and synthetic investigations were carried out by a few groups of workers[60]. The combustibility of this hydrocarbon[61], its pyrogenetic conversion to an artificial petroleum[62], and more recently, its polyisoprene character and relationship to rubber[63] have been extensively studied. Its biological significance has also been considered[55,64], but even at this stage little advance has been made.

Although among marine animals squalene occurs in gross quantities only in some elasmobranch types, it is not restricted to this sub-class, but has been found in cod-liver oil[64c,65], in herring and whale oils[49], and in other teleost fish oils[66].

A most interesting occurrence of this compound is that reported by Dimter, who found squalene in the fat of the human ovarian dermoid cyst in noteworthy amounts, although it could not be demonstrated in the unsaponifiable residue of the serum, or the depot and liver fats[67]. In the plant world squalene has been found, often in some quantity e.g. in yeast fats[68] (16.8%) and in other fungi[69]. Its probable occurrence in pinebark oil has also been reported.[70]

Many more usual vegetable fats have been examined by various workers utilising the chromatographic method[71], the formation of the hexahydrochloride[72], the high iodine value[73], or combinations of the first-mentioned method with either of the other criteria to detect and determine squalene[32,74,75*], but no generally applicable method was formulated.

Fitelson[32] has demonstrated the separation of hydrocarbons from the unsaponifiable fractions of fats by a chromatographic procedure, and, using the Rosenmund-Kuhnhenn[76] ** method, has determined the unsaturation of this fraction, from which the "squalene" content of the oil may be calculated. This technique is of particular importance in the analytical characterisation of olive oils, the unsaponifiable fractions of which are rich in squalene, and provides a means for the detection of olive oil in edible oil mixtures.

In the course of the investigation, Fitelson[32] and his colleagues[74] examined a large variety of fats, including however only two of marine origin. Since squalene is so widely distributed in marine animal oils[75], often in gross amounts, it was considered that this analytical method would be of importance in the general examination of fats of marine origin. Moreover, in these materials squalene may be associated with large amounts of a wide range of other substances -- fatty alcohols, α-glyceryl ethers, sterols, Vitamin A, etc. It seemed relevant, therefore, to record some results (Table I) of the application of the Fitelson method to a number of marine fats.

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* Contains a tabulated review of the literature on the occurrence of squalene.

** See Appendix C.

In the case of unsaponifiable fractions containing very large proportions of α-glyceryl ethers [Table I, (a) 89.2%; (b) 88.7%], of fatt· alcohols (c) and of sterols [(i) 91.6% calculated as cholesterol] effective retention of these substances in the simple chromatographic column recommended by Fitelson could be ensured if suitable quantities of material were used, and thus high results due to their passage through the column into the eluate avoided. With certain fats containing appreciable amounts of Vitamin A, however, [e.g. (g)(n)] two "abnormal" effects have been observed. In the one a faint yellow band moved rapidly down the tube into the eluate. This band could often be retained in the column if less material was taken, or if a longer column was used [e.g. (g)]. In the other, [e.g. (f)] a yellow "cloud" moved very rapidly through the column and into the filtrate. Both these yellow substances reacted with $SbCl_3$ in chloroform to give a blue colour, but neither of them was Vitamin A, since, after their removal from the column, the issuing filtrate failed to give the Carr-Price test until the elution of the Vitamin A fraction with benzene, or a benzene-alcohol mixture at a much later stage. Preliminary spectrographic observations carried out on the yellow material noticed in the first type of behaviour indicate that it is probably related to anhydro Vitamin A[77], either present in the original oil, or formed during the saponification in alcoholic medium[78,79] Both phenomena will be fully investigated using larger quantities of material.

It is in its application to unsaponifiable fractions containing large amounts of unsaturated hydrocarbons (e.g.

Basking shark, deepsea dogfish, spiny shark and chimaera liver oils) in which interference from Vitamin A or related substances is remote, that the Fitelson technique is of particular value, since the analyses indicate that the oils of a given species conform to a general type (cf. Basking shark and Centroscymnus liver oils in respect of "squalene" content of their unsaponifiable fractions). The unsaponifiable fractions of chimaera and spiny shark liver oils are interesting in that they contain very appreciable amounts of α-glyceryl ethers (9.5% and 14.5% respectively), which together with the squalene present make up 97.2% and 98.5% respectively of the total unsaponifiable matter. The "squalene" contents of the unsaponifiable fractions of the Centroscymnus oils are of interest as indicating the possible presence in these oils of hydrocarbons of greater unsaturation than squalene itself. In contrast, detailed studies of Cetorhinus liver oils have shown that in this case more saturated hydrocarbon material[80] accompanies the squalene. The flesh oil of a Centroscymnus gave a surprisingly high result for "squalene".

TABLE I

Specific name[41]	Common name	Oil from	Unsaponifiable matter content of oil %	Percentage unsaturated hydrocarbon calculated as squalene in Unsaponifiable fraction	Oil
a) Lepidorhinus pectoralis	Seven-gilled shark	liver	15.4(3)	1.2(3)	0.22)
b) Hexanchus griseus	Six-gilled shark	"	22.1(2)	1.1(3)	0.27
c) Physeter macrocephalus	Sperm whale	head	67.1(2)	0.2(3)	0.1(3)
d) Congermuraena australis	Cape eel	liver	11.2(4)	1.3(1)	0.1(2)
e) Lophius piscatorius	Angler fish	"	2.2(2)	6.6(1)	0.1(2)
f) Galeus melastomus	Kabeljauw	"	8.7(3)	6.6(3)	0.4(1)
g) Galeorhinus canis	Soup-fin shark	"	2.6(3)	1.7(7)	0.05
h) Carcharodon carcharias	Man-eater	"	55.1(3)	82.6(3)	53.56)
i) Cetorhinus maximus (1)	Basking shark	"	67.7(3)	82.6(3)	39.31)
(2)		kidney	23.2(1)	3.9(3)	1.03
(3)		liver	32.7(3)	74.0(3)	24.62)
(4)		"	56.3(1)	81.6(3)	20.63)
(5)		"	49.7(2)	*84.7(9)	41.63)
(6)		"	41.1(3)	80.2(7)	37.07)
j) Echinorhinus spinosus	Spiny shark	"	43.2(1)	87.7(2)	43.01)
k) Centroscymnus fusca (1)	Deep-sea dog-fish	"	81.2(1)	83.7(2)	90.33)
(2)		"	79.2(1)	**101.3	80.02)
l) Centroscymnus owstoni (1)	+Cape	"	96.1(1)	101.2	95.23)
(2)		flesh	22.3(3)	12.2(2)	2.00)
(3)		liver	91.2(1)	***100.1	91.32)
m) Chimaera monstrosa	Common Chimaera	"	76.3(1)	84.0(1)	62.63)
n) Arctocephalus pusillus (Sobrebar)	Seal	"	29.2(1)	1.7(1)	0.50

* Iodine Value of unsaponifiable fraction = 333.3
Iodine Value of eluted hydrocarbon = 330.0

** Iodine Value of unsaponifiable fraction = 350.9

*** Iodine Value of unsaponifiable fraction = 359.3
A solution of this unsaponifiable fraction gave no precipitate with 1% alcoholic digitonin

Calculated Iodine Value of Squalene = 371.5

+ From the Angola Coast.

EXPERIMENTAL

Preparation of Unsaponifiable Matter

The unsaponifiable matter was extracted by the S.P.A. method , using four extractions . Fitelson's recommendation that the unsaponifiable fraction be extracted in two steps with petroleum ether was not followed, because in all cases the total unsaponifiable matter was required for other purposes.

Estimation of Squalene

The directions of Fitelson were followed exactly* except that 2 gm. of oil usually provided sufficient unsaponifiable matter for at least one determination. The following quantities gave good results, for a 10 x 0.8 cm. column of alumina :

Unsaponifiable fraction high in content of	Weight of material used per chromatogram	Volume of Rosenmund-Kuhnhenn reagent used
Fatty alcohols	Not more than 120 mg.	10 ml. probably sufficient
α-glyceryl ethers	" " " 200 "	" " " "
Sterols	" " " " "	" " " "
Vitamin A	About 50 - 60 mg.	" " " "
Unsaturated hydrocarbons	25 - 35 mg.	15 - 20 ml.

Note : B.D.H. "Aluminium oxide for chromatographic adsorption analysis" was used throughout.

* See Appendix B.

It is recommended that the whole determination (i.e. chromatogram and titration) be carried out in duplicate at least. In the case of oils containing considerable amounts of Vitamin A some adjustment of the amount of material taken, or height of the column, was necessary, if the rapidly moving yellow band was to be retained. Where a yellow "cloud" passed down the column retention was virtually impossible.

Extent of Interference by Yellow Material

Some idea of the extent of the inaccuracy due to elution of Vitamin A-related matter may be gained from the figures for the seal liver oil given in Table I. The unsaponifiable fraction of this oil (unsaponifiable matter content = 29.61%; percentage of Vitamin A in the unsaponifiable fraction = 50%) was chromatographed (0.1888 gm.) and the moving band allowed to pass into the filtrate, which was distinctly yellow. Despite this, the "squalene" content, based on the unsaturation of the residue after removal of the petroleum ether was only 0.50% of the oil. An identical result was obtained using 0.0944 gm. of material. The error involved is thus small where the method is used to estimate more than traces of unsaturated hydrocarbons.

Precision of the Method

In cases of low unsaponifiable matter content where two separate preparations of unsaponifiable matter were chromatographed, agreement was of the order indicated by Fitelson. If aliquots of the same unsaponifiable matter solution were taken for analysis, the precision of results was of course greater.

THE UNSAPONIFIABLE FRACTION OF THE LIVER OIL OF THE SEVEN-GILLED SHARK - Heptranchias pectorosus

A tentative report on the α-glyceryl ethers present in this species

Fatty acid studies on several marine oils are going forward in these laboratories, and it was considered of interest to attempt analogous fractionation studies on the α-glyceryl ether fractions of oils containing those compounds[cf. 81]. Lovern[56] has regarded the existence of α-glyceryl ethers in marine oils as being a special case of hydrogenation, and if the α-glyceryl ethers are formed by the hydrogenation of the corresponding esters, the distribution of the various α-glyceryl ethers should bear some relationship to the fatty acid distribution in the same oil. It is, therefore, the object of the present studies to examine the α-glyceryl ethers of several oils, and to determine whether there is any correlation between the straight-chain alcohol residues of those compounds and the fatty acids of the oils.

The liver oil of Heptranchias pectorosus is one admirably suited to this investigation, since the livers of this species are rich in oil and high in unsaponifiable matter content -- mainly α-glyceryl ethers. The particular oil selected for the present study had the following characteristics.

Unsaponifiable matter content						=	19.5%
α-glyceryl ether content of the unsaponifiable fraction						=	*89.2 %
Squalene	"	"	"	"	"	=	1.1 %
Vitamin A	"	"	"	"	"	=	0.5 %
Cholesterol	"	"	"	"	"	=	6.4 %

* as selachyl alcohol.

It is clear that any fractionation of an unsaponifiable fraction of this nature should be carried out on the material rendered sterol-free, and acetylated. However, only a very small quantity of digitonin was available, and the total unsaponifiable matter was acetylated and subjected to a preliminary distillation at high vacuum (0.1 mm.Hg) in an attempt to remove the bulk of the α-glyceryl ether acetates from the steryl acetates, and the small amount of Vitamin A acetate. The former object was not realised, but the distillate was Vitamin A-free, and the small residue, which was black and tarry, was discarded. No distillate was obtained at a temperature which would have indicated the presence of squalene or of fatty alcohol acetates. A fractionation of a fully hydrogenated sample gave no better result as far as the removal of sterols was concerned.

The distillated acetates were then carefully fractionated, and the iodine value and saponification equivalent of each fraction determined in the usual way. A further analytical figure, the "equivalent weight" of each fraction determined from its consumption of periodic acid was obtained, referred to as the "periodic acid equivalent". It is clear that, for pure α-glyceryl ether acetates, the saponification equivalent and the "periodic acid equivalent" are the same, but for fractions containing also acetates of other hydroxy bodies (e.g. sterols, fatty alcohols) which are not α-glycols, the "periodic acid equivalent" will exceed the saponification equivalent, since periodic acid is specific for α-glycols.*

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* This unsaponifiable fraction has been found to give close agreement for glyceryl ether content by both volumetric and gravimetric methods, i.e. no interfering substances are present.

From these analytical figures it became clear that steryl acetates started distilling over with the α-glyceryl ether acetates at quite an early stage.

However, from these figures, the amount of cholesteryl acetate in each fraction could be calculated, and consequently, the true weight of glyceryl ether acetate. From this latter weight and the periodic acid titre it was possible to calculate the true saponification equivalent of the α-glyceryl ethers in each fraction. That this method has a legitimate basis has been shown by check estimations on several fractions with digitonin, from which the amounts of cholesteryl acetate found agreed moderately well with those calculated (Table I). That the sterol present is almost pure cholesterol has been shown by splitting the digitonides obtained, and examining the melting points and mixed melting points with pure cholesterol, of the once recrystallised sterol fraction. The corrected saponification equivalents also show reasonable agreement when the correction has been made by both methods (Table II).

TABLE I

Fraction	Steryl acetate content (% weight)	
	Digitonin method	Volumetric difference method
10	5.2	5.9
11	9.0	8.9
12	15.1	16.0
13	-	19.2
14	27.5	27.2

TABLE II

Fraction	Corrected saponification equivalent	
	from digitonin results	from titration results
10	214.3	211.9
11	216.0	213.8
12	214.5	211.5
13	-	213.2
14	213.8	213.0

From these figures then, it has been possible to calculate the weights of α-glyceryl ether acetates present in each fraction, the true iodine value of those compounds (Table III), and, finally, the amounts of the various glyceryl ethers present (Tables IV and V).

TABLE III

No. of Fraction	Weight of glyceryl ether acetate	Saponification equivalent	Iodine value
1	3.2698	200.4	34.8
2	5.8552	205.3	33.6
3	7.4913	208.0	37.5
4	7.3784	208.2	38.5
5	8.8471	208.1	39.5
6	6.8968	208.1	41.1
7	7.1733	208.6	43.0
8	7.4141	209.2	45.9
9	7.0112	209.3	48.9
10	6.5471	213.1	52.6
11	5.4821	214.9	55.8
12	5.0469	213.0	61.2
13	1.0799	213.2	67.5
14	1.4254	213.4	66.1
Saddles	3.2573	225.0	87.3

TABLE IV

Glyceryl ether acetates (gm)

Fraction No.	Saturated Sidechain			Unsaturated Sidechain		
	C 14	C 16	C 18	C 18	C 20-2H	C 22-4H
1	1.342	0.029	-	1.899	-	-
2	0.463	2.105	-	3.287	-	-
3	-	2.454	0.343	4.694	-	-
4	-	2.296	0.335	4.747	-	-
5	-	2.797	0.211	5.839	-	-
6	-	2.155	0.005	4.737	-	-
7	-	1.957	0.062	5.154	-	-
8	-	1.664	0.064	5.686	-	-
9	-	1.282	-	5.729	-	-
10	-	0.717	-	4.590	1.240	-
11	-	0.281	-	3.735	1.466	-
12	-	-	-	4.322	0.674	0.053
13	-	-	-	1.012	0.016	0.052
14	-	-	-	1.305	0.051	0.057
Saddles	-	-	-	0.532	2.108	0.616
	1.805	17.789	1.020	57.168	5.555	0.778

TABLE V

Component α-glyceryl ethers (% weight)

Calculated (1) as acetates on the acetylated glyceryl ethers of the unsaponifiable matter distilled
(2) as free glyceryl ethers on the glyceryl ether fraction of the unsaponifiable matter distilled

Sidechain	Saturated C 14	C 16	C 18	Unsaturated C 18-2H	C 20-2H	C 22-4H
	skesyl[82] alcohol	chimyl alcohol	batyl alcohol	selachyl alcohol		
(1)	2.1(5)	21.0(9)	1.2(1)	67.9(2)	6.6(0)	0.9(3)
(2)	2.0(3)	20.8(4)	1.2(3)	68.2(1)	6.6(3)	0.9(6)

A calculation on the last three fractions regarding them as mixtures of two unsaturated components (selachyl alcohol and a $C_{22-2\alpha H}$-sidechain component) showed a decrease in α, the average unsaturation of the non-selachyl alcohol portion, as follows : fraction 13 α = 3.8
" 14 α = 3.1
Saddles α = 2.2

It was therefore considered more desirable to calculate the results as a mixture of three unsaturated components -- selachyl alcohol, a component with a C_{20-2H}-sidechain, and one with a C_{22-8H}-sidechain.

Comparison of the above results with those obtained from a fractionation of the fatty acids of the same oil[83] is interesting. It would appear that there is a far greater proportion of C_{18}-sidechain glyceryl ethers than C_{18} fatty acids. The C_{14} and C_{16}-sidechain glyceryl ethers are of the same order as the corresponding acids, but the C_{20} and higher acids are more plentiful in the acid fractions than C_{20} -- and higher -- sidechain α-glyceryl ethers in the unsaponifiable fraction.

TABLE VI

Fatty acid figures for the liver oil of Heptranchias pectorosus[83]

	Saturated					Unsaturated					
	C 14	C 16	C 18	C 20	C 22	C 14-2H	C 16-2H	C 18-2,6H	C 20-5.4H	C 22-8.7H	C 24-10H
%	1.6	16.6	6.9	1.4	1.6	0.7	11.0	30.3	15.6	12.9	1.4

It is noteworthy that the very highly unsaturated, high molecular weight α-glyceryl ethers found by Japanese workers[81] were not detected, and it is possible that such substances may have been present in the material under examination and discarded with the tarry residue of the preliminary distillation. The above workers precipitated the highly unsaturated glyceryl ethers as their bromo-addition compounds, regenerated them, distilled the acetates and computed the components. The compounds were characterised by identification of the fatty acids obtained after treatment of the glyceryl ethers with HI, and oxidation of the fatty alcohols subsequently obtained.

The saddle fraction in the present distillation, a light-coloured semisolid material, gave, after saponification and removal of the cholesterol, a viscous liquid fraction. This material gave no positive results on chromatographic treatment, and the white solid obtained after catalytic hydrogenation and several recrystallisations, melted over a big range. The 3,5-dinitrobenzoates were no easier to deal with. Insufficient material was available for further work on this, or on the head fraction containing skeeyl alcohol.

The existence of β-glyceryl ethers in this unsaponifiable fraction seems unlikely from the fact that there was agreement between sterol determined with digitonin and that from the titre-difference. This agreement suggests that the sterols and the α-glyceryl ethers are the only hydroxy bodies present.

These preliminary results have, it is felt, been sufficiently interesting to warrant a further attack on the problem, now in progress. Digitonin is now available, and

it is essential that a sterol-free unsaponifiable fraction of this oil be fractionated under more accurate conditions than those here employed, and the range of α-glyceryl ethers recalculated. Isolation of the several compounds will be attempted (in the present study only chimyl and batyl alcohols were obtained pure, former m.p. 61.6°C. latter m.p. 71°C.). It is also intended to prepare certain of the compounds appearing in the calculated range for possible comparisons.

EXPERIMENTAL

Extraction of Unsaponifiable Matter

It has been found that the large scale saponification, and extraction of the unsaponifiable matter of this oil is a matter of some difficulty,* so that batch saponifications and extractions using 50 gm. of oil at a time, and six extractions with ether, all according to S.P.A. ratios were carried out, and proved satisfactory. The material obtained was resaponified in the same way.

Removal of traces of Fatty Acid

A solution of the unsaponifiable fraction (about 130 gm) in ether (1500 ml.) was passed through a column of alumina (20 cm. x 2.0 cm.) and the ether then removed.[84]

Acetylation of the Unsaponifiable Fraction

The unsaponifiable matter was boiled gently under reflux for 90 minutes with 2 - 3 times the theoretical amount of acetic anhydride, and the excess removed on a water-bath under vacuum, first at the water-pump, and later at a high-vacuum pump.

Distillation of the Acetylated Material

A preliminary distillation was carried out on about 100 gm. of acetylated material, from a Claisen flask, at high vacuum (0.1 mm. Hg.). The residue (2.7 gm.) was a dark tarry mass, with separating crystalline material. This residue analysed at 36.7% cholesteryl acetate, and the cor-

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* Information by courtesy of Dr. N.J. van Rensburg.

rected saponification equivalent of the glyceryl ethers present calculated as 269.5. (Note :- In future work, similar fractions will be chromatographically treated, and the results obtained from the separated glyceryl ethers included in the total scheme.)

The fractionation was carried out in a simple form of fractionating flask as described by Hilditch.[2c] The bottom half of the column was lagged with asbestos string. The distillation was smooth, but the range of temperature extremely small. Fraction 1 came over between 190 - 206.5°C. (0.1 mm. Hg) and fraction 2 at 200 - 202°C. After this, the temperature remained constant between 206 - 214°C., and for fractions 11 - 14 was falling, slowly at first, but rapidly in fraction 14. The bath temperature was raised from 275°C. for fraction 1 to 345°C. in fraction 14.

Determination of Iodine Value

The Wijs method was used, with a contact time of 1 hour.*

Determination of Saponification Equivalent

The saponification equivalent was determined on samples of about 0.8 gm. using 20 ml. of $\frac{N}{2}$ alcoholic NaOH, and back-titrating with $\frac{N}{2}$ 50% alcoholic HCl, to phenolphthalein or alkali-blue 6B indicator.

Note :- The use of alcoholic NaOH in preference to KOH prevented precipitation of periodate in later stages of the analysis; 50% alcoholic HCl gave final titrated solutions which did not deposit sterols or glyceryl ethers. Washing was carried out with alcohol.

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* See Appendix C.

Determination of the "Periodic Acid Equivalent"

The titration mixture from above, after titration, was carefully transferred to a 100 ml. standard flask, and made up to volume with alcohol; 5 ml. aliquots were then oxidized with periodic acid exactly as has been described , except that 2 ml. of water were added to the aliquot before the periodic acid reagent, to prevent precipitation of sodium periodate. Blanks were carried out in an identical fashion on the blanks from the saponification equivalent determination above.

The results were not of the standard of reproducibility of the ordinary volumetric determination of α-glyceryl ethers. The indicator used in the previous determination did not interfere.

Determination of Sterols

(1) With digitonin. The method outlined by Dam[26] was applied to the 100 ml. solutions above, digitonin solutions of a suitable strength being added to give final concentrations of this reagent similar to those recommended.* Digitonides where prepared in greater quantity were split with pyridine[85].

(2) The following formula gave the weight of cholesteryl acetate in any fraction :-

$$\frac{428.4}{1000}\left[\begin{pmatrix}\text{volume of Normal NaOH}\\ \text{used in the saponifi-}\\ \text{cation (ml.)}\end{pmatrix} - \begin{pmatrix}\text{20 x volume of Normal}\\ \text{Periodic acid used in}\\ \text{the oxidation (ml.)}\end{pmatrix}\right] gm.$$

Example

A four-times re-distilled sample of acetylated unsaponifiable matter from early fractions of a previous distillation was examined.

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* See Appendix E.

Saponification equivalent = 209.7 (mean)
"Periodic acid" " = 212.3 (mean)
Cholesteryl acetate content
(1) Digitonin method 0.45%
(2) Titration " 0.36%
Corrected Saponification equivalent = 207.0 (mean)
Iodine value 38.50

This calculated to

64.32% selachyl acetate
3.44% skeayl "
32.24% chimyl "

Calculation of Component α-glyceryl ethers

The calculations were carried out according to Hilditch [2b] and details for similar calculations by Rapson, Schwartz, Stoy and van Rensburg[86].

A CONTRIBUTION TO A COLLABORATIVE STUDY OF THE LIPINS OF CETORHINUS MAXIMUS (Gunner), THE BASKING SHARK

A general study of the lipins of the Basking Shark is in progress in this laboratory, and the following results represent some of the data so far assembled on the constitution of the unsaponifiable fractions of fats from several organs of a single specimen. They are of interest as an illustration of the application of three analytical procedures — i.e. for α-glyceryl ethers, unsaturated hydrocarbons and sterols to the unsaponifiable fractions. It will be seen that in only one case is 100% exceeded* — that of the unsaponifiable fraction of the flesh oil. In the case of the unsaponifiable fraction of the liver oil, it is felt that the low value for the total percentage of sterols, glyceryl ethers and unsaturated hydrocarbons found, is due to the presence in that fraction of saturated hydrocarbon material — e.g. pristane[80].

The flesh oil is of outstanding interest. It was obtained in considerable yield (10.1%) and, since the unsaponifiable matter content is high and almost entirely α-glyceryl ethers, the true glyceride content of the oil is abnormally low (calculated α-glyceryl ether di-oleate in oil = 58.4%). That this result was not an isolated instance, nor due to any confusion in sampling is illustrated by the analysis of a flesh oil obtained from another specimen. The unsaponifiable fraction of this oil (19.25%) contained 60.0% of α-glyceryl ethers.

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* This may possibly be explained by the calculation of glyceryl ethers as selachyl alcohol, and sterols as cholesterol.

It was considered that this high glyceryl ether content of the flesh oils might be characteristic of species containing much squalene in their liver oils, and accordingly the flesh oil was extracted from a specimen of Centroscymnus. In this fish the flesh contained very little oil, and the unsaponifiable fraction appeared to be mainly steroid in nature, containing but little α-glyceryl ether. The examination of flesh oils of other species whose liver oils are high in squalene content will be carried out. A detailed examination of Basking shark flesh oil is in progress.

The tissue of the eye-socket was characteristically soft and spongy, and the oil extracted therefrom was surprisingly high in squalene content. As may be seen from the Table, the oils extracted from the spleen, kidney and epididymis had large unsaponifiable fractions, the bulk of which was steroid in nature.

TABLE I

Specific name	Common name	Oil from	%age of oil yielded (on wet weight)	%age unsaponifiable matter content of oil	%age glyceryl ether (as selachyl alcohol) in unsaponifiable fraction	%age unsaturated hydrocarbon (as squalene) in unsaponifiable fraction	%age sterol (as cholesterol) in unsaponifiable fraction	Total %
Cetorhinus maximus 3/3/45	Basking shark	liver (1350 lbs)	72	47.7(3)	0.9	82.1(9)	0.4(1)	84.0
		flesh (from tail region)	10.1	21.3(6)	96.2	3.1(6)	2.8(6)	102.7
		spleen	0.5(2)	59.5(3)	8.5	6.0(4)	81.1(9)	94.7
		kidney	0.2	45.1(6)	2.8	3.1(3)	89.0(8)	95.8
		epididymis	0.5(2)	53.3(6)	5.6	2.1(8)	89.0(2)	96.9
		eye socket tissue	0.05	30.7(3)	-	13.6(7)	-	-
		*"Crisle" (directly under the outer skin)	-	21.1(3)	-	2.5(3)	-	-
Cetorhinus maximus 18/4/46	Basking shark	liver	78	35.5(8)	2.3	***81.5(5)	-	-
		flesh (from head region)	5.4	19.1(5)	60.0	3.6(1)	-	-
Centrescymnus species	** Cagao	liver	90.0	94.1(6)	1.2	101.2	-	-
		flesh	0.4	22.3(3)	2.2	12.9(2)	-	-

* A hard sheath-like tissue (about ¾" thick).

** From the Angola coast.

*** For "squalene" content of oils of other specimens see [3].

Vitamin A content of all oils above is negligible

EXPERIMENTAL

Extraction of Oils

All the oils, except the flesh oil of the Basking shark 18/4/46, were extracted by the alkali digestion technique[87]*. The exception was extracted, after desiccation with Sodium sulphate, in a Soxhlet extracter with ether.

Extraction of Unsaponifiable Matter

The unsaponifiable fractions were extracted and estimated by the S.P.A. method using four extractions with ether .

Determination of α-glyceryl ethers

The periodic acid oxidation method described previously was used , and the α-glyceryl ether content calculated from the dimedone-formaldehyde complex obtained.

Determination of Unsaturated Hydrocarbons

The Fitelson technique , the application of which to marine oils was discussed previously , was used.

Determination of sterols

The digitonin method was used, according to the details prescribed by Dam[26], i.e. 100% excess of 1% digitonin in alcohol, was added to 5 ml. of a 0.5% solution (with

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* See Appendix D.

respect to the sterols) of the unsaponifiable fraction in ethyl alcohol. The mixture was brought to the boil in a water-bath, allowed to stand overnight, the precipitate filtered off on a tared sintered glass crucible, washed with a small volume of alcohol several times (total = 8 ml.), dried at 100° C., and weighed.

Note :- Pure cholesterol gave, on analysis in this way, a result of 102.4%. The conversion factor (sterol : digitonide) 0.2431 was used.

THE PROVITAMIN D CONTENT OF SOME SOUTH AFRICAN MARINE INVERTEBRATES

The provitamin D content of the lipins of invertebrates, particularly marine forms, has been shown to be high in general[88], and the extraction and processing of their fats to yield crystalline provitamin D preparations has been the subject of several patents[89]. A few South African invertebrate types have been examined, and the results are recorded in Table I. All were molluscs, with the exception of the Cape Spiny lobster, an arthropod.

The unsaponifiable fractions of the fat of the blue mussels examined (Mytilus meridionalis) have been found to contain rather more provitamin D (as measured spectrographically by the absorption in alcoholic solution, at $E^{1\%}_{1cm}$ 281.5 mu) than those of the so-called "white mussel", or clam (Donax serra). The results obtained for the former animal were somewhat higher than those recorded for the mussel by Bock and Wetter[88] or Fantl , although of the same order as the provitamin D content claimed in the patent literature[89a].

The oils of the larger forms (Haliotis, Turbo and Octopus) appear to contain moderately little provitamin D; the crude sterols of even the viscera of Haliotis and Octopus gave low results. The figures obtained for the Crayfish, the oils of which were extracted from the separate organs, were similar. Of the species examined, Oxystele tigrina proved the best source of provitamin D and contained 33.1% of this substance in the crude sterol fraction.

TABLE I

Name	Common name	Part extracted	Percentage of total weight	Method of extraction	Oil yield on wet weight %	Unsaponifiable matter content of oil %	$E^{1\%}_{1cm}$ 281.5 mμ of unsaponifiable fraction	Percentage Provitamin D (on "crude sterols")
Mytilus meridionalis (Krauss)	Blue mussel	Total "meat"	17 (up to 35 when quite fresh)	Alkali digestion[87] ether extracted	0.9	22.3(7)	45.9	13.7
		Total "meat"	"	Pepsin digestion ether extracted	1.4	20.6(9)	45.5	14.2
		"	"	Minced, dried at 65° C. Extracted in Soxhlet with acetone	1.2	26.2(0)	48.0	15.0
Haliotis midae (Linn.)	Abalone (Venus ear)	Total viscera	-	Alkali digestion ether extracted	1.9	13.5(0)	19.8	6.0
		Total flesh	-	"	0.21	61.5(6)	4.2	1.3
Patella granularis (Linn.)	Limpet	Total "meat"	30.8	Desiccated with sodium sulphate ether extracted	1.3	22.6(1)	19.4	6.1
Donax serra	Clam ("white" mussel)	Total "meat"	30.0	"	1.4	20.4(5)	20.0	6.3

Oxystele tigrina (Dillwyn)	Periwinkle	Whole animal crushed	total meat = 36.8	Desiccated with sodium sulphate ether extracted	1.3 on total (3.5% on "meat")	13.5(0)	106.0	33.1
Turbo sarmaticus (Linn.)	Top shell	Total "meat"	28	"	1.3	20.7(4)	17.9	5.6
Octopus rugosus (Bosch)	Rock octopus	Digestive gland	-	Alkali digested ether extracted	13.3	7.4(5)	10.5	3.3
		Rest of viscera	-	"	1.0	27.5(6)	10.3	3.2
Jasus lalandii (H.Edw.)	Crayfish (Cape spiny lobster)	flesh	-	"	0.5	23.1(2)	4.5	1.4
		stomach	* 1.1	"	1.3	11.9(1)	7.7	2.4
		intestine	-	"	0.3	38.2(4)	14.0	4.4
		digestive gland	* 6.0	"	21.4	5.3(0)	6.7	2.1
		gonad	* 2.7	"	6.5	8.4(7)	10.1	7.8

* Calculated on crayfish "waste" i.e. (body minus tail)

EXPERIMENTAL

The oils were extracted from the tissues by the methods indicated in the Table. They were saponified, the unsaponifiable matter isolated by the S.P.A. method, and the technique, substantially, of Gillam and Heilbron[88] followed thereafter. A Hilger E3 Quartz spectrograph was used in the measurement of the ultra-violet absorption by alcoholic solutions of the crude sterol fractions. The bands were in most cases only moderately well defined, that at 281.5 mu being the clearest. The value obtained for the once re-crystallized unsaponifiable fraction did not appear to differ greatly from that obtained on a solution of the unsaponifiable fraction itself.

Several re-crystallizations from alcohol produced a crystalline material, from solutions of which the absorption curve of provitamin D could be accurately plotted. The results were calculated as ergosterol, using the figure $E^{1\%}_{1cm}$ 281.5 mu = 320 for a pure provitamin D preparation.

SUMMARY AND CONCLUSIONS

I. It has been shown that in the extraction and determination of the unsaponifiable matter of marine oils by the Society of Public Analysts' method[1], at least four extractions, and not the specified three, are required in order to obtain quantitative results. The effect of the nature of the constituents of unsaponifiable fractions on the efficiency of their extraction has been studied, and marked differences in the ease of their removal from the saponification mixtures found — ranging from the hydrocarbons, which are most easily extracted, to the α-glyceryl ethers which are not easily removed. The effects of the nature of the soaps, and their concentration in the saponification mixture have also received attention.

II. A method has been developed for the estimation of α-glyceryl ethers in unsaponifiable matter -- depending on their α-glycol nature. Periodic acid was selected in preference to lead tetra-acetate on account of its greater specificity. Results have been calculated from reagent consumed and formaldehyde generated, and the latter method is regarded as less subject to interference. It has been found in many cases that the amount of reagent consumed has been in excess of that required for the formaldehyde produced. In particular, Vitamin A, and its esters, have been found to be susceptible of attack by periodic acid -- and even by iodic acid — with the liberation of iodine. This anomalous reaction is worth further study. The presence of other sub-

stances capable of reducing periodic acid has been demonstrated, and the technique here elaborated should provide a weapon for their study.

This new method is also being applied to the direct study of the metabolism of the α-glyceryl ethers in rats.

III. A survey of the occurrence of α-glyceryl ethers has been carried out by the periodic acid oxidation method above, and α-glyceryl ethers detected and estimated by the formaldehyde produced. 118 fats have been examined, ranging from mollusc to mammalian fats, and including several vegetable oils. The occurrence of α-glyceryl ethers has been found to be general in fish fats.

From the many data it is now possible to select favourable raw materials for detailed studies. In addition these substances have been found in marine invertebrate and land animal fats, but not in any of the five vegetable fats examined.

IV. The conditions of a method for the chromatographic separation of squalene from vegetable oils have been developed as a method for the examination of the more complex unsaponifiable fractions of marine oils. The routine examination of marine unsaponifiable fractions for unsaturated hydrocarbons (squalene) has been carried out on 28 oils. It is believed that an extension of such a chromatographic technique will be possible for the determination of saturated hydrocarbons -- e.g. by "partition chromatography".

V. The unsaponifiable fraction of the liver oil of Heptranchias pectorosus has been examined and a tentative report is given on the various α-glyceryl ethers present — calculated from fractionation data. The oxidation method of II above has been extensively used in the determination of equivalent weights. The preliminary analytical approach described is being followed by studies aimed at the isolation of the various new glyceryl ethers whose presence is indicated by the analytical data.

VI. The unsaponifiable fractions of the oils from various organs of Cetorhinus maximus have been examined. Squalene has been found in several oils other than the liver oil, in which it is predominant. α-Glyceryl ethers do not occur to any great extent in any of the oils examined except that of the flesh, where the unsaponifiable fraction in one case was overwhelmingly composed of these compounds. Certain visceral oils have yielded unsaponifiable fractions of extremely high sterol content, but that of the liver contains only small amounts of sterols.

As new specimens become available, further studies will be carried out, and it is believed that such data will provide information on the physiological significance of squalene and the α-glyceryl ethers particularly.

VII. A short report is given of the Provitamin D content of the oils of some South African marine invertebrates, and as a result of this, the commercial production of lipin extracts from these animals is being undertaken.

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APPENDIX A

Abstract of :

The Society of Public Analysts' Shortened Method for the Determination of Unsaponifiable Matter in Fats and Oils[1]

Reagents :- Alcoholic KOH solution, approximately $\frac{N}{2}$, not darker than "pale straw".

Ethyl ether; acetone.

Saponification:- 2.0 - 2.5 gm. of oil or fat, accurately weighed is boiled for one hour, with occasional swirling, under a reflux condenser with 25 ml. of approximately, but not less than, $\frac{N}{2}$ alcoholic KOH solution.

Dilution:- After the saponification, during which no loss of alcohol should occur, the alcoholic soap solution is transferred to a separating funnel, and washed in with 50 ml. of water, in all.

Extraction:- The soap solution, while still just warm, is extracted successively three times with 50 ml. of ethyl ether. The first quantity of ether is used to wash out the saponification flask before it is added to the soap solution in the separating funnel.

Each extraction is made by shaking the separator vigorously, allowing the two layers to separate and clarify, running off the aqueous alcoholic layer at the bottom of the separating funnel, and pouring the ethereal solution from the top of the separating

funnel into another separating funnel containing 20 ml. of water.

If the ethereal extracts contain solid suspended matter, they are passed through a dry fat-free filter into the second separating funnel, and the filter subsequently washed with ether.

Preliminary water-washing:- The extracts are rotated, without violent shaking, with the 20 ml. of water, and after allowing to separate, the wash water is run off. The ethereal solution is then washed twice with 20 ml. of water, with vigorous shaking on each occasion.

Alkali and water-washing:- The ethereal solution is washed twice with 20 ml. portions of $\frac{N}{2}$ aqueous KOH solution by shaking vigorously on each occasion, and then with two or more successive quantities of 20 ml. of water, until the wash water no longer reacts alkaline to phenolphthalein.

Solvent removal:- The ethereal extract is transferred to a weighed flask, the ethyl ether distilled off, and the residue dried to constant weight, preferably with the aid of acetone, not allowing the temperature to exceed 80° C.

Note :- A "continuous loss (of weight) during drying" is mentioned in the method. Kirsten[7b] has recommended drying at 100° C., and in this laboratory, drying has been found to be quick and efficient under suction at a water pump at a temperature of 80-100° C.

APPENDIX B

Abstract of :

The Fitelson method for the "Detection of Olive Oil in Edible Oil Mixtures"[32]

Extraction of Unsaponifiable Fraction:-

5 gm. of oil are saponified, and the unsaponifiable matter extracted either by the S.P.A. method, above, or by the Grossfeld-Timm method[5f], using petroleum ether. (Note :- hydrocarbons are preferentially extracted in the latter method -- cf. Section 1. Since Fitelson prefers to calculate results on the weight of oil taken, the total unsaponifiable fraction is not required, and the use of the Grossfeld-Timm method becomes convenient.)

The unsaponifiable matter is dried thoroughly, and the last traces of solvent removed.

Adsorption :-

Reagents :-

Petroleum ether : Skellysolve

b.p. 65 - 70° C. or equivalent.

Aluminium oxide ; adsorbent :-

80 - 200 mesh.

("Adsorption alumina for chromatographic analysis"

Fisher Scientific Co., or equivalent.

Apparatus :- Adsorption column.

Prepared immediately before use. A glass tube 0.8 cm. inside diameter, and 30 cm. long, has a wad of cotton-wool placed in the constricted end. Alumina is added in small portions, and each portion gently tamped, with gentle suction, until a column 10 cm. long is formed. A small wad of cotton-wool is placed at the top of the column, and tamped lightly. The column is washed with ca. 15 ml. of petroleum benzin, and should be kept under a shallow layer of petroleum benzin until used.

Adsorption :- The dry unsaponifiable matter from 5 gm. of oil is dissolved in 5 ml. of petroleum ether, and quantitatively transferred to the adsorption column above. The filtrate is caught in a 250 ml. glass-stoppered iodine absorption flask, and should emerge dropwise at a rate of ca. 1 ml./60 secs., gentle suction being applied if necessary. When the 5 ml. solution has been nearly drawn into the column, 5 ml. of petroleum ether is added -- which has been used to rinse the vessel that contained the solution of unsaponifiable matter. The addition of solvent is continued in 5-10 ml. portions, the top of the alumina column always being covered, until a total of 50 ml. of petroleum ether has passed through the adsorption tube.

The petroleum ether is then removed by evaporation -- the last traces being taken off in a current of CO_2. The residue is then dissolved in $CHCl_3$ (5 ml.) -- and its unsaturation determined by the Rosenmund-Kuhnhenn* method, $\frac{N}{2}$ thiosulphate being used.

1 ml. 0.05 N. $Na_2S_2O_3$ ≡ 1.71 mg. Squalene.

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* See Appendix C.

APPENDIX C

Determination of Unsaturation, i.e. Iodine Value

Both the Rosenmund-Kuhnhenn (Section 4) and the Wijs (Section 6) methods have been used in this work.

(a) Rosenmund-Kuhnhenn or Pyridine-Sulphate Bromide method[76] (Abstract)

Reagent :- 8 gm. pyridine and 10 gm. of sulphuric acid are dissolved in 20 ml. of chilled glacial acetic acid. 8 gm. of bromine in 20 ml. of acetic acid are then added, and the mixture diluted to 1 litre with glacial acetic acid. (The reagent is about $\frac{N}{10}$)

Determination :-

The material under examination is dissolved in 10 ml. of $CHCl_3$, and treated with an excess of reagent (60% minimum), and allowed to stand for 5 minutes. The excess of reagent is then determined by adding potassium iodide solution, diluting the whole with water, and titrating the liberated iodine to starch indicator with standard thiosulphate.

Blanks are carried out.

(b) Wijs' method[90] (Abstract)

Reagent :- 9 gm. iodine trichloride is dissolved in 1000 ml. glacial acetic acid of at least 99% strength. Exactly 5 ml. of this solution is taken and its

halogen content determined by adding KI and water, and titrating with $\frac{N}{10}$ thiosulphate. 10 gm. of pulverised iodimix then dissolved in the bulk of the solution, and 5 ml. again taken, and titrated. The halogen content should be adjusted so that it is somewhat more than one-half more than it was originally. The solution is then filtered into a dark bottle with a glass stopper fitting tightly.

Determination :-

The material under examination is dissolved in carbon tetrachloride, and the reagent added. A contact time of 60 minutes has been allowed in this laboratory, and the excess halogen titrated with standard thiosulphate after addition of KI and water. Blanks are carried out. For a successful determination, not more than 30% of the halogen present in the amount of reagent used should be consumed.

APPENDIX D

Extraction of Oils from Fish Tissues[87]

Since the alkali-digestion method of extracting the oils from fish and other tissues was extensively used (particularly in Section 2) a brief abstract is given here.

The minced material is mixed with twice its weight of 1% Sodium hydroxide solution, and the mixture steamed until complete disintegration of the tissue has been effected. The mixture is then cooled, and extracted with peroxide-free ether — alcohol being added, where necessary to break emulsions.

The extracts are washed free of alkali, and the ether distilled off. The oils are dried under vacuum in a water-bath, alcohol being added to assist in the removal of last traces of moisture.

APPENDIX E

Determination of sterols with digitonin, according to Dam[10,20]

5 ml. of an alcoholic solution of sterols (0.3% with respect to the sterols) is treated with 10 - 10 of a 1% alcoholic digitonin solution [25 - 100% excess]. (The digitonin should be dried under vacuum at 98° C.)

Immediately after mixing the solutions, the beaker is heated in a boiling water-bath until the alcohol comes to the boil. The solution is then allowed to cool and stand overnight and the precipitate filtered off (on a tared sintered glass crucible) and washed with 25 - 30 ml. of alcohol saturated with cholesterol - digitonide. This solution must be freshly prepared.

The precipitate is then dried in the oven, cooled and weighed.

APPENDIX F

Determination of Vitamin A

Where figures for Vitamin A are given in this work they have been determined colorimetrically, using the Carr-Price antimony trichloride reaction, and an Evelyn photo-electric colorimeter[23].

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