The Analysis and Characterization of Trace Elements, in Particular Bromine, Selenium and Arsenic in Marine Organisms

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CONTENTS

PREFACE

The work that is summarized here was started about ten years ago, and it still continues. Eighteen papers that pertain to various facets of this study are included, together with some recent results that have not yet been published. The papers are indicated by Roman numerals and are listed chronologically in the section: "List of Papers".

As the work proceeded, its objectives have naturally come to fall into three major areas:

1. The determination of trace elements to be found in marine organisms.

2. The chemical states in which trace elements may occur.

3. Characterization of the organic compounds containing trace elements as a part of the molecule.

These three fields are all important to the understanding of distribution, absorption, accumulation and metabolism of trace elements in the marine organisms.

The growing interest in the determination of trace elements in marine organisms, due partly to their role in environmental problems and partly to a growing realization of the physiological role played by many of them in living organisms, has helped to point out the direction in which this work should be led. The interest shown by colleagues and laboratories throughout the conduct of this work has also been a great encouragement.

As a characterization of trace elements present in organisms, i.e. their chemical state, is highly depending on the analytical methods in use, the latter has been given a relatively broad treatment. It is the author's hope that this summary could initiate some new ideas or new approaches to problems in the field of trace element investigation.

Most of the experimental work has been carried out at the Central Institute for Industrial Research and the author wants to express his

gratitude to the Institute and its Director, Alf Sanengen, together with other members of the staff, for all kind cooperation.

The author is furthermore indebted to Kari Martinsen, Grete Tveten, Reidun Backe and Hilde Vagn Knudsen for their skilful technical assistance.

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Thanks are further due to Director E. Heen, Norwegian Fisheries Research Institute, for his interest in this work.

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January 1974 Gulbrand Lunde

LIST OF PAPERS

I Lunde, G. "Activation Analysis of Bromine, Iodine and Arsenic in Oils from Fishes, Whales, Phyto- and Zooplankton of Marine and Limnetic Biotopes". Int. Revue ges. Hydrobiol. 52 (1967) 265.

II Lunde, G. "Analysis of Arsenic in Marine Oils by Neutron Activation. Evidence of Arseno Organic Compounds". J. Am. Oil Chem. Soc. 45 (1968) 331.

III Lunde, G. "Activation Analysis of Trace Elements in Fishmeal". J. Sci. Fd Agric. 19 (1968) 432.

IV Lunde, G. "Water Soluble Arseno-organic Compounds in Marine Fishes". Nature 224 (1969) 186.

V Lunde, G. "Analysis of Arsenic and Selenium in Marine Raw Materials". J. Sci. Fd Agric. 21 (1970) 242.

VI Lunde, G. "Analysis of Trace Elements in Seaweed". J. Sci. Fd Agric. 21 (1970) 416.

VII Lunde, G. "Activation Analysis of Trace Elements in Lipids With Emphasis on Marine Oils". J. Am. Oil Chem. Soc. 48 (1971) 517.

VIII Lunde, G. "Analysis of Arsenic and Bromine in Marine and Terrestrial Oils". J. Am. Oil Chem. Soc. 49 (1972) 44.

IX Lunde, G. "The Analysis of Arsenic in the Lipid Phase from Marine and Limnetic Algae". Acta Chem. Scand. 26 (1972) 2642.

X Lunde, G. "Location of Lipid-soluble Selenium in Marine Fish to the Lipoproteins". J. Sci. Fd Agric. 23 (1972) 987.

XI Lunde, G. "The Presence of Volatile, Nonpolar Bromo Organic Compounds Synthesized by Marine Organisms". J. Am. Oil Chem. Soc. 50 (1973) 24.

XII Lunde, G. "The Analysis of Organically Bound Elements (As, Se, Br) and Phosphorus in Raw, Refined, Bleached and Hydrogenated Marine Oils Produced from Fish of Different Quality". J. Am. Oil Chem. Soc. 50 (1973) 26.

XIII Lunde, G. "The Absorption and Metabolism of Arsenic in Fish". Fiskeridirektoratets Skrifter. Ser. Tekn. Undersøk. No. 12 (1973) 1-16.

XIV Lunde, G. "Trace Metal Contents of Fish Meal and of the Lipid Phase Extracted from Fish Meal". J. Sci. Fd Agric. 24 (1973) 76.

XV Lunde, G. "The Synthesis of Fat and Water Soluble Arseno Organic Compounds in Marine and Limnetic Algae". Acta Chem. Scand. 27 (1973) 1586.

XVI Lunde, G. "The Presence of Lipid-soluble Selenium Compounds in Marine Oils". Biochimica et Biophysica Acta 304 (1973) 76.

XVII Lunde, G. "Separation and Analysis of Organic-bound and Inorganic Arsenic in Marine Organisms". J. Sci. Fd. Agric. 24 (1973) 1021.

XVIII Lunde, G. "Analysis of trace Elements, Phosphorus and Sulphur, in the Lipid and Non-lipid Phase of Halibut and Tunny". J. Sci. Fd. Agric. 24 (1973) 1029.

1. INTRODUCTION

Trace elements[x)] have met with a strongly growing interest in recent years. This is partly a consequence of the concern for protection of our environment, but is also, however, due to the increasing awareness of the role of trace elements in the physiology of living organisms. In recent years several new trace elements have been added as well, to the list of those elements that are essential to living organisms through a specific function in their metabolism.

Besides measuring the absolute amount of a trace element, it is clearly of equal importance to find out whether the elements are present in varying forms, e.g. as inorganic ions, chelated, or bound as integral parts of organic molecules. Depending on their form, they may in many cases show different effects in living organisms. A toxic element like mercury will, for instance, by going from the inorganic divalent ion to the organic compound methyl mercury, change its effect on mammals. Similarly the inorganic ions of copper and zinc which are rather toxic to fish, will have their toxicity reduced when chelated by organic compounds in water.

The recent development in this field has been made possible partly by the introduction of new methods of analysis, neutron activation analysis (NAA) and atomic absorbtion spectrophotometry (AA) should especially be mentioned, and partly by improved equipment. This has resulted both in higher sensitivity, and in a reduction of the time required for analysis.

Lately, multielemental analysis has been introduced, allowing the simultaneous determination of several elements in a sample without any chemical treatment. Besides NAA, X-ray fluorescence (XRF) and mass spectrometry should be mentioned as relevant methods for this type of analysis.

The most important results obtained on the analysis of trace elements in marine and limnetic (fresh water) organisms up to the early fifties have been summarized by A.P. Vinogradov (1) in "The Elementary Chemical

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x) Trace element: Element usually present in concentrations less than 100 ppm.

Composition of Marine Organisms". Several studies in this field have appeared later, reporting values of trace elements in plants and animals from the marine environment (2-11). A good part of the work has, however, been carried out on a control basis by public or trade institutions, and is not available in published form.

In addition to the work carried out in the author's laboratory, there is also in other groups a growing interest in investigating the form in which trace elements exist. Apart from extensive biochemical work on the occurence and function of trace elements in enzymes and coenzymes, work relating to trace elements in organic bound forms has been scarce. This is particularly so for trace elements in lipid-soluble form.

The importance of marine raw materials for food is likely to increase in the coming years. Hence, knowledge as to the amounts of trace elements to be found, and to the form in which they occur, should become more important as well. In this context the breeding of fish (aquaculture) should be mentioned. Further development in this area may require a closer control of feed composition including the content of trace elements, comparable to what is now practiced in husbandry. Long term effects and interactions of trace elements would appear to be important in this field as well.

2. METHODS

The analytical methods used in the eighteen articles summarized here can be divided into three categories:

1. Fractionation of biological sample prior to the determination of trace elements (prefractionation).

2. Determination of trace elements in absolute terms.

3. Experiments where a radioactive tracer of the element to be studied is added to a biological system under in vivo conditions.

Some important aspects of the methods used will be discussed in the following sections, in particular the prefractionation of samples as used in these investigations. Especially in connection with trace element investigations such methods have, to the author's knowledge, not been used extensively in other laboratories.

2.1 PREFRACTIONATION

A fractionation of the biological sample prior to determination of trace elements is essential to the study of how they are bound chemically. It is generally most important that dissolution of sample components, their extraction and fractionation, are carried out in a way that minimizes further reaction or decomposition. Methods that allow the isolation of particularly stable compounds only may, however, be most useful for special purposes (XVII). This is pertinent to both the lipid and the aqueous phase.

2.11 THE OIL PHASE. One of the simplest types of prefractionation consists of a separation of the oil phase from the rest of the sample. Water-soluble components, such as inorganic ions that are not complexed in the oil, are removed by washing (I,IIV). Trace elements still present in the oil may subsequently be determined, to give the amounts of trace elements either organically bound or made lipid-soluble by chelation.

Especially with NAA it is possible to study the efficiency of the washing procedure, i.e. the removal of inorganic ions from the oil. The level of sodium for instance, which is easily detected nondestructively by NAA, will indicate how efficient the washing process has been.

The oil can be further fractionated according to the differing polarity of the various types of lipids. This may be performed by chromatography on activated silica gel, eluting with increasingly polar solvents, (e.g. hexane, hexane/diethylether, etc.; finishing with mixtures of chloroform and methanol (VII)). In a somewhat simpler procedure the lipids are dissolved in chloroform and chromatographed through a silica gel column (XII,XV). Neutral lipids, i.e. aliphatic hydrocarbons, steroid esters, tri-, di- and monoglycerides and free fatty acids, will elute rapidly under these conditions. More polar lipids, phospholipids in the

main, may subsequently be eluted and fractionated by addition of methanol to the chloroform. The fractions obtained can then be assayed for trace elements (II).

One advantage that accrues from the analysis of series of fractions in this way, is that the variation between fractions within a series may be evaluated. This is particularly so when it serves as a check on contamination from inorganic ions when working with very low levels of organically bound trace elements. Usually contaminants of this type are adsorbed to the silica gel as well, and are eluted in well defined fractions. Contamination is thus limited to certain fractions only.

The oil phase or its fractions may furthermore be saponified, allowing observation of whether the trace elements in question follow the saponifiable or the nonsaponifiable part. In such work the total amounts of trace elements found should be continually compared to their level in the original sample, showing their eventual concentration (or dilution) in particular fractions (VII).

It has been shown that an oil containing phosphorus, i.e. phospholipids, will sequester inorganic cations such as iron, zinc, cobalt, and others, making them lipid-soluble (VII,IXV). This sequestering action may be studied through the use of radioactive tracers of the same ions. Dissolving such a tracer in water and contacting the oil phase with this solution under thorough mixing, the tracer will be transferred to the lipid phase. After mixing the two phases they are separated by centrifugation, and the radioactivity is measured in both phases. Exchange of the radioactive sequestered tracer can be studied by repeating the experiments with the same or other inactive ions (12). Figure 1, p. 22, illustrates the accomplishment of an experiment where transfer of $^{65}Zn^{2+}$ to the oil phase was measured relative to the amounts of phospholipids present.

Some preliminary experiments studying the chelating effect of the oil of selected anions (Br^-, Cl^-, SeO_3^{--}, and HPO_4^{--}) were also carried out (12).

A further development in the study of lipid-soluble trace elements is a closer evaluation of the way these compounds are bound in living organisms. As a first step it is of interest to find whether they are connected to lipoproteins, and hence are possibly located to cell membranes; whether they in other ways are associated with tissue, or simply are solved in the body fat. These problems may be studied through a fractionation of aqueous protein-peptide extracts (prepared for instance through the boiling of fish, or through enzymic digestion of fish tissue) by molecular gelfiltration (MGF) and subsequent extraction of lipid material from the different fractions (X). The level and the distribution of the trace element in the fractions are then determined. When very low amounts of lipids are produced, an "oil-carrier" should be used in this extraction (X). The carrier should consist of an oil that has previously been analyzed and found to have a nondetectable level of the trace elements under investigation. A certain amount of "carrier" (1 - 2 ml) is added to the sample prior to extraction. Care is taken that it blends homogeneously with lipids originally present. Yield of original lipids from the high-molecular fraction may be estimated from yield of carrier. The carrier should make up at least 95 % of total lipids.

The binding of lipophilic compounds containing trace elements in tissue has also been investigated through extraction with different solvents. Relatively nonpolar solvents such as chloroform or hexane do not to any great extent remove lipids that are closely associated with proteins. Polar solvents such as isopropanol or methanol on the other hand, release such lipids and transfer them to a nonpolar phase (XVIII). Information of this kind may also be obtained by extraction of lipids by a nonpolar solvent, or releasing them by heat treatment (60 - 100 ^{o}C), from sample material such as fish subjected to controlled degrees of degradation and breakdown through prolonged storage (X,XII).

In connection with pollution problems, particularly the release of volatile halogenated hydrocarbons, volatiles from marine raw material have been isolated through distillation, to check for volatile, lipid soluble bromo-organic compounds that might be mistaken for similar chlorinated ones of industrial origin. The fractionation was carried out by steam distillation of homogenized sample material in water, to which was added

cyclohexane as a "carrier". The absolute amount of chlorine and bromine in the cyclohexane extract is determined by nondestructive NAA (XI,13). If the distillation process is repeated, a picture of the volatility of the halogenated compounds may be obtained.

The same method, i.e. NAA following a selective extraction, may be used as well for determining the total content of nonpolar (hexane extractable) organic bound chlorine and bromine in water samples. There is today a particular requirement for a method that will specifically and absolutely determine chlorine, and also bromine, in organic compounds without the requirements that the halogens are located to specific compounds, or present in such amounts that they can be identified by for instance mass spectroscopy (14). The less volatile halogenated compounds in the hexane extract that may not be assayed by gas chromatography, may well be the more important ones from the point of view of damage to our environment.

2.12 THE WATER-SOLUBLE PHASE. In an aqueous solution prepared from a biological material, e.g. by heat treatment (glue water) or enzymic hydrolysis, separation of inorganic ions from complexes and organic compounds of the trace elements are more complicated than with the oil phase. Methods of separation that may be used are particularly those based on various forms of liquid chromatography. The fractions resulting from such separations may subsequently be assayed separately. Trace elements present in organic bound forms are indicated by a selective increase in the concentration of a particular element in one or more fractions (V). Also, inorganic ions are separated from organic bound forms of the element in such separations. Behavior of inorganic forms of a trace element through a separation sequence may in most cases be observed by addition of radioactive ions of the element at an early stage in the work-up procedure. The ions may then be followed through the various fractionation steps by measuring the radioactivity.

As described under Section 2.11 (concerning the use of inorganic radioactive tracers in the oil phase), sequestering reactions, exchange of ions among various fractions, and other reactions into which the ions might enter, can be studied in the water phase in this way as well (12).

Of milder chromatographic methods, molecular gel-filtration (MGF) should be mentioned. This technique essentially separates molecules and aggregates according to size (V,X). Dextran gels of the Sephadex type, however, do contain a certain amount of mainly negatively charged groups, and do to some extent perform separations according to charge. Such effects are mostly observed when the ionic strength of the eluting agent is less than approx. 0,02 M. They may be avoided, or substantially reduced by addition of salts to bring the ionic strength above this value (15).

The inorganic ions of arsenic and selenium, and also other ions, may be separated from high-molecular weight material by MGF. Under the same conditions arsenite is well separated from arsenate (resp. selenite from selenate) (16). MGF allows the study of exchange reactions of trace elements localized to specific fractions. Following MGF-fractionation fractions may be mixed with either radioactive or inactive ions of the same species, and again be subjected to MGF. Exchange may then be studied by measurement of radioactive, respectively inactive ions in those fractions where the exchanged ions are eluted.

In separating the aqueous phase into meaningful fractions some quite specific properties of the element under investigation may be used. When fractionating inorganic arsenic, i.e. arsenite and arsenate, from stable organic bound forms of this element, advantage may be taken of the fact that As^{3+} is quite volatile as arsenic-trichloride. When reducing arsenate to arsenite and distilling in the presence of hydrochloric acid, both ions will be removed, whereas organic bound arsenic is retained (VII,X,XVII).

Autoradiography should be mentioned as a useful method of detecting radioactive fractions on TLC, and it considerably improves the scope of this means of separation (XV). For most practical applications ordinary X-ray emulsions can be used. The technique has also been applied on biological material (fish tissue) in studying the distribution of radioactive isotopes in the fish (XIII).

2.2 DETERMINATION OF ELEMENTS

For the determination of trace elements in this work, neutron activation analysis (NAA), atomic absorption spectrophotometry (AA), and X-ray fluorescence (XRF) have been used. Both NAA, AA, and XRF are fully described elsewhere (17-19). The possibilities of performing nondestructively analysis of trace elements by NAA, especially in oils and other nonpolar extracts, should be emphasized in this connection as a versatile and useful method (XII).

NAA combined with autoradiography has been used for studying the distribution (homogeneity) of trace elements in oils (VII).

The determination of trace elements in an organic matrix by XRF, however, is still a relatively new technique. The method allows the detection of 2 - 4 ppm of those elements for which it is most sensitive, and is particularly useful in the screening for trace elements that occur in the concentration range of 5 to 10 ppm and above in organic and biological material. The method may be used on both solid and liquid samples. The latter should, however, not be corrosive or too highly volatile. The determination of sulfur in defatted fish tissue and in marine oils down to about 10 ppm should be mentioned in this connection (XVIII). The method is highly reproducible, nondestructive, and very convenient. It has been used mostly in the assay of zinc, iron, halogens, phosphorus, sulfur, arsenic, and selenium. Care is required in avoiding matrix effects, and in the preparation of standards. Minor variations in the organic matrix, e.g. salt content or consistency of samples such as fish meal, meal of seaweeds and dehydrated aqueous fish extracts, can cause problems. Determination of trace elements in oil requires about 5 ml of material. Smaller samples may be dissolved in a suitable solvent prior to analysis.

2.3 MODEL STUDIES

The growth and feeding experiments (IX,XIII,XV) were a natural extension of the analytical investigations to characterize further arseno-organic compounds. The main advantage of using tracers is that both evaluation of enrichments and identification of the newly formed radioactive compounds are greatly facilitated.

These studies, involving growth experiments with algae and feeding experiments with fish, were performed to learn more about absorption and metabolism of arsenic in particular. The radioactive arsenic isotopes ^{74}As (75 %) with half-life 17.7 days, and ^{73}As (25 %) with half life 70 days were used in the growth experiments. The radioactive arsenic was added to the culture medium, and as sufficient amounts of algal material was produced, it was fractionated and analyzed according to the procedures outlined above. In the fish-feeding experiments the same general scheme was followed in that the radioactive arsenic was added to the feed, respectively to the water. The radioactive, arseno-organic compounds formed were assayed for in a similar way after measuring the total amount of radioactive arsenic present in the samples both during the feeding period and in the subsequent depletion period. Further, the distribution of arsenic within the fish was evaluated by means of autoradiography.

The problems of where in the marine food chain the arseno-organic compounds are formed, the degree of concentration taking place, etc., were also studied in these feeding experiments. By varying the natural content of arseno-organic compounds in the feed, the influence of this source of arsenic was evaluated. Two feed compositions were used in this study, one prepared essentially from ground beef containing less than 1 ppm of arsenic (present mainly as inorganic arsenic), and one prepared from ground saithe and defatted cod liver. The "marine meal" contained ~ 15 ppm of arsenic, most of it in organically bound form. Samples were removed from groups of fish kept on the two diets throughout a four-month feeding period. Aqueous extracts (glue water) were prepared from these samples by boiling and analyzed for arsenic by [illegible].

The experience so far with both the feeding (fish) and growth experiments (algae) proved such model studies to be most suitable for studies over absorption and metabolism of trace elements. Studies of interactions between the various trace elements may be carried out along the same lines.

3. RESULTS

The results are presented and discussed in the following sections: Heavy metals, halogens, phosphorus and sulfur, selenium, and arsenic.

3.1 HEAVY METALS

Heavy metals in this context imply essentially those of biological importance which in their metal form show a specific weight in excess of 5. This means that both elements which are essential or significant to the growth of living organisms, and those which are toxic in fairly low concentrations, are included.

The following heavy metals have been analyzed for in fish, fish products and samples of seaweeds: Cobalt, zinc, tungsten, molybdenium, mercury, cadmium, lead, copper, and antimony. A comparison of some results obtained by different workers is presented in Table 1. Determinations of arsenic and selenium are included in this table, but are discussed in separate sections.

A comparison of the individual data shows partly large variations in the content of trace elements, for instance in the case of copper and zinc in mackerel, cod, and herring. This indicates large natural differences in the uptake of these elements. Some results, particularly those of early origin, may however be influenced by systematic errors or contamination during work-up of samples.

The different samples of tunny show large (absolute) differences in the content of trace metals and indicate that the supply of trace elements originating from lower levels in the food chain and from water can vary widely. The rather similar levels of trace elements in halibut caught at different localities indicate on the other hand that the supply of trace elements can be quite constant, even at quite different localities (XVIII). The relationship found in the case of arsenic, bromine and iodine present in oil from cod liver from the Barentz Sea should also be mentioned in this context (I). The amounts of these trace elements are surprisingly constant, also from one year to the next.

TABLE 1
THE CONTENT OF TRACE ELEMENTS (ppm on dry weight basis) IN FISH
(ref., see next page)

	Mackerel *Scomber scombrus*		Herring *Clupea harengus*		Capelin *Mallotus villosus*		Cod *Cadus morhua*		Tunny *Thunnus thynnus*		Halibut *Hippoglossus hippoglossus*	
Hg	0,15	5 a	0,20	5 a	0,08	5 a	0,41	6	1,25	3	3,8	5 d[x]
	0,20	6							5,3	5 d[x]		
Pb	0,35	5 c	0,40	5 c	0,44	5 c	2,5	4	3,70	3	0,72	5 d[x]
	0,70	6					1,9	6	0,66	5 d[x]		
Cd	0,40	5 c	0,10	5 c	0,22	5 c	0,90	4	0,5	3	0,06	5 d[x]
	0,03	6					0,05	6	0,15	5 d[x]		
Fe	37,5	1 j	29,5	1 j	48	5 c	200	1 a	60	1 m	46,5	1 j
	43,5	1 l	28,5	1 l			63	1 b	90	1 o	22	5 d[x]
	61,2	1 q	31,5	1 p			17	1 l	165	5 d[x]		
	12,9	5 a	19	5 a			25,9	1 q				
	62	5 c	60	5 c			7,3	6				
	22	6										
Zn	68,9	5 a	250	1 e	121	5 a	92	1 i	35,0	3	26,3	5 d[x]
	49	5 c	110	1 e	53	5 c	25,8	4	65,0	5 d[x]		
	13,7	6	69,3	5 a			20,5	6				
			81	5 a								
			54	5 c								
Cu	45,0	1 d	16	1 l	2,90	5 a	19,0	1 l	3,65	3	2,09	5 d[x]
	13,0	1 l	12,4	5 a	4,93	5 c	2,0	1 q	2,48	5 d[x]		
	5,75	1 q	4,0	5 a			3,25	4				
	3,68	5 a	4,0	5 c			1,9	6				
	3,87	5 c										
	7,6	6										
Co	0,04	2	0,03	2	0,09	5 a	0,33	1 r	0,058	5 e	0,05	2
	0,12	5 a	0,19	5 a			0,04	2			0,046	5 e
	0,10	6					0,14	6				
As	0,2	1 c	1,0	1 f	10,9	5 a	1,5	1 f	2,50	3	1,0	1 g
	2,0	1 g	3,3	1 h			2,0	1 g	7,2	5 d[x]	6,0	1 h
	72,5	1 n	15,0	1 k			5,0	1 h			3,2	5 d[x]
	6,6	5 a	7,7	5 a			65,0	1 k				
	5,8	5 b	4,4	5 a			16,5	1 m				
	4,5	6	7,8	5 b			4,4	5 b				
							16,3	6				
Se	5,2	5 a	2,1	5 a	1,5	5 a	1,5	5 b	3,0	5 d[x]	2,0	5 d[x]
	1,6	5 b	1,3	5 b	0,55	5 c	1,6	6				
	1,9	5 c	1,4	5 c								
	1,4	6										

x) Average of 6-7 samples.

Ref. to TABLE 1

1. Vinogradov, A.P. "The Elementary Chemical Composition of Marine Organisms" Sears Foundation for Marine Research, Yale University, New Haven 1953.

a)	Boussingault	(1872)	
b)	Kostychev	(1883)	
c)	Gautier and Clausman	(1904)	
d)	Maquenne	(1919)	
e)	Bertrand and Vladesco	(1921)	
f)	Cox	(1925)	Fish from Sweden
g)	Cox	(1925)	Fish from England
h)	Bang	(1925)	
i)	McHargue	(1925)	
j)	Peterson and Elvehjem	(1928)	
k)	Sadolin	(1928)	
l)	Parks and Rose	(1933)	
m)	Rose	(1933)	
n)	White	(1933)	
o)	Toscani and Reznikoff	(1934)	
p)	Shackleton and McCance	(1936)	
q)	Nilson and Coulson	(1939)	
r)	Maligua	(1946)	

2. Ovren, I. Naturv.rekke. Nr. 9, 1957, University of Bergen.

3. Taylor, D.J. J. Assoc. Publ. Analysts, 9, 1971, 76.

4. Portman, J.E. The levels of certain metals in fish from coastal waters around England and Wales. ICES. C.M. 1971/E, 13.

5. Lunde, G.

a) (1968) III
b) (1970) V
c) (1973) XIV
d) (1973) XVIII
e) (1973) Unpublished

6. Holm, K . og Lunde, G. (Unpublished)

Bearing in mind that trace elements may exist in various chemical forms with different properties, this influences their absorption. One would expect, too, that species of fish which differ widely in their feeding habits or fat deposition, will show great species variations in this respect.

A significant inter-species difference in absorptive behavior has been observed in the case of seaweeds, in that concentrations of trace elements in different species of seaweeds from the same localities are shown to differ widely (VI). A comparison is presented in Table 2.

TABLE 2

TRACE ELEMENT CONTENT (ppm) IN SEAWEED (REINE, LOFOTEN)

Sample[x]	As	Se	Cu	Mo	Mn	Zn	Co	Fe
Laminaria hyperborea[xx]	70	0,04	9	0,3	5	98	0,18	177
Ascophyllum nodosum	44	0,09	22	2,0	13	114	0,5	106
Fucus serratus	47	0,17	8	2,2	5	137	0,8	65
Fucus speralis	34	0,18	12	5,4	28	218	0,2	182
Fucus vesiculosus	65	0,17	45	4,6	11	188	0,6	33
Pelvetia canaliculata	22	0,12	8	0,96	7	137	0,6	115

x) collected in February - March
xx) *lamine*

Boiling of fish raw material gives a water-soluble part (glue water) containing approx. 20 % of dry matter, and an insoluble part (fish meal) making up the remaining. Comparison of the concentrations of the various trace elements in these two fractions clearly shows some elements, such as copper and cobalt, to be concentrated in the water-soluble part, whereas others, such as zinc, cadmium and molybdenium are enriched in the meal (III,XVIII). Some results are presented in Table 3.

TABLE 3

THE CONTENT OF SOME TRACE METALS (ppm) IN MEAL (M) AND DEHYDRATED GLUE WATER (G W) PRODUCED FROM THE SAME SAMPLE

Sample	Fe		Zn		Cu		Hg		Cd	
	M	G W	M	G W	M	G W	M	G W	M	G W
Mackerel	15,0	4,4	84	8,7	2,0	10,4	0,41	0,01		
Herring[x]			86	7,7	11,2	17,2	0,26	0,01		
Herring[xx]	23	4,0	100	6,7	3,5	6,2	0,16	0,02		
Halibut	14	11,6	20	3,7	0,9	2,6	0,9	0,07	0,03	0,04
Tunny	370	290	27	26	0,25	4,4	3,7	0,47	0,10	0,05

x) mature
xx) immature

Mercury (methyl mercury) is of particular interest in this respect in that more than 95 % of the element follows the meal (III,XVIII). The low amounts of mercury found in glue water must imply that methyl mercury is either associated with proteins other than collagen (the main protein found in glue water) or tends to adsorb to the solid phase, i.e. the undissolved proteins. There is also a possibility that the presence of mercury in an insoluble compound (a protein) protects the compound against hydrolysis.

Recent results may point to a mechanism involving adsorption of a methyl mercury containing compound to the solid phase, or to protection of the mercury containing compounds against hydrolysis, as the level of mercury is drastically increased in the solid phase after enzymic hydrolysis of meal, especially when the hydrolysis is extensive. The enzymic hydrolysis of fish meal has been described elsewhere (V). Some preliminary data from this work are presented in Table 4.

Cadmium is an element of particular interest since it is among the most toxic of the heavy metals. The level of cadmium (0,05 - 1,0 ppm) found in dehydrated fish sample is, however, rather low. Comparing the content of cadmium to that of zinc, one may find whether or not cadmium is enriched in fish relative to seawater. In seawater the ratio of cadmium

TABLE 4

THE CONTENT OF MERCURY (ppm ON DRY WEIGHT BASIS) IN RAW AND PROCESSED FISH MUSCLE

Sample		Raw material	Enzymhydr. raw material		Boiled material		Enzymhydr. meal	
			Solid ph.	Aqueous ph.	Meal	Glue water	Solid ph.	Aqueous ph.
Pike	Esox lucius	11,1	60,5	4,1	10,6	0,19	54,5	0,43
Cod	Gadus morhua	0,66	21,6	0,21	0,63	0,02	14,2	0,23
Halibut	Hippoglossus hippoglossus	1,4	14,0	0,77	1,6	0,10	14,2	0,31
Halibut	Hippoglossus hippoglossus	1,4			1,5[x]	0,11	2,9	0,08
Halibut	Hippoglossus hippoglossus	4,4			6,2[x]	0,17	8,4	0,07
Tunny	Thunnus thynnus	4,3	8,5	0,30	4,7	0,55	8,0	0,69

[x] Dehydratet at 110 °C.

to zinc is about 1:100 (20). Results so far obtained from fish show a cadmium/zinc ratio in the range of 1/100-1/1000, implying that no such enrichment occurs in fish. It should, however, be pointed out here that these results are based on analysis of fish fillets or whole fish, and that selective enrichment in particular organs (e.g. the liver or kidney) as is observed in mammals, still may occur.

As mentioned, a relationship between the content of cations such as Zn^{2+} and Fe^{3+} and the level of phospholipids in an oil have been demonstrated (12). The results of the model experiments where oils containing a certain amount of phospholipids are exposed to radioactive zinc ions confirm this hypothesis (see Fig. 1). In these investigations it was shown that radioactive zinc, complexed in oil in this way, can be exchanged with inactive zinc and that trivalent ferric ions are even more effective in this respect (12). In oil with a fair content of phospholipids, an appreciable part of the total content of heavy metals like zinc and iron may be associated with the oil phase in this way, see also Figs. 2 and 3.

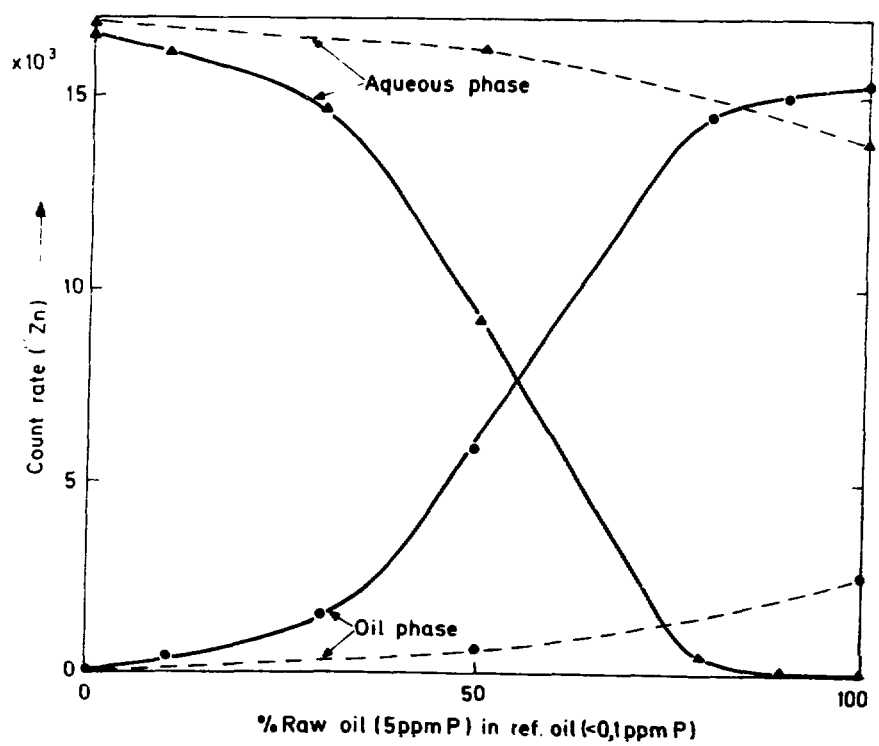

Fig. 1. The distribution of zinc in the lipid (•) and the aqueous (▲) phase as the content of phospholipids in the oil increases. (——): only $^{65}Zn^{2+}$ added, (----): $^{65}Zn^{2+}$ and 10 ppm Zn^{2+} added.

No corresponding absorption of anions in the oil phase was observed.

3.2 PHOSPHORUS AND SULFUR

Neither in the oil nor in the water phase are phosphorus and sulfur included among the typical trace elements in marine organisms. Both are, however, of interest in relation to the trace elements included in this study. They will therefore be discussed briefly in this context.

The content of phosphorus as phospholipids in industrially produced marine raw oils is partly dependent on the state of the fish raw material at production time. Preliminary results show that phospholipids are released nonlinearly under conditions of anaerobic storage of fish (XII). Small amounts only may be removed with the neutral fat by heat treatment during the first three to four days of storage, followed by a tenfold increase in released phospholipids in the next couple of days. Concurrent with this release of phospholipids, an increase in the content of heavy metals such as zinc is also observed in the oil (see Fig. 2 and 3).

Sulfur was determined in fish meal and oils in order to follow the relationship between selenium and sulfur. It is commonly assumed that selenium-containing amino acids may be synthesized along the same routes as sulfur analogues (21), see also Section 3.4. Few data are available on the amounts of sulfur in marine oils, and furthermore, those that are available have partly been measured on refined oils. The data indicate a level of 5 to 20 ppm of sulfur (22). There is also the question of whether these lipid-soluble sulfur compounds are originally present in the lipid phase or whether they are converted to such during storage. Results from this study prove raw oils from fresh, marine raw material to contain much higher levels of sulfur than those previously reported. Sulfur has been determined in the oil phase from halibut and tunny (XVIII), and in the same way in oils from mackerel, herring and cod liver, isolated both by heat treatment and by extraction with hexane/isopropanol and chloroform/methanol (23). Amounts of sulfur from < 10 to 900 ppm were found in these oils (see Table 5). At this point assumptions as to the nature of these compounds are guesswork only.

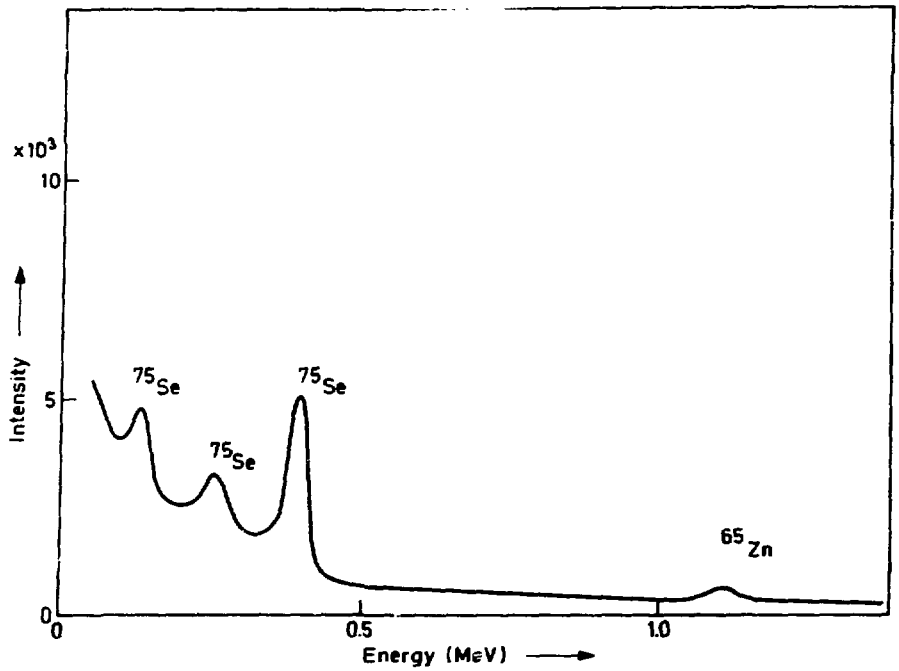

Fig. 2. Gamma spectrum of neutron activated mackerel oil produced from fresh raw material. The spectrum was registered about 1 month after the neutron activation.

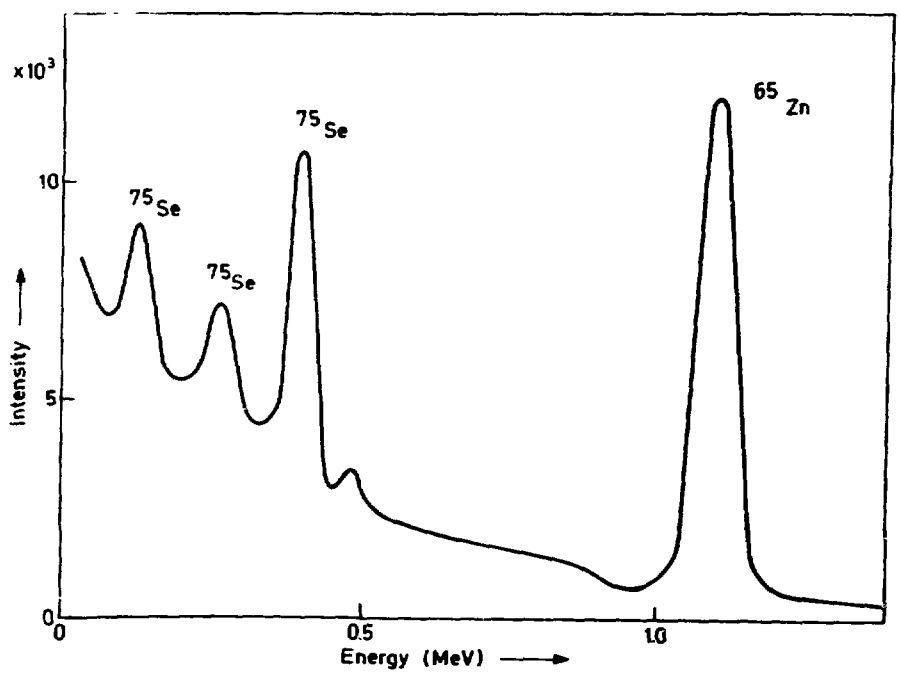

Fig. 3. Gamma spectrum of neutron activated mackerel oil produced from raw material of the same origin as described in Fig. 2, but stored in 7 days at 18 °C. The measuring conditions are as in Fig. 2.

TABLE 5

THE CONTENT OF SULFUR (ppm) IN MARINE OILS
PRODUCED FROM FRESH RAW MATERIAL

Sample	Method of production	S
Capelin	Heat treatment 100 °C	10
Herring	" " "	15
Mackerel	" " "	35
Cod liver	" " "	10
Capelin	Chloroform/methanol (2:1)	143
Herring I	" "	56
Herring II	" "	408
Mackerel I	" "	890
Mackerel II	" "	640
Red fish	" "	122
Cod liver	" "	40
Halibut	Hexan/isopropanol (1:1)	125
Tunny	" "	66

3.3 HALOGENS

Bromine was firstly described in marine oils in the 1830's (24), but has not received any further interest until the investigations reviewed here were started. This work has mainly dealt with the amounts and distribution of oil-soluble bromo-organic compounds. The results obtained indicate that bromine is present in a wide variety of organic compounds in marine oils. Unspecific addition of bromine to double bonds in fatty acids would seem to be a likely route of formation of such compounds. But the results indicate that they are not associated with unsaturated fatty acids in particular (VII). Bromine-containing organic compounds have been found as well in birds and terrestrial animals that mainly have consumed feeds of a marine origin. They are, however, not found in the lipids of mammals which have lived on a terrestrial diet, or in various edible oils of terrestrial origin (VIII).

Bromine and iodine were determined in cod liver oil from different localities. In cod coming in towards the coast of Northern Norway to spawn,

the level of both iodine and bromine is surprisingly constant. Samples from other localities, Møre and Skagerak, among others, show quite different values (I). Some recent results (25), including also values for the chlorine content, are summarized in Table 6.

TABLE 6

THE CONTENT OF CHLORINE, BROMINE AND IODINE (ppm) IN FISH OIL

Sample	Locality		Cl	Br	I
Capelin	Lopphavet	1972	33	4,3	0,91
"	"	1973	29	4,1	0,55
Herring	Steinkjer	1972	54	2,1	1,3
"	Rørvik	1972	60	2,6	3,2
"	Hommelvik	1972	50	1,9	0,6
"	Hvaler	1973	85	3,2	4,3
"	Oslofiord	1973	51	2,8	4,4
"	Shetland	1972	38	4,2	1,1
Mackerel	Bergen	1971	125	2,9	4,3
"	Kristiansand	1972	97	3,1	4,2
Cod liver	Lofoten	1972	28	6,0	9,2
"	Møre	1972	28	9,1	5,0

In connection with determination of chlorinated hydrocarbons in marine organisms and in seeawater as a result of the industrial release of such compounds (mostly from the manufacture of polymers), it was of interest to check whether bromo-organic compounds might contaminate and disturb the registration of these chlorinated hydrocarbons (XI). The results of the determination of bromine in the cyclohexane concentrates collected after steam distillation show that 0,1 to 1 % of total fat-soluble bromine was collected under these conditions. The analysis of such volatiles by gas chromatography with electron capture detection (ECD) showed a number of ECD-sensitive compounds to be present. Some of them were detected by flame ionization detectors as well, and these were further analyzed by coupled gas chromatography/mass spectroscopy. None of the compounds that could be analyzed in this way was, however, found to contain bromine (XI).

These investigations have later been expanded to include chlorine and iodine as well, and are presently continuing with samples of fish from areas that are either typically contaminated or expected to be relatively free from contamination. Very recent results show the presence of fairly volatile chlorinated hydrocarbons, and somewhat less volatile iodinated ones (25). Even though a fair amount of the lipid-soluble chlorinated organic compounds found in marine raw materials today originate from human activity (and presumably some of the brominated ones as well), there are undoubtedly also such compounds of a "natural" origin present in marine organisms. Further characterization of the latter type is of great interest.

3.4 SELENIUM

Toward the end of the fifties selenium was characterized as one of the essential elements (26,27). Selenium deficiency in domestic animals results in a variety of typical symptoms, necrosis of muscle tissue being perhaps the best known. At the same time selenium is extremely toxic and may well be compared with mercury or cadmium in this respect. Selenium compounds are among the most potent antioxidants known in living organisms, and they show a marked effect in capturing free radicals in the same way as thiols and other "radical scavengers" do (28).

Certain extracts, e.g. prepared from yeast (Torula), were shown to contain at least two selenium-organic compounds with improved therapeutic effect as compared to selenite, seleno-amino acids or other known selenium compounds tested against selenium deficiency diseases (29). Isolation and identification of these compounds from yeast have, however, not so far proved feasible (30).

Marine organisms appear generally to have a higher content of selenium than what is found in terrestrial ones (with the exception of some plants growing in seleniferreous areas) (V,27,31-33). The level of selenium in fish is in the range of 1 - 4 ppm, usually about 2 ppm (see Table 1). Disregarding some very high levels detected in some tunny samples (XVIII), the selenium content in fish thus appears to be rather constant.

The selenium/sulfur ratio in meal prepared from halibut and tunny, and in other fish meal as well, is quite constant. It may indicate that

synthesis of the major part of these substances proceeds along the same lines (XVIII,XIV). As most of the sulfur in the fish meal is present as sulfur amino acids, it is likely too, that most of the selenium is present as corresponding seleno amino acids in such samples. Some meal samples produced from tunny have shown very high contents of selenium; about ten times the amounts usually found in fish meal (XVIII). In glue water produced from the same samples a level of 70 ppm was observed. As sulfur contents in these samples were normal, synthesis of selenium compounds in this case must have been preferentiated or have proceeded along different routes. This is especially evident when recognizing the enrichment of selenium in glue water (see Table 7).

TABLE 7

THE CONTENT (ppm) OF ARSENIC, SELENIUM AND BROMINE IN DEHYDRATED MEAL (M) AND GLUE WATER (G W) PRODUCED FROM THE SAME SAMPLE

Sample	As		Se		Br	
	M	G W	M	G W	M	G W
Mackerel	4,5	15,2	2,7	15,1	8,3	130
Herring[x]	3,6	24	1,3	5,6	2,2	63
Herring[xx]	2,3	13	1,9	2,8	4,7	65
Halibut	8	186	1,6	2,1	-	-
Tunny	5	21,8	2,7	10,3	-	-

x) mature
xx) immature

In connection with the work on selenium a new group of lipid-soluble seleno-organic compounds was detected. Selenium in the marine oils varied from 0,05 to about 10 ppm (X,XVI,XVII). The selenium to sulfur ratio is much less constant as well (XVIII). It should be pointed out, however, that the nature of both the lipid-soluble selenium compounds and the lipid-soluble sulfur compounds are so far unknown, and that the conclusions are quite limited until more becomes known about their nature. Varying chemical properties may, for instance, influence their distribution between oil and water phase even though their mode of formation may be the same.

The lipid-soluble seleno-organic compounds described above appear to be partly associated with lipoproteins, and are extracted quantitatively only by total extraction of lipids by mixtures of polar and nonpolar solvents. They appear to be rather stable, and they are found in the lipids of commercial fish meal as well (XIV). Such meal is usually exposed to high temperature drying as well as to prolonged storage in presence of air prior to their use as feeds.

Further evaluation of therapeutical aspects of selenium compounds from meals and oils with particularly high contents of selenium would seem to be of particular interest, as would studies of their toxicity. The very high levels of selenium found in some samples of fish may indicate that there exists selenium-containing compounds that are not as toxic as compounds so far tested (32). The toxic level in feed for domestic animals is of the order of 3 - 4 ppm for selenite. Lipid-soluble seleno-organic compounds of natural origin would furthermore be expected to show differences in absorption and effects, as well, compared to selenite or other known water-soluble seleno-organic compounds.

Fish meal is recognized as an important source of selenium in the feeding of domestic animals. Usually about 5 % of meal is added to the feed composition. A clear relationship is found between amounts of selenium given as fish meal and the level of selenium in blood from sheep and lambs half a year later (33). This study was undertaken in an area deficient in selenium.

Recent results indicate that selenium seems to relieve the toxic effects of methyl mercury in Japanese quail (34). The selenium present in tuna is shown to replace known selenium compounds as a source of selenium in this respect. It is here suggested that fish may use selenium as a detoxification agent against methyl mercury, and that the absorption of selenium is regulated by the mercury content in the fish. As to the latter suggestion the results so far show that the level of selenium is much more constant than that of mercury, and as the major amount of selenium in fish apparently is present as selenium-amino acids, this hypothesis appears somewhat dubious.

3.5 ARSENIC

Arsenic as an element, appears to hold a quite unique position in the biosphere. Results thus far show that marine organisms synthesize arseno-organic compounds from inorganic arsenic, whereas no evidence points to a corresponding ability in terrestrial organisms (V,IX,XIII, XV,35). Accordingly more than one hundred ppm of arsenic (in the form of arseno-organic compounds) may be found in the first group whereas levels in the latter rarely exceed one ppm (36).

Arsenic was first detected in marine organisms in the 1890's (37). It received a great deal of interest in the following decades, particularly during the period 1920-1940, presumably as a result of the arsenic-containing pharmaceutics in use at the time (38).

Some of this interest was focused on arsenic in marine organisms as well. In Table 1 are given some of the results so far obtained. Some of the lower values given here may be caused by insufficient analytical techniques. The content of arsenic in fish from coastal areas appears to be lower than in fish of a pelagic origin (I). One reason for this may be a reduction in available arsenic in coastal areas due to a rather heavy absorption by marine plants in these areas. Considerable variations may occur depending on the localities where the fish has been caught, however.

The presence of arsenic as compounds other than arsenite and arsenate has been indicated by Vinogradov (1) among others. Studies were undertaken in the period up to 1940 to further characterize these compounds (39,40). It was found for instance that arsenic could be extracted quantitatively from marine raw material (shrimp) by ethanol (39)[1]. In a feeding experiment with rats in which arsenic in shrimps was compared to inorganic arsenic, it was found that arsenic in the two cases was metabolized differently, indicating that arsenic in shrimps did not exist as inorganic arsenic or as compounds broken down to this stage in metabolism (41).

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[1] These results could not be reproduced in the author's laboratory.

The effects of both tri- and pentavalent inorganic arsenic in mammals are rather well understood (38). Trivalent arsenic (arsenite) is much the more toxic, and reacts with e.g. sulfhydryl groups in enzymes to block essential metabolism. A similar mode of action has also been found for some trivalent arseno-organic compounds. Pentavalent arsenic (arsenate) acts as an "uncoupler" in that it competes with phosphorus in formation of organic esters in the main metabolic pathways. The arsenate esters formed are unstable in water, however, and hydrolyze to release arsenate again.

In addition to arsenite and arsenate, arsine and trimethyl-arsine should be mentioned as very toxic compounds.

As in the case of mercury, selenium, and tellur, arsenic may be methylated by microorganisms under certain conditions (42,43). The ensuing compounds are quite volatile and hence may be distributed via the atmosphere. Only in one case has a volatile arsenic compound been encountered in these investigations; possibly di- or trimethyl arsine. This was found in sample material from shrimps, and was isolated by steam distillation and subsequent extraction of the distillate by hexane. The amounts of arsenic detected in this way, however, amounted to only a few percent of total arsenic in this material.

Interpretation of the significance of the content of arsenic in marine organisms is presently under discussion. There is broad agreement to the view that most of the arsenic is not present as inorganic ions (arsenite/arsenate), and that the organic forms of arsenic probably are less toxic than the inorganic ones. The chemical state, i.e. whether the arsenic is present as pentavalent or trivalent in the arseno-organic compounds, is however, not known. Also, few data exist about pharmacological properties or long term effects of these arseno-organic compounds in terrestrial organisms. A discussion in 1972 in Fishery News International clearly states the confusion of this matter (44,45).

The main goal of the studies summarized concerning arsenic in marine organisms was to shed some new light on the chemistry and the synthesis of these compounds. Some of the most important results obtained in the studies reviewed are presented in the following:

Arsenic is present in marine organisms as both water-soluble and lipid-soluble arseno-organic compounds. The level of arsenic is established in this work to be between 1 and more than a 100 ppm (I,IV,VI,VIII,XIV, XVIII). Some other results are mentioned in Table 1, p. 17. The data indicate enrichment factors from 200 - 300 to 30 000 - 40 0000 in dehydrated raw material compared to arsenic in water, ~ 3 ppb (20). It should be noted also, that the arsenic is enriched in the glue water, see Table 7.

Work-up of marine raw material, e.g. by heat treatment or by enzymic hydrolysis, and followed by fractionation of the water-soluble part by MGF, results in the major part of the arsenic being found in a fraction with higher molecular weight than the amino acid fractions, but well separated from the high-molecular weight material, i.e. the void volume. This indicates that arsenic is not associated with the proteins in fish. For such an association to be present in vivo, it has to be weak enough to be completely destroyed by such methods as are described here (V).

Growth of unicellular algae with radioactive arsenic added to the growth medium shows both oil and water-soluble arseno-organic compounds to be formed (XV). The oil-soluble compounds are transformed to a water-soluble one on treatment with hydrochloric acid. This compound is very stable and shows chemical properties similar to the one found in highest concentrations in the aqueous extracts. This compound is ninhydrin positive, and has been studied in more detail by MGF and TLC.

Among the most important results obtained from the fish-feeding experiment was the finding that fish too has the ability to synthesize lipid-soluble and water-soluble arseno-organic compounds. Only a small part of the inorganic arsenic present in the feed was, however, converted to arseno-organic compounds, and as the content of inorganic arsenic in natural waters is low compared to the marine organisms, this direct synthesis must therefore be of minor importance as a source of arseno-organic compounds in fish. Absorption of arsenic from feeds with a varying content of organically bound arsenic, on the other hand proves lower levels in the marine food chain to be the major source of arsenic for fish (XIII).

When inorganic arsenic (arsenite/arsenate) is added to the water it is absorbed by the fish; however, no arseno-organic compounds were detected by the methods used for studying this process (XIII).

All results so far indicate the nature of these arseno-organic compounds to be similar in unicellular algae and in fish. Recent results show that lipid-soluble arseno-organic compounds extracted from meal of seaweeds and treated with HCl, give a water-soluble arseno-organic compound with properties similar to the ones most prominent in fish and algae (46).

The results of the fractionation of organic bound arsenic from inorganic compounds or those broken down to inorganic arsenic during the HCl treatment, show that under these conditions 0,3 to 3,0 ppm of the arsenic present in marine samples is removed during the distillation. The remaining arsenic consists essentially of stable arseno-organic compounds (XVII).

The stability of the arseno-organic compounds may also be illustrated by some preliminary investigations carried out to check for presence of organically bound arsenic in sediments (47). The analysis has been performed on clays taken 10, 20 and 30 cm below the seabottom in the outer Oslofjord (Hvaler) and from cores (1 - 10 m) drilled in the North Sea. After distilling off the inorganic arsenic, the undistilled was evaporated, extracted with ethanol and fractionated on TLC before assaying arsenic in the different fractions by NAA. The results show that approximately 50 percent of the arsenic in sediments taken from the Oslofjord and about 15 percent of the sample from the North Sea are present as organic compounds.

Further studies over the chemical nature of the main arseno-organic compounds are continuing at present. Enrichment of arsenic to concentrations of 1 - 4 percent As (dry weight) has been achieved through ethanol extraction of dehydrated water-soluble marine raw materials (and from lipids treated with hydrochloric acid as well) followed by MGF and preparative TLC. Suitable conditions for further enrichment by ion exchange chromatography have been achieved quite recently (46). A ninhydrin developed TLC fractionation is presented in Fig. 4. The

spots were scrapped out and analyzed by NAA. Spot A only contains arsenic. All arsenic present in the sample was found in this spot.

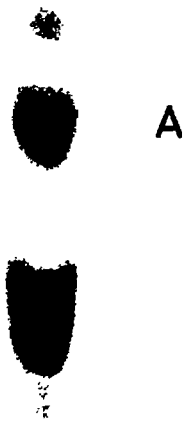

Fig. 4.

Ninhydrin developed thin layer chromatogram of an aqueous extract (produced from cod liver) enriched in organic bound arsenic. Only compound A contains arsenic.
TLC system:
Substrate: Cellulose F (Merck)
developing solvent:
Chloroform/methanol/ammonia 2/2/1.

Preparation of derivatives of the arseno-organic compounds has been tried on the concentrated material, and acetylation has so far met with success as indicated by disappearance of ninhydrin reaction and the appearance of a compound with a different (and higher) Rf-value in TLC. This compound is, however, still water-soluble and attempts to improve the volatily by further derivation, e.g. by using methylation and silylation, have not succeeded.

4. SOME ECOLOGICAL ASPECTS OF TRACE ELEMENTS IN THE AQUATIC ENVIRONMENT

Although trace elements and their significance to aquatic organisms have been investigated to a much smaller extent than have the corresponding terrestrial systems, it is generally assumed that trace elements function similarly in both.

Aquatic organisms are, however, much less exposed to deficiencies in these elements. This is particularly so in the marine environment, where supply and availability of trace elements are rather abundant, relatively speaking. Deficiencies in specific elements may, however, be encountered in eutrophic lakes in high production, and in coastal areas where iodine, for instance, is strongly enriched in seaweeds.

Absorption of trace elements in aquatic organisms, and perhaps passive absorption in particular, may, however, follow routes quite different from those common in terrestrial life. Besides the uptake of trace elements from food, they may be absorbed through the skin and the respiratory system of the organism. Of equal importance is excretion through the same organs. (It should be emphasized that absorption and excretion in water occur to an extended medium, rather than to a confined one as it is for terrestrial plants.)

It will carry too far to go into the aquatic ecological aspects of each trace element here. Rather, some comments based on the results obtained in the present work will be presented.

The results with respect to arsenic is the most important in the context of aquatic ecology. Methylation of arsenic, analogous to the methylation of mercury by microorganisms seems to play a very minor part in the aquatic environment.

When inorganic arsenic is absorbed together with the food, the result is a synthesis of arseno-organic compounds that are very resistant to degradation, while inorganic arsenic absorbed through the skin and gills (fish) does not seem to be converted to such compounds. Due to a high accumulation of the arseno-organic compounds in the different

steps in the aquatic feed chain compared to that of inorganic arsenic in the water, the amount of inorganic arsenic present in the aquatic organisms is negligible. Results furthermore indicate that organisms low in the feed chain synthesize the major part of the arseno-organic compounds, and thus are the main originators of such compounds.

Even though it has not been shown conclusively that the arseno-organic compounds found in algae are identical to those found in fish and other marine organisms, all results obtained so far (such as thin layer chromatographic behavior, stability, molecular weight, ninhydrin reaction, etc.), point to a common base. The fact that these compounds are formed in both algae and animals further confirms this view.

Aquatic organisms may have an intensified need for protection against microbial invasion, particularly in areas of mucous membranes. Synthesis and excretion of these arseno-organic compounds may be associated with such a function. Both the need and the possibility of obtaining such substances are drastically reduced on land, and may be the reason why terrestrial organisms cannot synthesize these compounds.

Of other results pertinent in this connection should be mentioned the ability of marine organisms to form lipid-soluble compounds containing the trace elements arsenic, bromine, chlorine, iodine, and selenium. Sulfur may be included here, although it is not usually considered a trace element. Such lipid-soluble compounds are not known in terrestrial organisms.

Unlike the arseno-lipids, halogens do not appear to exist as specific compounds or classes of compounds, but rather to be nonspecifically bound to different lipid-soluble compounds. The possibility that such matter may contain substances that are stable, and may be harmful to terrestrial ecological systems, should be considered.

Of particular interest are the lipid-soluble seleno- and sulfo-compounds that have been detected. The large variations in the level of these compounds imply either that they protect or make up for other essential compounds, or that they are localized in the tissue, and in varying degrees are accessible during the isolation of fatty matter. The rather

constant content of selenium in fish meal, and of sulfur in sea-water indicates that the supply of both selenium and sulfur to marine organisms is quite constant.

EPILOGUE

The coming years appear to bring a greatly enriched activity in the research on trace elements, their nutritional and pharmacological effects, the forms in which they are present in living organisms, and their interactions in nature.

Modern analytical chemistry would seem to make this development possible. However, the importance of concerted efforts by biologists and analytical chemists in cooperation in the attack on this type of problem is becoming increasingly evident.

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Activation Analysis of Bromine, Iodine and Arsenic in Oils from Fishes, Whales, Phyto- and Zooplankton of Marine and Limnetic Biotopes

Contents

1. Introduction

The first analyses of bromine and iodine in marine organisms were performed in the years from 1830—1840. SARPHATI (1934) and others noted the presence of bromine and iodine in fishes. The content of arsenic in fishes was observed by THIERGARDT (1897) and others around 1900. After these first studies more systematic investigations were initiated early in the 1920. These investigations were now primarely concerned with the determinations of iodine and arsenic in fishes. However, no analyses of bromine were made in this period or later. A survey of this development can be found in 'The Elementary Chemical Composition of Marine Organisms' by VINOGRADOV (1953). Some of the results discussed in the latter publication pertaining the distributions of iodine and arsenic in fish have interest in connection with the present work and will therefore be mentioned.

Most of the determination of iodine in fishes refer to muscular tissues and different organs. It is reported that species of the family Gadidae contain more iodine than species of other fish families such as Salmonidae, Clupeidae, Scombridae and Pleuronectidae. Determinations of iodine in fish oils, especially in

cod liver, *Gadus morrhua*, show a iodine content of 1.5—16.5 ppm. Corresponding determinations of the iodine content in fresh water fishes indicate that these contain 10—100 times less iodine than the marine forms. Also it was observed that the iodine content in fishes from the coast of Norway were richer in iodine than the same species of fishes or species from the same fish family caught along the Pacific coast of the United States. This indicates a connection between the iodine content in the fishes and their nutrition. In some experiments reported, fish tissues have been extracted succesively by HCl, cold and hot alcohol, chloroform and ether. All of these fractions contained iodine. Therefore it is concluded that most of the iodine in the fishes is present as an unknown organic compound.

Reviewing the results of the arsenic determinations in fishes it is of interest to note that this element accumulates in liver oil and in oil from other organs. In the cod liver oil it is found from 0.3—0.45 mg arsenic per 100 cm^3 oil, and in the liver fat of herring 9 ppm arsenic.

The amount of arsenic found in many different fish species in salt and fresh water show considerable variations, but no results point out any significant difference in the arsenic content. It also seems probable that the arsenic occurs in the fishes as an organic compound.

2. Methods. The Principles for the Neutron Activation Analysis

Many excellent surveys, publications, and proceedings concerning activation analysis are available wherein the theory and the different fields of applications are described. Therefore only a short genreal introduction to the method will be given here, together with some particular problems concerning the irradiation of oils. For a more thorough study of activation analyses one may refer to JENKINS and SMALES (1956), ATKINS and SMALES (1959), BOWEN and GIBBONS (1963), KOCH (1960), and to proceedings from conferences in activation analyses, VIENNA (1959), TEXAS (1963), SALZBURG (1964), and TEXAS (1965).

If an amount $W(g)$ of an element is irradiated the radioactivity A (desintegrations sec^{-1}) produced in a time, t, can be established by the following equation:

$$A = \frac{\varphi \cdot \sigma \cdot \theta \cdot W \cdot N}{M} (1 - e^{-0.693\, t/T_{1/2}})$$

wherein φ is the neutronflux in neutrons cm^{-2}, Θ is the percentage abundance of the isotope to be activated and σ ist the activation cross section (in barns) for this particular isotope. N is Avogadros number, M is the atomic weight of the element and $T_{1/2}$ is the half-life of the radioactive isotope formed. The expression $1 - e^{-0.693\, t/T_{1/2}}$ is called the saturation factor.

From this equation it can be seen that with an irradiation time of $t = T_{1/2}$ the saturation factor is 0.5, and when it is increased, the factor will approach 1. At this time the same amount of radioactive isotopes formed, will disintegrate and the maximum sensitivity is attained. In practice the irradiation time depends on the element to be analysed and may vary from less than one hour to many days. The radioactive isotopes which are formed will usually desintegrate by emitting both γ- and β-radiation and can therefore be detected by measurement. From the previous formula for calculating the induced activity it is seen that A is proportional to the weight of the element. An absolute determination can now be performed by irradiating a known amount of this element (a so-called standard) under the same condition as the sample. The activity induced in the

Table 1. Half lives, sensitivities[1]), and characteristic γ-energies[2]).

Element	Activated form	Half life $T_{1/2}$	Sensitivity g/g sample	Characteristic γ-energies (MeV)
Na	Na – 24	15.0 h	$1 \cdot 10^{-10}$	1.38, 2.76
Cl	Cl – 38	37.3 m	$5 \cdot 10^{-9}$	1.64, 2.15
P	P – 32	14.3 d	$1 \cdot 10^{-9}$	no γ
Mn	Mn – 56	2.6 h	$1 \cdot 10^{-11}$	0.84, 1.8, 2.11
Fe	Fe – 59	45.1 d	$5 \cdot 10^{-7}$	1.10, 1.29
Co	Co – 60	5.2 y	$1 \cdot 10^{-9}$	1.33, 1.17
Cu	Cu – 64	12.8 h	$5 \cdot 10^{-9}$	0.51
Zn	Zn – 64	245 d	$1 \cdot 10^{-8}$	1.11
Zn	Zn – 69	13.8 h	$1 \cdot 10^{-9}$	0.438
As	As – 76	26.7 h	$5 \cdot 10^{-11}$	0.55
Br	Br – 82	36 h	$1 \cdot 10^{-10}$	0.55, 0.78, 1.04, 1.30
Mo	Mo – 99	66 h	$1 \cdot 10^{-8}$	0.141, 0.181, 0.74
I	I – 128	25 m	$1 \cdot 10^{-9}$	0.455

Notes to the table: The irradiation is performed in a uranium reactor at a neutronflux at 10^{12} cm^{-2} sec^{-1} for one month or to saturation. The measurements are supposed to start two hours after the end of the irradiation with an end window G.M. tube. The effeciency is 10% for β^- and β^+ with maximum energy 0.2 MeV and 1% for β-particles with lower energy.
1) from JENKINS and SMALES (1956)
2) from CROUTHAMEL (1960)

standard is then compared with the corresponding activity in the sample. As the weight of the standard is known, the amount of the element in the sample can be determined.

Table I contains half-lifes and sensitivities, from JENKINS and SMALES (1956) for some elements of interest. Characteristic γ-energies are taken from CROUTHAMEL (1960). The sensitivity described in Table I is based on theoretical calculations on β-measurements of induced activities. This technique will give the highest sensitivity, but it is necessary to perform thorough separation of the radioactive isotope to be measured. In practice the registration is performed by a γ-spectrometer where the demand to radiochemical purity of the sample is not that high as for the previous method. Several radioactive isotopes can here be analysed in one sample. This latter technique may reduce the sensitivity, but on the other hand it will improve the precision of the analysis because the number of separation steps is reduced.

Neither carbon, hydrogen, oxygen, nor nitrogen will give any measureable radioactivity if the activation is performed in a nuclear reactor. For this reason it is therefore possible to register the activity induced in trace elements in many organic and biological samples without chemical separations. Sodium is one of the elements that may interfere in this procedure. In this type of material sodium is usually present in a relative high concentration. It has high sensitivity for neutron activation, and its radioactive isotope Na-24 emits high energy γ-rays. The registration and interpretation of other radioactive isotopes can therefore be impeded. For radioactive isotopes with a long half life (2 days or more), the interference from Na-24 can be avoided by waiting until this isotope has

decayed to a level where the other active isotopes can be measured. Elements forming radioactive isotopes with shorter half-liefes (~5 hours) can be analysed either by selecting a short irradiation time, or it may be necessary to remove sodium by chemical methods.

When the irradiation takes place in a nuclear reactor with thermal neutrons, the most common activation process will be a (n, γ) reaction where an isotope captures a neutron with a subsequent simultaneous emission of a γ-quantum. The γ-emission will give the activated isotope a recoil in the opposite direction. Usually the kinetic energy gained in this process will be higher than the binding energy, and the atom may diffuse out in the matrix. This is especially true if the sample is in liquid phase or it is a biological material. These atoms, or "hot atoms" as they are called, can here form volatile compounds and escape from the sample. In the present investigations both the bromine, iodine, and arsenic will leave the molecules where they originally were located and diffuse into the oil. These atoms are very reactive and it is highly probable that the bromine and iodine will abstract hydrogen from organic molecules forming hydrogen-bromide and hydrogeniodide. Both of this compounds have a definite vapor pressure at the irradiation temperature (about 40 °C) and therefore these can escape as a gas. However, the probable reaction will be that they add to double-bonds and remain in the oil.

In order to investigate this type of reaction and also the behaviour of the arsenic activated, 5 different oils were neutron activated and extracted with water. Each of the liquid phases were then separated and the radioactivity measured in each fraction with a scintillation detector. The result showed that 1–4% of activity induced in the bromine and arsenic in the oils could be extracted into the waterphase. From disintegration curves of oil and corresponding water-fractions it was furthermore observed that they contained nearly the same mixture of Br-82 and As-76. This shows that the water extracts about the same relative amount of arsenic and bromine from the oil, and confirms the fact that bromine and arsenic almost quantitatively will stay in the oil after activation. Due to the half-life of I-128, the activated iodine could not be determined in these experiments, but will certainly follow the same reactions scheme as the bromine.

3. Experimental

a) Production of the Oils

Many of the herring oils and some of the other marine oils were produced in factories. They were all analysed as received. The other samples of fish were obtained at the commercial fish market or caught by local fishermen. The plankton was collected with a phytoplankton tow net. From these samples the oils were produced in the laboratory either by boiling with water or by extraction with a chloroform-methanol mixture (2:1), or with petrolether. The last method of extraction was only used on dried materials. The oil produced by extraction contained some impurities, especially sodium. In order to remove these it was necessary to wash the oil with water.

The fish oils were produced either from whole fish, liver or tissue (whole fish without intestine). No further treatment was performed before the extraction of the oils. The plankton samples were dried at 50–60 °C before the extraction.

b) Irradiation and Registration

Ordinary about 0.5 g from each oil sample was used for the determination of bromine, iodine, and arsenic. The method of analysis and the handling procedure employed here will put a lower limit of about 25 mg oil for each activation.

The oils were sealed into polyethylene ampoules and irradiated in JENER's nuclear reactor JEEP I, Kjeller Norway. The neutronflux was about $2 \cdot 10^{12}$ n cm^{-2} sec^{-1}. The irradiation time was from 1—2 hours when all three elements were determined. If only bromine and arsenic are to be analysed, it is reasonable to prolong the time of irradiation to 1—2 days. This will increase the sensitivity for the bromine and arsenic determinations.

After irradiation the oils were poured from the ampoules into 5 ml test-tubes and measured. The weight of the oils were determined by weighing the tubes with and without the activated oil.

As standards for the quantitative determinations of bromine, iodine, and arsenic NH_4Br, KI and As_2O_3 (Merch. pro analysis) were employed. A 400 channel γ-spectrometer (Intertechnique) with a 2 · 2" thalliumactivated NaI crystal was used to register the induced radioactivity in the oils. Due to the short half-life of I-128 ($T_{1/2}$ = 25 min) it is necessary to start the registration as fast as possible, within 1—2 hours after irradiation. During 4—5 hours this activity is reduced and the registration of the radioactivity from bromine and arsenic can start.

The determination of iodine was performed by comparing the intensity of the 0.45 MeV γ-peak from the activated oil with the activated iodine standard. The I-128 activity in the oil sample was also controlled by following the decay of this isotope. Bromine-82 and As-76 have both of their prominant γ-intensities

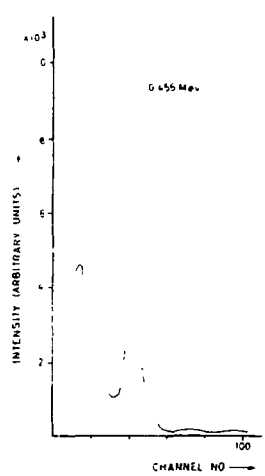


Fig. 1

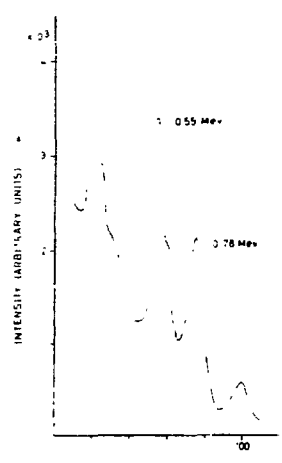


Fig. 2

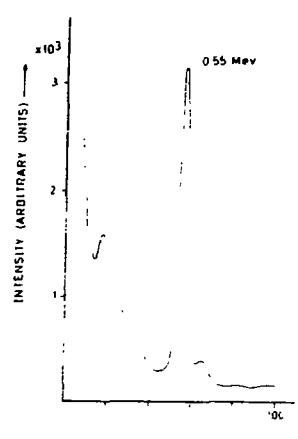
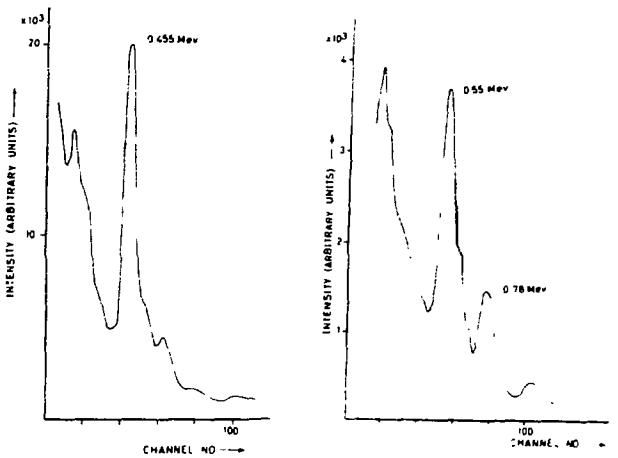


Fig. 3

Fig. 1. γ-spectrum of activated iodine standard, recorded 1 hour after activation.
Fig. 2. γ-spectrum of activated bromine standard.
Fig. 3. γ-spectrum of activated arsenic standard



A B

Fig. 4. A γ-spectrum of activated cod liver oil (sample No. 5, Table 4), recorded 1 hour after activation.

Fig. 4. B The sample as in Fig. 4. A recorded 1 day after activation

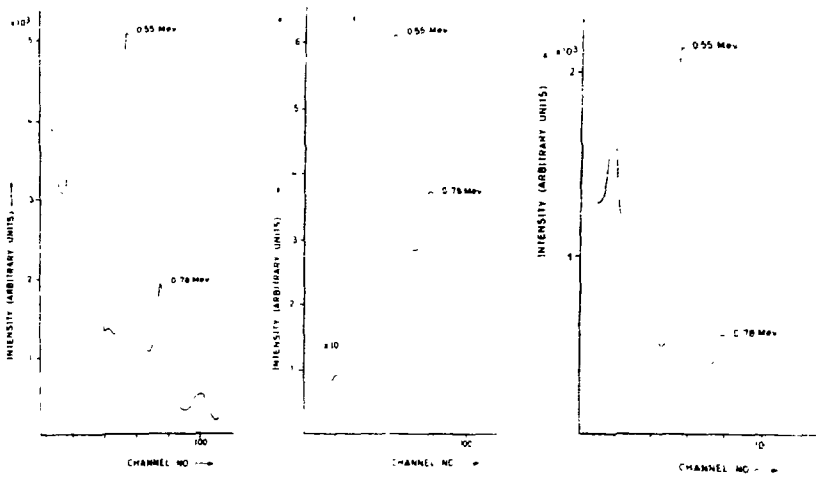


Fig. 5 A Fig. 5 B Fig. 6

Fig. 5. A γ-spectrum of oil extracted from salmon tissue (sample No. 1, Table 3).

Fig. 5. B γ-spectrum of salmon liver oil (sample No. 1, Table 3).

Fig. 6. γ-spectrum of activated herring oil (sample No. 16, Table 5)

at 0.55 MeV and these will therefore be superimposed in the γ-spectrum recorded. Also Br-82 has γ-energies of 0.78 and 1.04 MeV which can be used for quantitative determination. If the measurements of bromine and arsenic are made within a day after the activation there will be some contribution of 0.51 MeV and 0.62 MeV γ from Br-80 m[1]). Bromine-80 m has a half-life of 4.4 hours and will disintegrate to Br-80 with a half life of 18 min. In this period it is important to measure the bromine-standard so as to control the disintegration of Br-80 m. After the decay of about one day the influence from this isotope is negligible.

The 0.78 MeV γ-peak of Br-82 was used for the determination of the bromine. The arsenic was determined by using the 0.55 MeV γ-peak from As-76 after the contribution from Br-82 in the 0.55 MeV γ-peak was subtracted.

The figures 1–3 show bromine, iodine, and arsenic standards, and in fig. 4–6 are presented some typical γ-spectra of activated cod liver oil, herring oil, and salmon oil.

4. Results

Tables 2–7 give the result from determination of the bromine, iodine, and arsenic content in the different oils. In addition to the fish and plankton oils also some samples of whale oil were activated and analysed.

Besides radioactive isotopes of bromine, iodine, and arsenic, other radioactive isotopes could be observed after the activation. Among the latter the radioactive phosphorus and chlorine isotopes P-32 and Cl-38 may be mentioned. The radioactive phosphorus isotope, P-32, has a half life of 14.3 days and is a pure β-emitter. In a γ-spectrum this isotope will be recorded as bremsstrahlung. The chlorine isotope, Cl-38, has a half life of 38 min and can also be determined in a mixture of bromine, iodine, and arsenic with a γ-spectrometer.

Some samples of vegetable and animal oils were irradiated to see if any of the trace elements found in fish oils were present. The following oils were irradiated and measured with the same technique as described previously: safflowerol, peanutoil, soyaoil, shefat, pigfat, and fats from cow and hen liver. The results of these experiments showed that no bromine, iodine, or arsenic could be detected.

As reported by SADOLIN (1932) the arsenic content in the cod liver oil can be partly removed by washing with a sodium carbonate solution. The results from similar experiments in this study, where the oils were washed with other basic solutions, showed that the arsenic could be completely removed. The latter being accomplished by washing with a 0.1 M NaOH solution, whereas 0.1 M NH_3 had no influence. Neither the bromine nor the iodine content was changed by this treatment. Herring oil and cod liver oil were used for these experiments.

In some other experiments the different methods of oil production were investigated. Here it was of interest to see if the distribution of bromine, iodine, and arsenic was dependent of how the oils were produced. Determinations of the three elements in different oil fractions, from the same cod liver, produced by water boiling and extraction with chloroform-methanol (2:1) showed no significant variations in the contents of these elements. The same result was also obtained when water boiling and petrolether extraction of cod liver were employed and compared.

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[1]) Bromine-80 m is an isomeric state of the isotope Br-80.

Table 2. Oil from fresh water fish

Sample	Locality [1])	Caught	Method of production [2])	Organ	Comments	Br ppm	As ppm	I ppm [3])
Perca fluviatilis 1	Björnsjöen (l), Ak.	Autumn-63	C m	Tissue	Fish 0.1 kg	7.7	0.8	-
Perca fluviatilis 2	Langevatn (l), Ak.	March-64	P e	Tissue	Fish 0.2 kg	0.4	0.2	1.8
Perca fluviatilis 3	Öyeren (l), Ak.	May-64	B w	Tissue	1 fish 1.05 kg	1.8	<0.1	4.4
Perca fluviatilis 3	Öyeren (l), Ak.	May-64	B w	Liver	From P. fluviatilis	0.9	0.2	1.5
Lucioperca lucioperca	Öyeren (l), Ak.	May-64	B - w	Tissue	1 fish 4.43 kg	0.9	0.3	3.7
Esox lucius	Öyeren (l), Ak.	1. 5. 64	B w	Tissue	1 fish 4.23 kg	0.9	<0.1	1.1
Aspius aspius	Öyeren (l), Ak.	24. 4. 64	B w	Tissue	1 fish 3.3 kg	0.3	0.9	5.3
Abramis brama	Öyeren (l), Ak.	24. 4. 64	B w	Tissue	1 fish 1.6 kg	0.9	0.3	0.6
Leuciscus idus	Öyeren (l), Ak.	24. 4. 64	B w	Tissue	1 fish 1.6 kg	0.4	<0.1	0.1
Leuciscus cephalus	Öyeren (l), Ak.	24. 4. 64	B w	Tissue		0.8	0.7	1.1
Thymallus thymallus	Glomma (r), He.	Aug.-63	P – e	Tissue		3.6	<0.1	-
Salmo alpinus	Valdres (d), Op.	Autumn-63	P e	Tissue		0.6	<0.1	9.1
Salmo alpinus	Valdres (d), Op.	April-64	B w	Tissue		0.9	<0.1	1.9
Salmo eriox	Jaren (d), Op.	Autumn-63	P - e	Tissue		4.5	<0.1	-
Salmo eriox	Glomma (r), He.	Aug.-63	P – e	Tissue		2.1	<0.1	-
Salmo eriox	Kivsjöen (l), He.	July-63	B - w	Tissue		6.0	<0.1	1.0
Coregonus lavaretus	Jaren (d), Op.	Autumn-63	P - e	Tissue		1.0	<0.1	1.0

[1]) Ak. Akerhus county, He. Hedmark county, Op. Oppland county, (l) lake, (r) river, (d) district.
[2]) C m Extracted w/chloroform-methanol, P e Extracted w/petrolether, B w Boiled w water.
[3]) Not determined.

Table 3. Oil from salt water fish

Sample	Locality [1])	Caught	Method of production [2])	Organ	Comments	Br ppm	As ppm	I ppm [3])
Brevoortia tyrannus	North America	-62	F	Whole fish		2.0	15.5	
Scomber scomber 1	Agder, S. N.	Dec.-63	C m	Tissue		29.3	7.3	
Scomber scomber 1	Agder, S. N.	Dec.-63	C m	Liver	From S. scomber 1	47.0	5.0	
Scomber scomber 2	Agder, S. N.	Jan.-64	C m	Tissue		14.8	4.5	
Scomber scomber 3	Agder, S. N.	May-64	C m	Tissue		5.4	4.2	2.7
Mallotus villosus	Finnmark, N. N.	March-63	F	Whole fish		7.5	13.2	
Sebastus marinus	Vesterålen, N. N.	Oct.-62	F	Liver		5.5	4.3	
Hippoglossus hippoglossus	Vesterålen, N. N.	May-62	F	Liver		8.2	5.3	
Gadus virens	Vesterålen, N. N.	Oct.-62	F	Liver		7.5	3.2	-
Brosmius brosme	Vesterålen, N. N.	Wint.-63	F	Liver		8.1	1.8	
Gadus esmarki	Halten Bank	4.4.-64	F	Whole fish		12.7	3.2	
Anguilla anguilla 1	Oslo Fiord, S. N.	Febr.-64	C m	Tissue		10.5	0.5	
Anguilla anguilla 1	Oslo Fiord, S. N.	Febr.-64	C m	Liver	From A. anguilla 1	25.4	8.9	-
Pleuronectes platessa 1	Agder, S. N.	Dec.-63	C m	Tissue		5.0	2.0	
Pleuronectes platessa 1	Agder, S. N.	Dec.-63	C m	Liver	From P. platessa 1	26.4	1.0	
Pleuronectes platessa 2	Denmark	Jan.-64	C m	Tissue		7.5	2.2	
Pleuronectes platessa 2	Denmark	Jan.-64	C m	Liver	From P. platessa 2	31.8	4.0	
Salmo salar 1	Finnmark, N. N.	Autumn-63	C m	Tissue	1 fish ab. 15 kg	6.4	3.1	-
Salmo salar 1	Finnmark, N. N.	Autumn-63	C m	Liver	From S. salar 1	21.9	6.7	--

[1]) S. N. Southern Norway, N. N. Norther Norway.
[2]) F Produced in factory, C m Extracted w chloroform-methanol.
[3]) Not determined.

Table 4. Oil from cod *(Gadus morrhua)*

Sample No.	Locality [1]	Caught	Method of production [2]	Organ	Comments	Br ppm	As ppm	I ppm [3]
1	Mehavn, Fi., N. N.	22. 4. 63	F	Liver	Mature arctic cod	8.5	3.4	11.7
2	Mehavn, Fi., N. N.	29. 4. 63	F	Liver	Mature arctic cod	7.0	3.9	20.1
3	Stö, No , N. N.	19 12. 62	F	Liver	Mature arctic cod	7.1	2.4	10.8
4	Stö, No., N. N.	28. 1. 64	F	Liver	Mature arctic cod	7.9	2.4	10.7
5	Stö, No., N. N.	17. 2. 64	F	Liver	Mature arctic cod	8.4	2.6	13.7
6	Alsvåg, No., N. N.	13. 5. 63	F	Liver	Mature arctic cod	8.0	2.3	12.2
8	Siger Fiord, No., N. N.	12. 3. 64	F	Liver	Mature arctic cod	7.6	2.2	19.4
9	Hadsel Fiord, No., N. N.	Oct.-63	F	Liver	Coastel immature cod	6.4	2.4	6.0
10	Möre, W. N.	1. 3. 64	C - m	Liver		5.6	4.2	6.1
11	Möre, W. N.	Dec.-63	C - m	Tissue	1 fish 4 kg	14.1	10.0	
11	Möre, W. N.	Dec.-63	C - m	Liver	From No. 11	59.6	4.0	-
12	Möre, W. N.	2. 3. 64	C - m	Liver	1 fish 4 kg	12.2	1.0	5.4
13	Möre, W. N.	2. 3. 64	C - m	Liver	1 fish 4.4 kg	12.6	0.7	5.6
14	Möre, W. N.	2. 3. 64	C m	Liver	1 fish 2.7 kg	17.9	1.3	9.4
15	Oslo Fiord, S. N.	3. 4. 64	C - m	Liver	1 fish 2 kg	32.5	1.4	60.4
16	Oslo Fiord, S. N.	3. 4. 64	C - m	Liver	1 fish 2 kg	9.6	0.8	25.6
17	Skåne, Sweden	Dec.-63	C - m	Tissue	1 fish 4 kg	14.5	26.0	—
17	Skåne, Sweden	Dec.-63	C m	Liver	From No. 17	3.7	1.7	-

[1]) Fi -- Finnmark county, No. - Nordland county, N. N. Northern Norway, W. N. Western Norway, S. N. -- Southern Norway.
[2]) F Produced in factory, C - m Extracted w/chloroform-methanol.
[3]) Not determined.

Table 5. Oil from herring (*Clupea harengus*)

Sample No.	Locality [1])	Caught	Method of production [2])	Organ	Comments	Br ppm	As ppm	I ppm [3])
1	Iceland	Autumn-62	F	Whole fish		3.0	8.2	
2	Iceland	8. 7. 63	F	Whole fish		0.7	9.0	
3	Iceland	6. 8. 63	F	Whole fish		0.7	9.0	
4	Iceland	20. 9. 63	F	Whole fish	Mature herring	0.7	9.0	
5	Vest Fiord, N. N.	14. 3. 64	F	Whole fish	Mature herring	5.4	11.3	-
6	Vest Fiord, N. N.	March-64	F	Whole fish	Mature herring	3.5	13.2	
7	Vest Fiord, N. N.	March-64	F	Whole fish	Mature herring	5.0	12.5	
8	Vest Fiord, N. N.	May-64	F	Whole fish	Mature herring	9.6	14.3	
9	Möre, W. N.	3. 3. 64	B — w	Whole fish	Immature herring	5.5	8.0	3.3
10	Ramsöy Fiord, W. N.	21. 3. 64	F	Whole fish	Mature herring	5.5	13.4	-
11	Kristiansund, W. N.	5. 4. 64	F	Whole fish	Mature herring	5.6	10.5	
12	Bergen, W. N.	Spring-64	B — w	Whole fish	Mature herring	3.4	13.0	
13	Bergen, W. N.	Nov.-64	C — m	Whole fish	Immature herring	2.7	3.6	
14	Bergen, W. N.	Autumn-64	B — w	Whole fish	Immature herring	5.1	8.4	
15	Bergen, W. N.	Jan.-64	B — w	Whole fish	Immature herring	4.4	8.4	
16	Egersund, S. N.	9. 4. 64	F	Whole fish	Mature herring	6.1	11.7	
17	North Sea	5. 4. 64	F	Whole fish	Mature herring	5.8	11.1	
18	Skagerak	Dec.-63	C — m	Whole fish	Immature herring	22.5	3.8	
19	Skagerak	Dec.-63	C — m	Tissue	Immature herring	14.6	3.6	
19	Skagerak	Dec.-63	C — m	Liver	From No. 19	24.0	6.7	-
20	Skagerak	Jan.-64	F	Whole fish	Immature herring	12.0	12.7	-
21	Oslo Fiord, S. N.	Jan.-64	C — m	Whole fish	Immature herring	8.3	3.1	
22	Oslo Fiord, S. N.	Dec.-63	C — m	Tissue	Immature herring	2.2	4.5	1.2
22	Oslo Fiord, S. N.	Dec.-63	C — m	Liver	From No. 22	13.7	4.4	-
23	Oslo Fiord, S. N.	Feb.-64	B — w	Whole fish	Mature herring	3.6	6.6	
24	Oslo Fiord, S. N.	3. 3. 64	B — w	Whole fish	Mature herring	1.0	9.0	5.1

[1]) N. N. Northern Norway, W. N. Western Norway, S. N. - Southern Norway.
[2]) F Produced in factory, B — w Boiled w/water, C — m Extracted w/chloroform-methanol.
[3]) Not determined.

[1]) W. N Western Norway, S. N. Southern Norway.
[2]) F Produced in Factory, B w Boiled w water, C m Extracted w/chloroform-methanol.
[3]) Not determined.

Table 6. Oil from whale

Sample [1])	Locality	Caught	Method of production [2])	Organ	Comments	Br ppm	As ppm	I ppm [3])
Whale x_1	Antarctica	Season 61 62	F	Blubber	Color 4.5	0.8	2.2	
Whale x_2	Antarctica	Season 61 62	F	Blubber	Color 5.8	0.7	2.8	
Whale x_3	Antarctica	March-63	F	Blubber		0.8	2.5	
Balaenoptera physalus	Antarctica	March-63	F	Blubber		1.0	1.8	-
Balaenoptera physalus	Antarticac	March-63	F	Meat-bone-blubber	-	0.8	2.3	
Balaenoptera physalus	Antarctica	March-63	F	Meat		0.5	2.4	
Physeter macrocephalus	Antarctica	Season 62 63	F	Blubber		5.6	0.6	

[1]) x mixed sample from tank.
[2]) F Produced in factory.
[3]) Not determined.

Table 7. Oil from marine and limnetic plankton

Sample No.	Locality [1])	Date of collection	Method of production [2])	Comments The dominating plankton	Br ppm	As ppm	I ppm [3])
1	Gjersjöen, Ak. (F. w.)	13. 8. 63	P e	Diatome plankton	150	n. d.	
2	Steins Fiord, Bu. (F. w.)	8. 8. 63	P e	Diatome plankton	120	n. d.	
3	Maridalsvatnet, Os., (F. w.)	15. 8. 63	P e	Bluegreen algae plankton	75	n. d.	-
4	Pollen, Ak. (F. w.)	26. 6. 64	P e	Dinoflagellat plankton	95	n. d.	n. d.
5	Årungen, Ak. (F. w.)	26. 6. 64	P e	Rotifer plankton	20	n. d.	n. d.
6	Nerevatnet, Ak. (F. w.)	26. 6. 64	P - e	Diatome plankton	24	n. d.	n. d.
7	Oslo Fiord (S. w.)	23. 3. 64	P e	Diatome plankton	15870	n. d.	
8	Oslo Fiord (S. w.)	23. 3. 64	P e	Dinoflagellat plankton	10900	n. d.	

[1]) Ak Akershus county, Bu Buskerud county, Os Oslo county, F. w. Fresh water, S. w. Salt water
[2]) P e Extracted w/petrolether.
[3]) n d. Not detected, Not determined.

The oils produced from plankton were compared with fish oils by IR-spectrometry. The spectra showed that the plankton oils contained more free acids, otherwise the spectra were similar.

With oil samples of about 0.5 g it is assumed a accuracy of $\pm$ 10—15% or better. When smaller amount of oil is to be analysed the accuracy may be poorer. The possibility that some of the activated isotopes will disappear as hydrogen compounds is higher in small samples. This is especially the case for the oils extracted from plankton where the content of bromine is essentially higher than in fish oils.

5. Discussion

The principal goal for this investigation was to develop a method for determination of the bromine, iodine, and arsenic content in oils from marine and limnetic organisms. Due to the selection of the oils analysed, it was also possible to draw some conclusions concerning the concentration and distribution of these elements in the different samples of oil.

The values for the bromine and iodine contents in the oils analysed are in good agreement with results from earlier investigations. Whereas no quantitative determinations of bromine in fish oils are available in previous literature.

The present work confirms that the nutrition of the fishes seems to be an important factor for the bromine, iodine, and arsenic content in the oils. It probably also indirectly reflects the content of the elements in fresh and sea water except for bromine in the latter.

It is seen that oils from limnetic organisms contain generally less bromine, iodine, and arsenic than oils from marine organisms. This is especially the case for the arsenic content in fish oils from inland localities where the concentration of this element was below 0.1 ppm.

In cases where fishes of the same species seem to have the same living conditions, the value for these elements are nearly constant. Mature cod coming from Barentz Sea and into the coast of the North Norway to spawn, have about the same content of bromine, iodine, and arsenic in the liver oil whereas the concentration of these elements in coastal cod from North Norway and from other localities along the coast of Norway is significantly different. A similar relation can be seen for mature herring caught just before and during the spawning period. Samples of oil from this type herring ("storsild") from Vest Fiord, More, and the North Sea have a bromine content of 3—6 ppm and an arsenic content of 10—14 ppm in spite of the highly different localities. Samples of oil from herring caught near Iceland with intervals of about 1 month show similar constant bromine and arsenic contents: Br, 0.7 ppm and As, 9.0 ppm. Most of the samples of oil from local herring caught along the coast have values quite different from those quoted above. It seems from the results that fishes located along the coast and in the fiords have a higher bromine content in relation to the arsenic content, than the fishes from open sea.

The bromine content in the oils from marine and fresh water organisms is present at a more constant level than the other two elements. This is of interest, especially when looking at the high bromine content found in plankton oil, pointing out that the element may have some connection with the production of the oils.

From determinations of liver and tissue oils in the same fishes it is observed that there is a higher bromine content relative to the arsenic content in the liver as compared with the tissue.

It is difficult to say whether the arsenic is present as an arsenorganic compound or if the element is a substitute for phosphorus in one or some of the phospholipids. The fact that the unknown compound containing arsenic seems to exist as a weak acid also excludes the connection with many of the phospho-lipids.

It can also be concluded that both the bromine and iodine seem to be parts of organic molecules which are highly soluble in the oils.

The analytical method established in this investigation has proved to be especially fitted for analyzing trace elements in oils. One interesting feature of the method is the possibility of studying the relation between two ore more trace elements in one sample and compare this with the same relation in other samples. This can be done with a high degree of precision if the sample is analyzed non-destructively. Any accumulation of one element compared with the other can then be detected.

Generally, the neutron activation analyses may also be applied for other problems related to trace elements in biological systems.

6. Summary

A method for analysing bromine iodine, and arsenic in oils from marine and limnetic organisms has been developed. It is based on neutron activation of the oils and the measurement of the induced radioactivity by a γ-spectrometer without any chemical treatment of the oils. Ordinary about 0.5 g of each oil was used for determination of all three elements. The lower limit here is about 25 mg oil for each determination. This is set by the sensitivity of the method and difficulties in handling the oil samples of this size.

The oil samples which were analysed contained: In fish oils, 0.3 – 60 ppm bromine, 1 – 60 ppm iodine, and <0.1 – 26 ppm arsenic. In oils from limnetic plankton, 20 – 170 ppm bromine and in oil from marine plankton, $1.0 - 1.5 \cdot 10^4$ ppm bromine. The results for arsenic and iodine are in reasonable agreement with published data obtained by other analytical methods. The results also support earlier assumption that the iodine and arsenic are present as organic compounds. This also seems to be the case for the bromine. No sign of bromine, iodine, and arsenic was observed in oil samples extracted from terrestric plants and animals.

7. Acknowledgement

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Analysis of Arsenic in Marine Oils by Neutron Activation. Evidence of Arseno Organic Compounds

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Abstract

The arsenic content of phospholipid fractions separated from codliver oil (*Gadus morrhua*) and herring oil (*Clupea harengus*) was analyzed by means of neutron activation. The fractions were separated on a silicic acid column by chloroform methanol mixtures as eluting agents. The results indicate that the arsenic appears as arseno organic compounds. Two such compounds were evident in herring oil.

Introduction

ARSENIC HAS EARLIER been observed in marine oils (1–4). There seems to be a certain agreement that the arsenic appears as one or more arseno organic compounds (4). A work by Sadolin may be mentioned in this connection (5). By extracting codliver oil by alcohol, it was possible to enrich the arsenic content from 3.9 mg per kg to 100 mg per kg. After further treatment a fraction was obtained, which contained 1000 mg arsenic per kg. On the basis of these studies it appeared that the compound containing the arsenic has a closer resemblance to the phospholipids than the neutral lipids.

The purpose of this work was to study, in more detail, whether the arsenic replaces phosphorus in the phospholipids or whether it exists as one or more independent arseno organic compounds. Codliver oil (*Gadus morrhua*) and herring oil (*Clupea harengus*) were used in this investigation. The phospholipids from these oils were fractionated, and each fraction was analyzed by neutron activation. A description of the method is given elsewhere (4).

Preparation of Samples

Oil from codliver and herring was extracted by a mixture of chloroform methanol (2:1 v/v) after homogenization of the samples in a mechanical mixer. After removal of the solid phase the solvent was evaporated at 50–60C. The phospholipids were separated from the neutral lipids by using activated silicic acid (E. Merck AG, Darmstadt), 0.2–0.5 mm, for chromatography. The oil was dissolved in chloroform and mixed with silicic acid. About 4 g of silicic acid to 1 ml of oil were used. In this step the phospholipids were adsorbed to the silicic acid, and the chloroform phase, containing the neutral lipids, was filtered out.

TABLE I

Arsenic Content in Phospholipid Fractions

Fraction	Eluating agent	Eluating volume (ml)	As (ppm) in Phospholipids: Codliver oil	As (ppm) in Phospholipids: Herring oil
1	Chloroform	500	1.0	0.8
2	Acetone	150	18.0	23.0
3	0.5% Methanol in chloroform	200	26.0	34.0
4	7 % Methanol in chloroform	250	11.2	34.0
5	12 % Methanol in chloroform	250	24.3	30.0
6	17 % Methanol in chloroform	250	85.0	225.0
7	22 % Methanol in chloroform	500	308.0	900.0
8	27 % Methanol in chloroform	250	718.0	911.0
9	above 27% Methanol	250	427.0	590.0

TABLE II

Eluating Agents Used in Fraction Collector Experiment

Fraction		Eluating agent	Eluating volume
1	A[a]	Chloroform	210
2	B	Acetone	150
3	C	5% Methanol in chloroform	210
4	D	15% Methanol in chloroform	300
5	E	25% Methanol in chloroform	300
6	F	40% Methanol in chloroform	300
7	G	65% Methanol in chloroform	210
8	H	90% Methanol in chloroform	270

[a] See Fig. 1.

It was then washed twice with chloroform prior to the filtering of the phospholipids by a chloroform methanol mixture (1:20 v/v). The phospholipids were fractionated on a silicic acid column (height 50 cm, inner diameter 2.2 cm) by means of chloroform methanol mixtures.

The fractions were eluted by gradually increasing the methanol content in the chloroform with a speed of about 4 ml per minute. The first elution processes were carried out with chloroform methanol mixtures, as described by Froines et al. (6) in their studies of the phospholipid distribution in menhaden tissue (Table I). Neutral lipids, not separated during the initial purification stage, were eluted first with pure chloroform. An acetone elution was done to remove oxidized products (7). To obtain a more exact elution diagram, a fractionation with an automatic fraction-collector was carried out. Each fraction was 30 ml, and the chloroform/methanol mixtures (Table II) were almost the same as used by de Koning (8). The phospholipids in the various fractions obtained were not analyzed.

Irradiation of Samples

After evaporation of the solvent the phospholipid fractions were weighed, transferred to polyethylene ampoules, and sealed. The ampoules were irradiated in the nuclear reactor JEEP I (Kjeller, Norway)

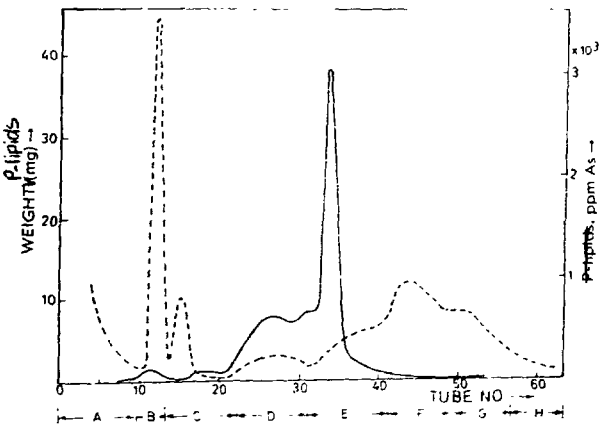

FIG. 1. Phospholipids ------, arsenic ———. Chromatographic separation of phospholipids and arsenic (herring oil). The composition of the eluating mixtures A, B, etc., is given in the text.

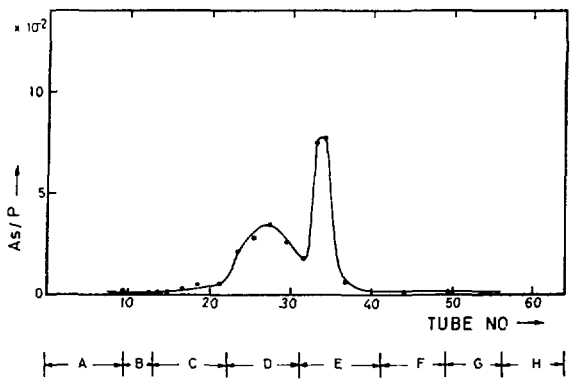

FIG. 2. The ratio between arsenic and phosphorus (w/w) in fractions of phospholipids from herring oil. From the same chromatographic separation as shown in Fig. 1.

with a neutron flux of approximately 2.10^{12} neutrons cm^{-2} sec^{-1} for 24 hours. Arsenic and phosphorus standards (As_2O_3 and $(NH_4)_2HPO_4$ PA-reagents, E. Merck A. G., Darmstadt) were irradiated at the same time. A 400-channel *gamma* spectrometer (Victoreen Scipp 400), with a 2 × 2-in. sodium-iodide crystal, was used for recording the induced activity in the samples. As it was difficult to avoid sodium contamination of the samples during fractionation of the phospholipids, it was expedient to wait about five days before recording the activated arsenic, arsenic-76. The activity of sodium-24 [$T_{1/2}$ (half life) = 14 hours] was then reduced sufficiently to allow recording of the arsenic-76 ($T_{1/2} = 26$ hours). The arsenic photo peak of 0.55 MeV was used for the quantitative determination of arsenic in the phospholipids. This was compared with the same photo peak from the arsenic standard.

When the measurement of radio-active arsenic is undertaken without removing the lipids from the activated polyethylene ampoules, impurities from the polyethylene ampoule can disturb the recording of the arsenic-76. This applies especially when the arsenic content in the samples is below 0.1 μg. These problems can be avoided by subtracting, during the recording of the samples, the *gamma* spectra from an identical, empty polyethylene ampoule which is irradiated under the same conditions as the samples. After the arsenic-76 activity has decayed, the induced phosphorus isotope, phosphorus-32, is measured directly by means of a proportional counter. As phosphor-32 is a pure β-emitter, this method gives a poor degree of precision owing to the uneven distribution of the lipids in the polyethylene ampoules.

The phosphorus determinations were used for calculating the As/P ratio in the different phospholipid fractions. For this purpose a high accuracy was not necessary.

Results and Discussion

The results of the first experiments appear in Table I. The arsenic content of the various fractions is calculated in ppm. The results indicate that the arsenic content in the fractions is highest in the fractions eluted with 20–30% methanol. From the experiment where the fractions were collected with an automatic fraction-collector (Table II and Fig. 1) it was evident that there were two arseno organic compounds present, one that was eluted with about 20 volume % methanol and the other with about 25–30 volume % methanol. The arsenic content in one of the fractions was about 3,000 ppm. This should have been sufficient to characterize the compound by other analytical methods. The phosphorus content in the fractions (fraction 20–50) was from 1.9–3.9%. By means of the phosphorus determination the As/P ratio was calculated (Fig. 2). The two peaks indicate also the presence of two arseno organic compounds. If the arsenic had replaced the phosphorus in the phospholipids, this would probably have resulted in an approximate constant As/P ratio in the different fractions.

On the basis of the results obtained, two arseno organic compounds or arseno lipids are indicated and their chemical properties resemble the properties of the phospholipids. It is most probable that it is the fish itself or other organisms contained in the food intake of the fish, which synthesizes these arseno organic compounds. The arsenic in sea water (about 0.003 ppm) appears mainly as inorganic anion (9).

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ACTIVATION ANALYSIS OF TRACE ELEMENTS IN FISHMEAL

By G. LUNDE

Fishmeals produced from herring (*Clupea harengus*), mackerel (*Scomber scomber*), capelin (*Mallotus villosus*) and Norway pout (*Gadus esmarki*) were analysed by neutron activation. The elements tested were mercury, bromine, arsenic, selenium, antimony, copper, cobalt, iron, zinc, molybdenum and tungsten. The distribution of these elements in the solid and aqueous phases in boiled fish used as raw material for fishmeal was also studied.

Introduction

Interest in trace elements and their physiological significance has increased considerably in recent years, for example in relation to animal feedingstuffs. Feed mixtures should contain sufficient amounts of trace elements necessary for the animals. Of these elements the following may be mentioned specially: molybdenum, zinc, fluorine, copper, manganese, cobalt, chromium and selenium. There is also a certain amount of interest in arsenic; although this element has not been localised in any metabolic process, additions of certain arseno-organic compounds have a favourable influence on growth of chickens and pigs.[1]

Relatively few data are available for trace elements in feeds of marine origin.[2,3] The purpose of this work was to analyse the most common types of fishmeal commercially produced in Norway.

Fishmeal is usually produced by boiling the fish at 95–100°. The material is then pressed in a screwpress where the insoluble phase (the press cake) is separated from the liquid phase consisting of oil and nitrogenous liquor. The oil is separated by centrifugation and the nitrogenous liquor is concentrated by evaporation.

In Norway the normal procedure for making fishmeal is to mix the press cake and the solubles and dry the mixture to so-called full meal. The dehydration of the meal is normally carried out at 60–80°.

In connexion with the project it was also of interest to investigate more thoroughly how the various trace elements are distributed in the press cake and the nitrogenous liquor. For this reason some samples of press cake and N liquor were produced from the same raw material.

Experimental

Neutron activation was used as analytical method. This method is sensitive for several among the typical trace elements. It is also suitable when several elements are to be analysed simultaneously in the same sample. Owing to a high content of Na and P in the fishmeal it is necessary to perform a chemical separation after the irradiation. This separation was carried out according to the directions given by Samsahl.[4-6] A general introduction to the method has been given by Bowen & Gibbons.[7]

The following elements were analysed or looked for: Hg, Br, As, Se, Sb, Cu, Co, Fe, Zn, Mo and W. All of these elements, with the exception of Fe, show high sensitivity by this method. The radioactive isotopes induced in these elements by pile neutrons disintegrate, giving off gamma emissions; they can therefore be recorded by means of a multi-channel gamma spectrometer after a relatively simple chemical separation.

Sample preparation

The common types of fish used in the production of fishmeal in Norway are herring (*Clupea harengus*), mackerel (*Scomber scomber*), and capelin (*Mallotus villosus*), but smaller landings of trash fish, such as Norway pout (*Gadus esmarki*), are also used. The samples for the analyses were obtained directly from factories or they were produced in the laboratory. The raw material for the laboratory samples was obtained from the commercial fish market and treated as follows: about 2 kg fish were ground in a blender, and boiled in a glass apparatus for about half an hour. Some distilled water was added. The oil was then separated and the residue was filtered and washed once on the filter with distilled water. The insoluble portion, the press cake, was dehydrated at 50–60° and finally homogenised in an agate mortar. The water-soluble portion was concentrated by evaporation at 80–90°. Prior to the neutron irradiation all the samples were dehydrated at 105° to constant weight. The yield of press cake and N liquor from the samples produced in the laboratory is presented in Table I.

Irradiation and registration

Approximately ½ g fishmeal was used in each irradiation. The samples of the concentrated N liquor were diluted with distilled water (1 : 2) in order to facilitate the transfer of the N liquor after irradiation to the dissolution flasks. All samples were sealed in quartz ampouled and irradiated for 3 days at an approximately constant neutron flux $2 \cdot 4 \times 10^2$ neutron cm^{-2} sec^{-1} in the nuclear reactor JEEP I, Kjeller, Norway. Standards (PA reagents from A. Merck, Darmstadt) for the elements to be analysed were irradiated simultaneously.

The chemical separation of the trace elements to be analysed[4-6] was based on a wet dissolution (H_2SO_4), distillation and ion exchange technique. By varying the eluting conditions groups of elements were adsorbed in different ion-exchange columns. After the separation the fractions of resin were collected in measuring glasses and the activities were recorded by a 400-channel γ spectrometer (Victoreen

Scipp 400) with 2 × 2′ sodium iodide crystal. The standards were subjected to identical treatment; they were dissolved and adsorbed on to ion-exchange resin in the same way as the corresponding elements from the activated samples. A precision of ±10–15% can be expected by this method when the concentrations of the respective elements are not close to the limit of the sensitivity

Results and Discussion

The analytical results for commercially produced fishmeal samples are given in Table II, and those for samples produced in the laboratory are in Table III. Zn and Fe are adsorbed to the same ion-exchange resin, and owing to the high activity of the 1·11 MeV γ-peak from ^{67}Zn the activity from 1·29 MeV of ^{59}Fe was difficult to measure. Only a few such measurements were performed, and low precision was to be expected (±40–50%). The variations in the results of the Fe determinations may also be due to contamination by this element throughout the processing of the meal. The concentrations of Co, Zn and Cu in the fishmeal are in agreement with other analytical results.[2,8] Results concerning Hg, As, Br and Sb content in fish can be found in 'The elementary chemical composition of marine organisms' by Vinogradov.[9] The Br content was only determined in the samples produced in the laboratory (Table III). No previous determinations of Se in marine fish have been found.

Table III indicates how the various trace elements are distributed in the solid and liquid phases (i.e. press cake and concentrated N liquor). It may be noted that among the essential elements, Zn and Mo seem to occur mainly in the press cake while Co and Cu are more concentrated in the N liquor. The way in which the different trace elements are distributed on the two phases may have some effect on the living organism's ability to resorb these elements. The Se, As and Br contents are greater in the liquid phase than in the solid phase. In some preliminary studies using the radioactive isotope, ^{76}As, it has been shown that the As does not exchange with inorganic arsenite and arsenate (Lunde, G. unpublished results). The As may therefore exist as an arseno-organic compound.

From the analyses there does not appear to be any significant difference in the trace element content of fishmeal produced from different types of fish. The variations found seem to be as much an expression of the difference in basic food intake and habitat of the fish as of species differences.

TABLE I

Yield of press cake and nitrogenous liquor produced from fish raw material in the laboratory

Sample	Raw material, g	Press cake, dehydrated, g	N liquor, conc., g
Herring (Mature	2140	232	80
Herring (Immature)	1520	358	47
Mackerel	1460	258	49

TABLE II

Trace elements ppm in press cake and concentrated N liquor

Sample	Fraction	Locality	Se	As	Sb	Br	Co	Cu	Zn	Fe	Hg	Mo	W
Herring (Mature)	Press cake	Western Norway	1·9	2·3	0·061	4·7	0·17	11·2	86	—	0·26	0·26	<0·005
Herring (Mature)	N liquor	Western Norway	2·8	13·0	<0·01	65	0·28	17·2	7·7	—	<0·01	<0·05	<0·005
Herring (Immature)	Press cake	Western Norway	1·3	3·6	0·013	2·2	0·087	3·5	100	23	0·16	0·13	<0·005
Herring (Immature)	N liquor	Western Norway	5·6	24·0	<0·01	63	0·11	6·2	6·7	4·0	<0·02	<0·05	<0·005
Mackerel	Press cake	North Sea	2·7	4·5	0·017	8·3	0·10	2·0	84	15·0	0·41	0·10	<0·005
Mackerel	N liquor	North Sea	15·1	15·2	<0·01	130	0·22	10·4	8·7	4·4	<0·01	<0·05	<0·005

— Not measured

TABLE III

Trace elements ppm in factory-produced fishmeal

Sample	Locality	Se	As	Sb	Co	Cu	Zn	Fe	Hg	Mo	W
Herring	North Sea	2·9	4·9	0·029	0·51	3·0	88	—	0·18	0·05	<0·005
Herring	North Sea	3·2	4·0	0·028	0·07	2·8	76	—	0·26	0·30	<0·005
Herring	Shetland	3·7	15·0	0·040	0·25	2·0	84	—	0·36	0·03	<0·005
Herring	Western Norway	2·7	4·0	0·015	0·19	1·6	75	14	0·18	0·07	<0·005
Herring	Western Norway	2·2	2·7	0·029	0·15	2·3	85	106	0·09	0·13	<0·005
Herring	Western Norway	2·0	4·0	0·017	0·18	1·6	74	—	0·11	0·07	0·014
Herring	From stock, Oslo	3·6	5·3	0·012	0·34	2·4	65	—	0·13	0·16	<0·005
Mackerel	North Sea	5·3	3·8	0·015	0·17	1·2	88	—	0·21	<0·05	<0·005
Mackerel	North Sea	2·2	2·7	0·047	0·23	2·1	77	87	0·09	0·15	<0·005
Capelin	Northern Norway	1·3	2·6	0·015	0·05	1·7	108	—	0·026	0·2	<0·005
Capelin	Northern Norway	1·7	19·1	0·018	0·13	4·1	134	—	0·13	0·06	0·008
Norway pout	Western Norway	1·5	3·9	0·20	0·82	2·9	180	—	0·40	<0·05	0·030

— Not measured

Acknowledgment

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IV

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Water Soluble Arseno-organic Compounds in Marine Fishes

MARINE fishes have been shown to contain arsenic in both the lipid and non-lipid phase[1-2]. In the first case it is probably in the form of one or more arseno-organic compounds[3]. Previous work has established that when raw fish is boiled the arsenic is enriched in the water soluble phase (the N-liquor)[4].

We have investigated whether the arsenic is present as an organic or an inorganic compound in the N-liquor. This is especially interesting in connexion with the use of fish products as food. There may be an effect similar to that reported for the arsenicals used as additives in feed for domestic animals[5].

Our method has been to study the exchange between the arsenic originally present in the N-liquor and added radioactive arsenite–arsenate. Comparison of the arsenic content in the N-liquor before and after elution through an anion exchange resin can reveal the origin of the arsenic.

We used samples of mackerel (*Scomber scomber*), capelin (*Mallotus villosus*) and herring (*Clupea harengus*), which were boiled for approximately 20 min to give the N-liquor. After removing the solid by filtration, the concentration of dry matter in the N-liquor was adjusted to about 2 per cent with distilled water. To 100 ml. of this solution was added 2 ml. of a radioactive arsenite–arsenate solution (0·02 μg/ml. of As). We used the arsenic isotope ^{76}As, produced by irradiation with thermal neutrons in a nuclear reactor. The solution was heated to approximately 80° C for about 5 h, cooled and eluted through an anion exchange column ('Dowex-2' 1 × 8 200/400 mesh). The radioactive arsenic in the anion exchange resin and the N-liquor were then measured in a well-type NaI scintillation counter. Results showed that all radioactive arsenic was adsorbed to the anion exchange resin.

The N-liquor, from before and after the treatment just described, was analysed by neutron activation. The samples (1 ml.) were sealed in polyethylene ampoules and irradiated together with standards of arsenic for 2 h in a nuclear reactor at a flux of 5×10^{12} n/cm^2 s. A simple radiochemical separation was then performed to purify the induced radioactive arsenic sufficiently for it to be registered by a γ-spectrometer. We added 10 mg arsenic carrier and "hold-back" carriers of sodium and phosphorus together with HCl to make the N-liquor about 6 N in HCl. The solution was heated to 70°–80° C for 2 h. This treatment brought about an exchange between the

arsenic carrier and the arsenic to be analysed. Arsenic was then precipitated as a sulphide, and the precipitate was washed. (If radioactive sodium and phosphorus, ^{24}Na and ^{32}P were still present at this point and impeded the registration of ^{76}As, the As_2S_3 was dissolved in NH_3 and additional hold-back carriers were added before reprecipitating the As_2S_3 with HCl.) The samples were then ready for registration.

Table 1. ARSENIC CONTENT IN N-LIQUOR BEFORE AND AFTER ION EXCHANGE TREATMENT

Sample	Locality where caught	When caught	Arsenic before ion exchange (p.p.m.)	Arsenic after ion exchange (p.p.m.)
Herring	West coast of Norway	Spring 1968	8·3	7·8
Herring	North coast of Norway	Spring 1968	6·4	5·2
Herring	North Sea	Summer 1968	19·7	19·2
Herring	North Sea	Summer 1962	22·0	21·6
Mackerel	North Sea	Summer 1968	3·2	3·1
Capelin	North coast of Norway	Spring 1968	10·3	7·9

The results in Table 1 are calculated as p.p.m. of As as dry matter in the N-liquor before anion exchange. They indicate that the content of arsenic in the N-liquor was only slightly reduced during the treatment I described and diminished far less than the dry matter in the N-liquor during ion exchange—on average 20–25 per cent.

This investigation has shown that arsenic is present in the N-liquor chiefly as one or more arseno-organic compounds in which the organic arsenic does not exchange with inorganic arsenic. The reduction of the arsenic content observed after ion exchange treatment may be a consequence of adsorption to the resin.

I thank the Norwegian Herring Oil and Meal Industry Research Institute for samples of N-liquor.

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ANALYSIS OF ARSENIC AND SELENIUM IN MARINE RAW MATERIALS

By G. LUNDE

The content of arsenic and selenium has been analysed in the following marine organisms: cod (*Gadus morhua*), herring (*Clupea harengus*), mackerel (*Scomber scomber*), Norway haddock (*Sebastes marinus*), lobster (*Homarus vulgaris*), mussel (*Mytilus edulis*), clam (*Pecten maximus*), oyster (*Ostrea edulis*), squid (*Ommastrephes sagittatus*) and whale (*Balaenoptera physalus*). The analyses were performed both on the raw material and in the water-soluble phase after the samples had been boiled (the N liquor). An enrichment of arsenic is observed in the N liquor, compared with the raw material. The results indicate that selenium is also enriched in the N liquor from fish, but not from the invertebrate animals analysed.

The N liquor and the water-soluble phase of the enzyme-hydrolysed presscake (the water-insoluble phase after boiling) were fractionated by molecular gel filtration. Fractions from these elutions were analysed for arsenic and selenium. Selenium was present in the fractions with a molecular weight above about 5000. Arsenic was connected with the lower molecular weight fractions, and may be present as more arseno-organic compounds.

Introduction

In previous work it has been shown that marine organisms contain about 1–20 ppm arsenic[1,2] and 1–5 ppm selenium.[2,3] Arsenic appears in fish both in the lipid and in the water-soluble phase,[2,4,5] whereas selenium seems only to be present in the non-lipid fraction. Since the arsenic content in ocean water is about 3 μg kg and the corresponding value for selenium is about 0·1,[6] a strong accumulation of these elements evidently occurs in fish. No corresponding arsenic enrichment in plants or animals of terrestrial origin has so far been indicated, either in the lipid or in the non-lipid phase. Some results show that terrestrial animals contain about 0·1–0·3 ppm of selenium and in some organs more.[7,8] The content of this element also seems to be somewhat higher in marine organisms.

Arsenic and selenium are the subject of considerable interest in the feeding of domestic animals. Both elements have a favourable influence on growth and health.[9] Selenium is an essential trace element and may be present both as an inorganic and as an organic compound, the latter mainly by replacing sulphur-containing amino acids, i.e. seleno-methionine, seleno-cysteine etc. Too low a concentration of this element leads to severe diseases in cows, pigs and other animals. Arseno-organic compounds (arsenilic acid and others) promote growth in the same animals. However, any physiological significance of this element is not known. In large concentrations both arsenic and selenium will have a toxic effect. This is particularly true of selenium. The toxic effect of these elements will depend on the form in which they appear. Organic arsenic compounds are recognised as less toxic than inorganic compounds such as AsH_3, As_2O_3 etc.[11] Notably, the arsenic has an antagonising effect in the case of selenium poisoning of domestic animals.[12]

The analyses of the trace element content in the water-soluble (N liquor) and the water-insoluble fraction (the presscake) from boiled fish showed that the concentration of arsenic and selenium was higher in the water-soluble portion than in the presscake.[2] Arsenic also seems to be present as organic compounds in the N liquor.[13] In this case the arsenic present in N liquor did not exchange with inorganic arsenic, i.e. arsenite–arsenate.

The purpose of this work was to analyse some selected samples of marine organisms and N liquors produced from these, and to study in more detail the form of the arsenic and selenium in these samples. Of particular interest is whether or not any similarity exists between the selenium in marine organisms and the selenium-organic compounds found in terrestrial organisms.

Experimental

Molecular gel filtration

The investigation was based on molecular gel filtration of the N liquor and the water-soluble phase of enzyme-hydrolysed presscake, following analysis of arsenic and selenium in individual fractions. The position of the fractions containing these elements will provide information concerning the molecular weight, and whether or not they are present in the same way in the different marine organisms. This method of fractionation has previously been used for studying trace elements connected to serum proteins;[14,15] likewise organic iodine and inorganic iodine have been separated by means of gel filtration.[16]

Molecular gel filtration is generally used for the separation of water-soluble organic molecules. The fractionation is based on the difference in molecular weight. This method can also be applied to the fractionation of solutions where organic compounds with different molecular weights are to be separated from inorganic ions. It should, however, be noted that the ions will not behave similarly to non-ionic molecules with identical molecular weight. The reason for this is that gel made of dextran has a certain number of carboxyl groups that will cause the resin to behave like a weak cation-exchange resin. Here both inorganic and organic anions may elute together with neutral compounds with higher molecular weight, especially when the ionic strength is low. For the same reason the cations may be retarded. The fractionation method should be tested first to establish where the inorganic ions of the elements to be analysed are eluted, in order to ensure no contamination of these. A more detailed description of such a study is to be presented later. Only results pertinent to this investigation will be presented. In Fig. 1 the position of some inorganic cations and anions are shown eluted together with N liquor made of herring. Radioactive isotopes were used for tracing the ions in the molecular filtration.

The analyses of arsenic and selenium in both raw material and fractionated samples were performed by neutron activation. This method offers great sensitivity for these elements.

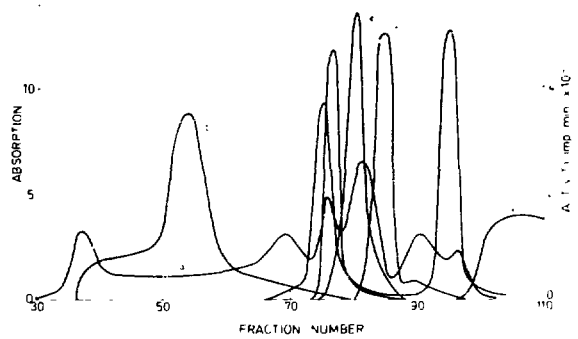

FIG. 1. *Position of some different inorganic ions when molecular gel-filtrated (Sephadex G-25) together with N liquor made from herring*

Elution was performed with 0·005 M-NH_3
(a) Absorption of the N liquor at 254 nm, (b) Cu^{2+}, (c) AsO_3^{3-}, AsO_4^{3-}, (d) SeO_3^{2-}; (e) PO_4^{3-}, (f) Br^-, (g) Na^+

Irradiation with thermal neutrons from a nuclear reactor will give the radioactive isotopes ^{76}As with a half-life of 26·5 h and ^{81}Se with a half-life of 121 days. They both disintegrate by emitting γ-photons suitable for registration with a multichannel γ-spectrometer. If no interfering radioactive isotopes are present in the fractions from the molecular gel filtration and the content of arsenic and selenium is not too low, it is possible to register these and also other induced activities without any chemical treatment, i.e. non-destructively.

A radiochemical separation of these elements is necessary for the analyses of arsenic and selenium in the raw material. The method used here is that described by Samsahl.[17–19]

Thin-layer chromatography was used for establishing where the main fractions of amino acids were eluted when gel filtrated.

Preparation of samples

Samples of fish and other marine species (listed in Table I) such as clams, lobster and whale, were acquired from the official fish distribution centre in Oslo, Norway, and from factories producing commercially available deep-frozen fish fillets. From two species of fish – cod and herring – samples were also taken of skin, bone and liver.

The raw material was first homogenised in a mechanical mixer to which double the sample's weight of distilled water was added; it was then boiled for 20 min in a glass apparatus. The mixture was subsequently cooled, filtered and washed once with distilled water. The oil was then removed. In order to convert part of the presscake into a water-soluble form suitable for gel filtration, the presscake was treated with a protease enzyme (Bioprase: Nagase and Co. Ltd., Japan), produced from the bacterium *Bacillus subtilis*.

To a tubular flask were added 10 g presscake, 40 ml distilled water, 0·1 g enzyme and 0·05 g preservative (methylentetramin). The pH was adjusted to 7–7·5 with $NaHCO_3$. The flask was placed in a thermostat at 50° and agitated for 24 h. The solution was then filtered and stored in the same way as the N liquor.

The samples of N liquor and hydrolysed presscake to be used for further analyses were transferred to polyethylene flasks and stored at −20°. The majority of samples were pale yellow.

Fractionation

The molecular gel filtrations of N liquor and the hydrolysed presscake were carried out by means of a dextran resin (Sephadex, type G-25 fine, from Fine Chemicals Pharmacia, Sweden). This resin will fractionate molecules in the molecular weight range 100–5000. Molecules with molecular weight above about 5000 will follow the void volume. This fraction will hereafter be called the protein fraction.

The pH of the solution to be fractionated was adjusted to 8·3–8·4 with NH_3. Before being applied to the column the solution had to be filtered, as some of the samples were slightly turbid following the period of storage. An asbestos filter (John C. Carlson Ltd., U.K., type K5, clarifying filter) was used for this operation. The first half of the filtrate was discarded. At each separation 50 ml of a solution containing 0·2–1·8% dry matter was used (see Table II). The resulting clear solution was added to the top of the column (1·17 m × 4·5 cm), and adsorbed on the resin. The elution was carried out with 0·005 M ammonia solution produced by mixing ammonia with distilled water. The rate of elution was from 1·7–3 ml/min. In all the experiments a fraction cutter (LKB, Sweden) was used and each fraction was approximately 20 ml. Immediately after the eluate left the column, the absorption at 254 nm of the eluate was recorded with a Uvicord spectrometer (LKB, Sweden). On the basis of the elution diagram, fractions were combined and evaporated in a Rotovapor at 40°. Figs 2–5 show examples of the absorption of some of the elutions.

Analyses of arsenic and selenium

The fractions obtained from the molecular gel filtration and used for the analyses of arsenic and selenium were usually 100–150 ml. These volumes were about double the amount necessary for resolving one peak in the gel filtration. The fractions intended for arsenic analysis and where there was a possibility of contamination of arsenite/arsenate (see Fig. 1), were treated at this stage with an anion-exchange resin in order to ensure complete removal of the inorganic arsenic.

All fractions to be analysed were evaporated to dryness, weighed and transferred to polyethylene or quartz ampoules, depending on the irradiation time. Because some of the evaporated liquid fractions could not be readily transferred after neutron irradiation from the irradiated ampoules, these samples were dissolved in 0·5–1 ml distilled water before the ampoules were sealed. All the samples of the raw material used for the production of N liquor and presscake were dried to constant weight (105°) and sealed in quartz ampoules before activation.

The samples were irradiated in Jeners nuclear reactor JEEP II (Kjeller, Norway). Usually an irradiation time, varying from 2 h at a neutron flux of approximately 5×10^{12} n/cm² sec to $1{\cdot}1 \times 10^{13}$ n/cm² sec for 20 h, was used, depending on the desired sensitivity of the elements to be analysed. Standards of arsenic and selenium were irradiated simultaneously. After irradiation the samples from the gel filtration were transferred to inactive powder vials and the activity was recorded on a multi-channeled γ-spectrometer (Victoreen Scipp 400) with a 2" × 2" in sodium iodide crystal. The standards were dissolved in water, diluted and recorded in the same way. As there was generally some sodium in the samples, it was expedient to wait for 4–6 days before the registration of arsenic could begin. After this period the induced sodium activity (^{24}Na with a half-life of about 15 h) was sufficiently reduced to enable recording of ^{76}As.

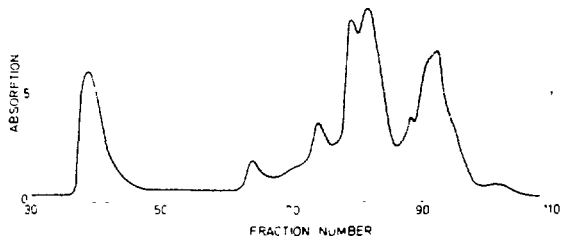

FIG. 2. *Absorption at 254 nm of molecular gel-filtrated N liquor of cod liver*

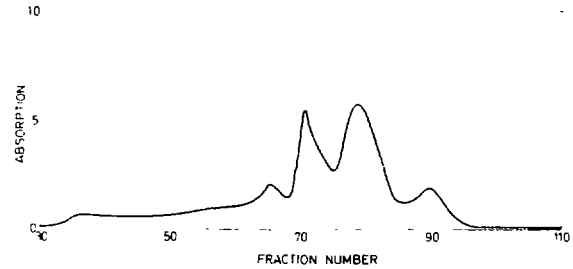

FIG. 3. *Absorption at 254 nm of molecular gel-filtrated enzyme-treated presscake of lobster*

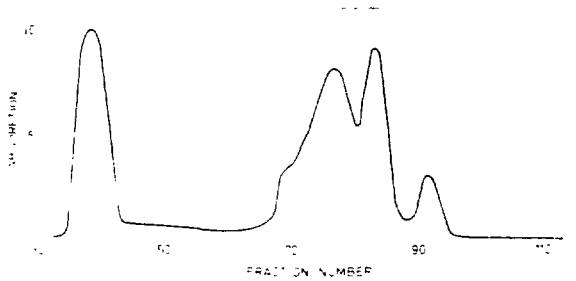

FIG. 4 *Absorption at 254 nm of molecular gel-filtrated N liquor of oyster*

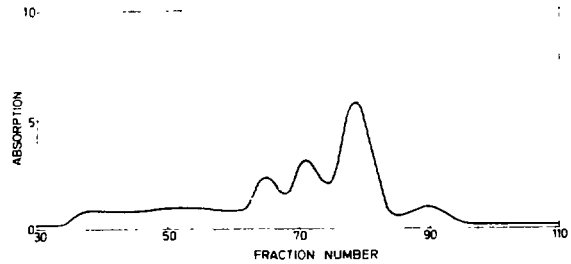

FIG. 5. *Absorption at 254 nm of molecular gel-filtrated enzyme-treated presscake of oyster*

The determination of phosphorus was also of interest in establishing whether or not there was a relationship between phosphorus and arsenic in the compounds. ^{32}P, induced during neutron activation, was recorded after the ^{76}As had disintegrated. The phosphorus isotope ^{32}P emits only β-particles and is recorded as brehmstrahlung in the γ-spectrum. The amount of phosphorus may be measured by totalling channels 20-30 in the γ-spectra.

The selenium, with a half-life of 121 days, was measured after waiting about a month, allowing all the relatively short-lived radioactive isotopes to disintegrate.

In some of the fractions the content of interfering activities was too high or the content of arsenic or selenium was too low, to enable a non-destructive analysis. A simple radioactive separation was therefore carried out. By treating the sample with 6 N-HCl at 50–80° the activated arsenic was exchanged for an arsenic carrier of arsenite–arsenate. The arsenic was then precipitated as a sulphide. The fraction containing selenium was treated with sulphuric acid and hydrogen peroxide until a clear solution was obtained. Selenium carrier was added and the selenium was precipitated as a sulphide. Standards for both arsenic and selenium were prepared in a similar way to the sample to be analysed.

The method for analysing the total amount of arsenic and selenium in the marine raw material and the N liquor was based on distillation of the two elements with hydrobromic acid, after dissolving the organic material in sulphuric acid and hydrogen peroxide.[17-19]

The approximate position of the amino acids in the gel filtration was established by means of thin-layer chromatography. Samples of various fractions from the gel filtration of N liquor from herring and cod fillets were used. Here chloroform–methanol–ammonia (2 : 2 : 1 by vol.) was used as solvent and cellulose as the thin layer.

Results and Discussion

The results of the analyses of the total amount of arsenic and selenium in the different samples of raw material and N liquor, produced from the raw material, are presented in Table I. They confirmed previous results of fish analyses, both as to the content of these elements in the raw material and also that they were enriched in the N liquor. For the other organisms analysed, a corresponding enrichment of arsenic in N liquor was observed, but not for selenium. Although not many samples were analysed in the latter case, a difference seems evident here for the fish species compared with the other organisms.

The arsenic and selenium content were significantly lower in the fish samples than in samples from the other organisms. This may have some connexion with the fish being at a higher level in the food-chain. If the inorganic arsenic present in the sea water is the main source of the production of arseno-organic compounds, the ability of the organisms to concentrate water may be of importance. Probably the arsenic content was also influenced by the locality in which the different organisms had been caught. This has been shown for the arsenic present in the lipid phase of fish.[20]

The results for the whale meat were an exception as they were significantly lower than the other results. More data need to be obtained.

In the elution diagram from gel-filtrated N liquor the first peak represents the void volume and consists mainly of proteins, i.e. compounds with molecular weight above about 5000. From the studies by thin-layer chromatography of fractions from gel-filtrated N-liquor of herring, mackerel and cod fillets, it was established that the amino acids start to elute in fractions 65–75. The section between the first peak and the amino acids usually consisted of a shoulder, and was mainly a mixture of different peptides. In some of the gel-

TABLE I

Arsenic and selenium content in dehydrated raw material and N liquor, ppm

Sample	Name	Raw material		N liquor	
		Arsenic	Selenium	Arsenic	Selenium
Cod fillet	*Gadus morhua*	2·2	1·2	13	2·6
Code bone	,, ,,	0·9	0·7	11	1·6
Cod liver	,, ,,	9·8	3·7	37	4·6
Cod skin	,, ,,	3·5	8·6	6·1	1·4
Herring fillet	*Clupea harengus*	3·8	1·0	24	2·4
Herring bone	,, ,,	1·9	0·8	n.d.	n.d.
Herring skin	,, ,,	7·2	0·8	n.d.	n.d.
Mackerel fillet (mature)	*Scomber scomber*	3·5	1·3	n.d.	2·9
Mackerel fillet (immature)	,, ,,	3·0	1·4	17	3·9
Norway haddock fillet	*Sebastes marinus*	3·3	1·5	23	4·5
Lobster fillet	*Homarus vulgaris*	5·3	1·5	14	2·9
Mussel	*Mytilus edulis*	8·0	3·9	9·7	1·1
Clam	*Pecten maximus*	11·6	2·6	18	1·0
Oyster	*Ostrea edulis*	7·6	n.d.	9·8	0·9
Squid	*Ommastrephes sagittatus*	6·5	3·0	17	0·6
Whale meat	*Balaenoptera physalus*	0·36	0·5	0·9	0·4

n.d. = not determined

filtration experiments peaks in this area could also be observed, especially in the lower molecular weight region. The water-soluble phase from the enzyme-treated presscake gave a similar elution diagram, except that the protein fraction was reduced; in some of the samples this fraction did not appear at all (Figs 3 and 5).

The fractions from the molecular gel filtration can generally be analysed non-destructively for arsenic up to fractions 70–75. This was particularly the case for fractionated N liquor where the arsenic content in general was higher than in the samples produced by enzyme-hydrolysed presscake. Above fractions 70–75 a radiochemical separation of the arsenic was usually necessary, owing to the presence of what was probably organic bromine. As seen from Table II, fractions 1–3 have been analysed in each of the gel-filtrated samples. They were all taken from fractions 60–75. The arsenic concentration in the fractions below no. 60 was too low to be analysed non-destructively.

When the results in Table II are compared with the total amount of arsenic found in the raw material and in the N liquor, only some of the arsenic can be accounted for in the analysed fractions. Therefore arsenic is also present in fractions above nos 60–75. Compared with the amount found in the raw material and the N liquor, no fractions were specially rich in arsenic, either calculated as absolute amount of arsenic or as μg As/g dehydrated matter, indicating that no distinct arsenic compound seems to be present. This leads to the assumption that the arsenic probably exists as part of more organic molecules in the N liquor, and in the water-soluble part of the enzyme-hydrolysed presscake. As these arseno-organic compounds are mainly neutral, it is not possible to use the position of the amino acids in the elution diagram as a standard for molecular weight determination. Because of the ion-exchange character of the gel-filtration resin, the speed of elution of amino acids was probably faster than corresponding neutral molecules. The arseno-organic compounds registered in the fractions 60–75 may therefore have molecular weights considerably higher than the amino acids.

Only preliminary studies were undertaken on the phosphorus content in the different fractions. Phosphorus was detected in some of the protein fractions and in fractions above about no. 70. No evidence was found of phosphorus and arsenic replacing each other.

From the results it should be concluded that the arseno-organic compounds analysed in this study may be present originally in the marine organisms or they may be formed from one or some few compounds by decomposition when the raw material is boiled. It is tempting to assume that these compounds are derivatives of a relatively simple arseno-organic molecule present in all marine organisms. This compound is probably synthesised by the same mechanism in one or more species of marine organisms. The arsenic in this arseno-organic compound cannot be linked to organic groups by ester bondings in the same way as phosphorus, as these are not stable in water.[21] Both the lipid and water-soluble arseno-organic compounds may, however, be of a phenylarsine type. Derivatives of such a compound provide possibilities both for lipid and water-soluble compounds. However, more work has to be done for more definite conclusions to be obtained.

The selenium was only analysed in the protein fractions (see Table III). This was done both in order to avoid any contamination from inorganic selenium (Fig. 1) and to see if selenium was present in the same way as sulphur in the sulphur-containing amino acids, thus being a constituent of the proteins. When the Sephadex G-25 resin was used all molecules with molecular weight above about 5000 will appear in this fraction. The presence of selenium in these fractions indicated that this selenium was probably present as one or more selenium organic compounds.

The concentration of selenium in the protein fractions seems mostly to be higher than in the raw material. The fraction of proteins, i.e. N × 6·25, constitutes 80–90% of dehydrated raw material after the fat is removed. It is therefore difficult to understand that in most of the analyses the amount of selenium connected to the proteins exceeds the amount found in the raw material by a factor greater than 1·1–1·2, especially if the seleno-amino acids are present in the different proteins, by replacing sulphur-containing amino acids, and follow the same way of reactions as those when decomposing etc. On the other hand this fraction may also be enriched in selenium by other water-soluble organic selenium compounds, either with a high molecular weight or by addition to high molecular

TABLE II

Arsenic content in samples fractionated by molecular gel filtration

Sample	N liquor			Enzyme-hydrolysed presscake		
	Wt. of sample to be fractionated, g	Wt. of fraction, g	As μg	Wt. of sample to be fractionated, g	Wt. of fraction, g	As μg
Cod fillet	1·0	0·067 0·024 0·024	1·1 5·4 0·28	0·6	0·16 0·12	0·42 0·80
Cod bone	0·2	0·010	0·42	0·7	0·12 0·055 0·060	0·15 0·81 0·42
Cod liver	0·8	0·073 0·026	1·2 2·3	0·4	0·056 0·054 0·078	1·1 0·14 0·50
Cod skin	2·5	0·049 0·036	1·1 2·1	1·1	0·18 0·26 0·62	0·63 0·10 0·68
Herring fillet	1·0	0·021	1·2	0·8	0·083	0·51
Herring bone	0·6	0·003	0·01	0·5	0·14	1·0
Herring skin	1·0	0·016 0·060	1·4 0·30	0·3	0·058	0·74
Mackerel fillet (mature)	1·0	0·047	2·3	0·6	0·17 0·079	0·06 0·52
Mackerel fillet (immature)	1·0	0·017 0·016 0·034	1·9 0·9 0·43	0·9	0·070 0·074	0·46 1·1
Norway haddock fillet	1·0	0·048	1·7	0·8	0·077 0·082	0·41 0·41
Lobster fillet	1·0	0·016	1·12	0·5	0·017	0·93
Clam	0·9	0·057 0·22	2·7 2·2	0·6	0·17	0·90
Squid	1·0	0·071 0·40 0 065	4·9 6·6 0·29	0·6	0·10 0·098 0·17	0·26 0·63 1·1
Whale meat	1·0	0·09 0·03	0·16 0·10	0·6	0·14 0·20	0·05 0·05

TABLE III

Selenium content in the protein fraction from molecular gel filtration

Sample	N liquor		Enzyme-hydrolysed presscake	
	Wt. of fraction, g	Se, μg	Wt. of fraction, g	Se, μg
Cod fillet	0·079	0·62	—	—
Cod bone	0·009	0·11	0·069	0·25
Cod skin	0·079	0·40	0·032	0·32
Cod liver	0·074	0·31	0·054	0·42
Herring fillet	0·006	0·05	0·036	0·40
Herring bone	0·0022	0·01	0·019	0·12
Herring skin	0·030	0·33	0·059	0·20
Mackerel fillet (mature)	0·010	0·13	0·019	0·08
Mackerel fillet (immature)	0·028	0·20	0·045	0·18
Norway haddock fillet	0·032	0·18	0·017	0·10
Lobster fillet	0·032	0·20	0·017	0·13
Clam	0·15	1·6	0·022	0·04
Oyster	0·13	0·47	0·037	0·09
Squid	0·098	1·2	0·008	0·04
Whale meat	0·07	0·10	0·037	0·38

weight compounds. It should also be noted that the difference in chemical behaviour of sulphur and selenium may have some effect on the distribution of selenium in the different sulphur-containing compounds and also the way in which compounds decompose during the production of the N liquor.

The results presented in this investigation should be of interest in connexion with the use of fish as a feed ingredient for domestic animals. Probably all the arsenic and at least part of the selenium are present as organic compounds. Arsenic especially, but also to some degree selenium, are, as such, less toxic than if they had been present in an inorganic state. In addition the arsenic may have the same beneficial effect on the growth and health of the organism as has been reported for organic compounds of arsenic. The fish raw material should also provide an adequate source of selenium.

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ANALYSIS OF TRACE ELEMENTS IN SEAWEED

By G. LUNDE

The following trace elements have been analysed in various algae samples: arsenic, copper, molybdenum, manganese, zinc, cobalt, antimony, selenium and iron. The ash content has also been determined. The samples were collected from two different localities in Norway, one (Trondheimsfjord) characterised by a relatively strong influence of river water, industrial waste etc.; the other (Reine in Lofoten) is free from this type of contamination. In the case of two species, *Ascophyllum nodosum* and *Laminaria hyperborea*, the variation in the content of trace elements has been studied during a one-year period, samples being taken every second month. Considerable differences in the content of trace elements was found between the Laminariaceae and Fucaceae.

Introduction

Most of the seaweed meal produced industrially today is utilised as feed additive for domestic animals. Many feeding experiments have been undertaken where the nutritional value of the seaweed has been studied.[1 3] However, the results obtained from these have not been conclusive. Variations in processing and storage of the meal have probably been important factors influencing its nutritional value. Although there have been contradictory results from these experiments it is generally agreed that the seaweed meal is of considerable importance as a source of minerals and some vitamins in compound feed.

In addition, there may also be trace elements appearing in chemical combinations that improve the nutritional effects of these elements. This may be the explanation in experiments where the addition of seaweed meal had a favourable effect compared with other sources of minerals.[3]

Relatively little work has been published to date on the analysis of the trace element content of seaweed. Winogradov[4] has summed up the main results obtained up to the beginning of the 1950s. In addition, Young & Langille[5] and Black & Mitchell[6] among others, have contributed with studies reporting both analyses of a number of trace elements and their physiological significance.

Recently more attention has also been focused on the uptake of inorganic ions in algae. This includes both trace elements[7,8] and elements appearing in larger amounts, for example the alkaline earths.[9] Considerable enrichment of many of these elements is known to occur in the plants, often by a factor of 1000 or more. Several relevant aspects present themselves in determining how this takes place. First, a passive reversible enrichment, in which the ions are absorbed to definite locations in the plant can be considered, often with a high degree of selectivity for certain ions.[9] In addition, an active transport of ions through the cell membranes, i.e. an irreversible enrichment, is also occuring.[7]

To date, spectrographic methods have been chiefly employed in the analysis of typical trace elements in seaweed, but other procedures have also been used.[5] Among the elements affording greatest problems are those forming volatile compounds at relatively low temperature, such as arsenic, selenium, mercury, antimony. Among other analytical methods activation analysis has proved to be an adequate method for analysing many of these elements both in the micro-and submicro-region (1–0·001 μg).

The purpose of this work was firstly to extend the knowledge on some of the trace elements present in seaweed by analysing samples from new localities, and secondly to see if the trace element content varied when samples of the same algae were taken from different localities where a difference in the trace element content should be expected. Also, seasonal variations in the level of trace elements were worthy of attention and samples of two species collected from the same locality were analysed every second month for a year.

Experimental

Collection and processing of samples

Samples of seaweed were collected from Reine in Lofoten and from Trondheimsfjord, Norway during the period 1951–1968. An abundance of fresh and uncontaminated seawater characterises Reine in Lofoten, while Trondheimsfjord is exposed periodically to trace element contamination from rivers, sewage and industrial waste.

Each sample contained at least 50 specimens. Free water on the seaweed was removed by shaking, and the samples were dried at 40–80°c. They were then homogenised in a porcelain ball mill after smaller samples had been quartered out. Some of the samples from Reine in Lofoten were ground on a Wiley laboratory mill. Special care was taken so that these samples were completely dry before grinding. Care was also exercised throughout this treatment to avoid contamination from the metallic parts during the processing.

Analytical methods

Analyses were carried out by means of neutron activation and atomic absorption. Arsenic, copper, molybdenum, zinc, cobalt, antimony and selenium were determined by neutron activation according to the methods described by Samsahl and others.[10-12] Samples of approximately 0·2 g dried material were activated in quartz ampoules for 20 h at a neutron flux of $1 \cdot 1 \times 10^{12}$ cm^{-2} sec^{-1}, together with standards of the elements to be analysed. The samples were dissolved in sulphuric acid and hydrogen peroxide. Thereafter the arsenic, antimony and selenium were distilled by means of hydrobromic acid and cooled in a sulphuric acid adapter. If the samples contain mercury this element will also appear in this fraction. Both the distilled elements and those remaining in solution were processed further and obtained in suitable fractions by an ion-exchange technique. These fractions contained radioactive isotopes of the elements to be analysed and could be recorded on a gamma spectrometer without interference from other radioactive nuclides. Standards of the elements to be analysed were recorded in the same way.

Copper and zinc were also analysed by atomic absorption measurement, together with manganese and iron. Samples of about 1 g placed on a water bath at 90–100° were dissolved in sulphuric acid and hydrogen peroxide. After the

solutions cleared, they were diluted to 25 ml before analysis. A Perkin-Elmer atomic absorption instrument, Model 303, was used for this purpose.

Ashing was performed in a laboratory furnace at 450°. The samples were heated slowly up to this temperature and kept there for about 20 h.

Results and Discussion

Marine plants undoubtedly have a more constant supply of minerals than terrestial ones, due both to better conditions for absorption of the elements (ions) and to the fact that the sea is a more reliable source of inorganics than the soil. The concentration of trace elements in the same species of algae would therefore be expected to keep within relatively close limits, and analytical results outside these limits are probably due to some kind of contamination.

The results of the analyses are presented in Tables I–IV. They confirm, in general, previous results, even though results for some of the elements vary. This is the case with arsenic, copper, molybdenum, zinc, cobalt and iron. No previous quantitative data are available for the selenium and antimony contents in algae.

The agreement between results for copper and zinc, analysed both by atomic absorption and by neutron activation was good, being within 20% for most of the samples. For these two elements the average value obtained by the two methods is used.

The iron, copper and zinc contents in the samples from Reine in Lofoten, ground in a Wiley mill, appear in the same range as the other, proving that the grinding process does not lead to a measurable increase of metal content in the samples. The high values found for some trace elements in a few samples is probably due to some kind of contamination. A sample from Flakk in Trondheimsfjord, *Fucus serratus* (Table IV) had high manganese and zinc contents, but normal iron and copper contents. This distribution probably reflects high manganese and zinc contents in seawater. Contamination from tools should also cause a high iron content. Some samples also contained a considerable amount of iron, without other trace elements, such as zinc, manganese and copper, being equally large, e.g., *Fucus spiralis* (Table III, 3.6.51) from Reine with 931 ppm iron, and *Laminaria hyperborea* (Table IV, 18.2.57) from Munkholmen with 856 ppm. These results indicate that the seaweed had been contaminated either in the sea or during the collecting and processing.

The selenium content in the samples analysed varied between ~0·04 and ~0·2 ppm. In general, selenium follows sulphur in biological processes and can replace sulphur in sulphur-containing amino acids and other sulphur compounds. Comparing the values of selenium found in algae with the corresponding figures for marine fish species, which are about 2·5 ppm selenium,[13] a significant difference between marine plants and animals in the ability to enrich selenium is indicated. This observation is also confirmed when the sulphur/selenium ratio in seaweed is compared with that of fish. The sulphur content in seaweed is about 3%[14] and in fish about 0·7%.[15] Based on these data the figures for the sulphur/selenium ratio are about 3×10^6 for algae and about 3×10^4 for fish. The corresponding ratio in milk products from dairy cows is about 8×10^4.[16] In marine algae the sulphur is mainly present as sulphated polysaccharides whereas the sulphur in fish (and in milk products) is located in the proteins. It should therefore be concluded that the accumulation of selenium in relation to sulphur is considerably higher when the elements are located in proteins than when they are linked to polysaccharides in the compared samples.

Comparison of trace element content in samples from the same algae species taken from Reine and Trondheimsfjord showed relatively little difference. The results indicate that the trace element content is more dependent on ability to accumulate these elements than to variation in trace element concentrations in the water at the two localities.

The analyses of *Laminaria hyperborea* and *Ascophyllum nodosum*, presented in Tables I and II, especially demonstrate how the various species enrich different trace elements. *Laminaria hyperborea* has the largest arsenic content while *Ascophyllum nodosum* exhibits a higher content of the other elements.

Two samples of red algae (*Gigartina mamillosa* and *Rhodymenia palmata*) (Table IV) were not particularly different from the brown algae with regard to the trace elements in question.

TABLE I
Trace element content (ppm) in *Laminaria hyperborea* lamina (Reine in Lofoten)

Date of collection	Ash, % of dry matter	As	Cu	Mo	Mn	Zn	Co	Sb	Se	Fe
28.2.57	32·7	70	9	0·3	5	98	0·18	0·10	0·04	177
30.4.57	31·8	68	8	0·3	6	96	0·16	0·15	0·04	130
30.6.57	24·4	66	6	0·6	5	60	0·10	0·07	0·04	71
27.8.57	20·0	53	6	0·4	4	53	0·14	0·07	0·05	118
27.10.57	19·3	53	22	0·7	4	73	0·12	0·05	0·07	49
30.12.57	25·6	71	8	0·6	4	88	0·12	0·10	0·05	81

TABLE II
Trace element content (ppm) in *Ascophyllum nodosum* whole plant (Reine in Lofoten)

Date of collection	Ash, % of dry matter	As	Cu	Mo	Mn	Zn	Co	Sb	Se	Fe
28.1.57	23·8	44	22	2·0	13	114	0·5	0·19	0·09	106
29.3.57	20·6	23	29	1·5	14	240	0·4	0·20	0·09	176
30.5.57	21·2	22	18	2·1	11	104	0·4	0·53	0·07	114
30.7.57	18·9	23	35	1·6	12	99	0·7	0·45	0·07	145
30.9.57	22·2	30	27	1·1	10	74	0·4	0·21	0·06	101
30.11.57	21·6	38	19	1·4	15	103	0·5	0·22	0·07	116

TABLE III
Trace element content (ppm) in seaweed (Reine in Lofoten)

Sample*	Date of collection	Ash, % of dry matter	As	Cu	Mo	Mn	Zn	Co	Sb	Se	Fe
Pelvetia canaliculata, Reine	1.3.51	23·6	22	8	0·96	7	137	0·6	0·38	0·12	115
Pelvetia canaliculata, Reine	3.6.51	17·6	21	11	0·45	8	118	0·4	0·52	0·14	129
Fucus serratus, Reine	1.3.51	27·6	47	8	2·2	5	137	0·8	0·23	0·17	65
Fucus serratus, Reine	1.6.51	23·8	40	20	2·3	41	113	0·8	0·31	0·24	238
Fucus spiralis, Reine	5.3.51	25·3	34	12	5·4	28	218	0·2	0·29	0·18	182
Fucus spiralis, Reine	3.6.51	21·6	26	39	5·8	24	135	0·6	0·27	0·11	931
Fucus vesiculosus, Øvre Reine	1.3.51	23·2	65	45	4·6	11	188	0·6	0·37	0·17	33
Fucus vesiculosus, Øvre Reine	1.6.51	20·6	26	12	2·6	5	152	0·6	2·5	0·17	126
Laminaria digitata lamina, Reine	25.4.52	36·2	73	14	0·6	6	138	0·10	0·10	0·17	59

* The samples consist of whole plants unless otherwise indicated

TABLE IV
Trace element content (ppm) in seaweed (Trondheimsfjord)

Sample* and locality	Date of collection	Ash, % of dry matter	As	Cu	Mo	Mn	Zn	Co	Sb	Se	Fe
Laminaria digitata lamina, Munkholmen	23.10.56	27·6	109	20	2·2	9	170	0·4	0·31	0·09	384
Laminaria digitata lamina, Flakk	12.4.57	38·0	107	34	1·1	5	100	0·1	0·05	0·09	166
Laminaria hyperborea lamina, Munkholmen	18.2.57	37·0	69	22	2·7	10	167	0·7	0·30	0·16	865
Laminaria hyperborea lamina, Munkholmen	9.1.62	36·9	55	12	2·6	6	276	0·3	0·34	0·14	240
Laminaria hyperborea stipes, Munkholmen	26.6.57	36·8	94	16	7·8	21	122	0·2	0·29	0·17	186
Ascophyllum nodosum, Flakk	30.9.68	19·9	22	63	1·6	17	273	2·0	0·22	0·04	198
Gigartina mamillosa, Flakk	25.4.52	30·5	10	15	1·1	10	183	0·6	0·06	0·06	86
Rhodymenia palmata, Flakk	28.3.52	31·5	13	24	0·6	11	143	0·5	0·05	0·17	153
Fucus vesiculosus, Flakk	28.3.52	20·0	39	18	1·7	54	195	1·6	0·14	0·16	145
Fucus vesiculosus, Flakk	30.9.68	17·9	24	35	1·0	70	297	2·2	0·19	0·05	61
Fucus serratus, Flakk	30.9.68	21·9	28	27	1·6	164	520	5·2	0·26	0·06	166
Fucus spiralis, Flakk	30.9.68	24·8	15	43	1·3	54	390	2·8	0·20	0·05	420
Pelvetia canaliculata, Flakk	30.9.68	23·7	15	41	1·0	10	112	0·7	0·12	0·06	226

* The samples consist of whole plants unless otherwise indicated

Little data exist on seasonal variation of trace elements in seaweed. Among other factors this variation is dependent on the form in which the trace element is present in the algae, i.e. as part of organic molecules or as inorganic ions, reversibly or irreversibly bound. Both destruction of organic material in the algae and seasonal variations in the concentration of trace elements in the water are important factors in this connexion. The results obtained in the present study demonstrate that such a variation does exist in such material. Arsenic, antimony and zinc contents in *Laminaria hyperborea* are higher in February–April than in September–November. This may have some connexion with the reduction of the ash content, but cannot account for it all.

When comparing the trace element content in seaweed and in plant material of terrestial origin,[17,18] the results indicate that the marine algae generally contain larger concentrations of the trace elements analysed in this study, e.g. zinc, copper and especially arsenic. This element is enriched in marine algae, particularly in the *Laminaria* species, by a factor 200–500 compared with arsenic in terrestial plant material. More attention should be focused on how this element occurs and whether or not it has any physiological role in marine algae.

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· Technical

SYMPOSIUM: METAL-CATALYZED LIPID OXIDATION

presented at the ISF-AOCS World Congress, Chicago, Illinois

REINHARD MARCUSE, Program Chairman

Activation Analysis of Trace Elements in Lipids With Emphasis on Marine Oils[1]

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ABSTRACT

A short introduction to the activation analyses is given where some of the main features of the method, especially when applied on biological material, are described. The following trace elements are analyzed in marine and vegetable oils: arsenic, bromine, sodium, copper, manganese, zinc, nickel and iron. Both arsenic and bromine are present as organic compounds. The arsenic is removed in the alkali refining process. The distribution of trace elements in oils has been studied by use of autoradiography and γ-spectroscopy. The results indicate that this distribution is dependent on the phospholipid content in the oil. A high phospholipid content seems to give a more homogeneous distribution of inorganic trace elements.

[1]One of 28 papers presented at the Symposium, "Metal-Catalyzed Lipid Oxidation," ISF-AOCS World Congress, Chicago, September 1970.

INTRODUCTION

The neutron activation analysis combines both high sensitivity and good accuracy for many elements. Today, when high sensitivity is required, the activation is mostly performed by thermal neutrons from a nuclear reactor. The activity is here induced by a (n, γ) reaction. Neutrons are captured by stable isotopes and these are then converted to new isotopes of the same element. At the same time a γ-quant is simultaneously emitted. Some of these isotopes may be unstable, i.e. radioactive, and will disintegrate by emitting characteristic γ and β radiation. The induced activity is measured either by recording the β particles with a Geiger Muller counter or the characteristic γ photons with a multichannel γ-spectrometer. The latter is by far the most used.

The sensitivity of the method will depend on several factors. Among these are the neutron flux (n/cm^2 sec), the irradiation time and the activation cross section, the probability for the isotope to capture a neutron (σ_{act}). In practical analytical work, that is to say, among the elements reported analyzed by this method, the sensitivity has been

INDEX

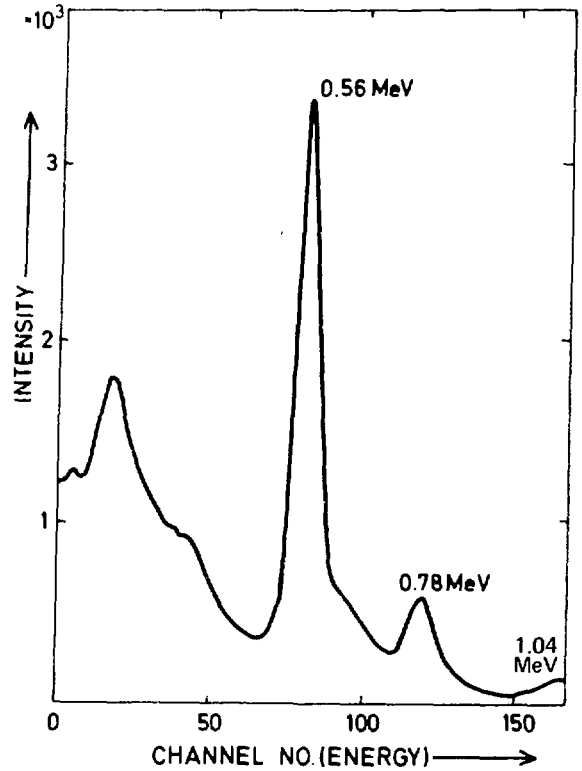


FIG. 1. Gamma-spectrum of neutron activated capelin oil recorded about one day after activation. The main peaks belong to As^{76} with 0.56 MeV and Br^{82} with 0.56 MeV, 0.78 MeV and 1.04 MeV.

from about 10^{-11} to about 10^{-4} g. In addition, the sensitivity will also depend on how the samples are prepared and how they are recorded. Many excellent review articles and books have been published both dealing with the theory for this analytical method and with practical applications (1-3).

In connection with the analysis of trace elements in oils, the following elements are well suited to be analyzed by this method: manganese, iodine, chlorine and nickel, with half lives from 25 min to 2 1/2 hr, copper, sodium, bromine and arsenic, with half lives from 12 to 36 hr, and zinc, cobalt, iron, chromium, with half lives from about 30 days to about 5 years. In addition phosphorus should also be mentioned. The radioactive phosphorus isotope P^{32} has a half life of about 14 days and emits only β-particles.

Some important features of this analytical method relevant to the analysis of oils should be pointed out in more detail.

1. The registration of the activity from an activated element is mostly performed by recording high energy γ-photons. This reduces matrix effects. As a result, it is not necessary to prepare the standards in the same way as the sample to avoid an uneven absorption of γ-photons in the standard and in the sample. Activities in biological materials may, for instance, be compared with standards dissolved in water.

2. Especially when trace elements in the sub-microgram area (1 ppb to 0.1 ppm) are analyzed, the risk of contamination during handling and analyzing the samples is considerable. For the activation analysis this is only a problem before the activation. After activation, the addition of impurities, even foreign sources of the elements to be analyzed, will be of no consequence for the results.

3. In many cases the matrix activity and also the activity induced in trace elements is negligible or does not interfere with the registration of the induced activity in the trace element to be analyzed. In these cases it is therefore possible to perform a nondestructive analysis of one or more elements. Examples of such matrixes are organic and biological materials. This type of nondestructive analysis is an interesting way to study the level of impurities in samples taken for instance from different steps during the processing of the oil, and also to study the relation between two or more impurities and how the different steps in the process may change this relation. This can be done by comparing γ-spectra of activated samples taken from the different steps.

If the γ-radiation (the γ-photons) from the induced activity in the element to be analyzed, is covered or interfered by γ-photons from other activities, it is necessary to carry out a radiochemical separation to purify the element enough to perform a registration with the γ-spectrometer. This is done usually by adding a carrier (milligram amount of the element to be analyzed). When the carrier has exchanged with the activated isotope, ordinary chemical separation methods are used to isolate this. Often only one or a few separation steps are necessary to get the sample pure enough to secure an adequate registration.

4. A distribution pattern of trace elements in flat sections (films, polished or flat surfaces etc.) can sometimes be obtained by using autoradiographic methods. A uniform exposure of the film reveals a homogeneous distribution whereas areas with a higher exposure indicate an inhomogeneous distribution of inorganic impurities or of particles. Due to high specific activity in these areas the corresponding exposed areas on the film may be rather large and no conclusion can be drawn as to the real size of the area. Also a variation in the background exposure may be observed when the oil is not evenly distributed. A γ-spectrum of the oil will indicate the main radioactive isotope present. This technique is especially useful for solids but also for frozen liquids or liquids with high viscosity.

TABLE I

Nondestructive Activation Analyses of Trace Elements in Unprocessed and Processed Marine Oils

Sample	As (ppm)	Br (ppm)	Na (ppm)
Crude oil	8-14	7-10	~0.1
After alkaline treatment and washing	<0.2	7-10	~0.3
After bleaching	<0.2	6-10	~0.2
After hydrogenation and deodorization	<0.2	4-7	~0.1

TABLE II

Destructive Activatonalyses of Trace Elements in Hydrogenated Marine Oils

Sample		Cu (ppm)	Ni (ppm)	Fe (ppm)
No. 1	10 samples	0.004-0.04	0.03-0.14	0.5-12
No. 2	6 samples	0.006-0.012	0.10-0.72	5-12

GENERAL INFORMATION ON ACTIVATION ANALYSIS OF OILS

The main elementary constituents of oils (carbon, hydrogen, oxygen, and in raw oils also phosphorus) will not give any detectable γ-radiation (γ-peaks) when irradiated with thermal neutrons. The β-particles from phosphorus, P^{32}, will be recorded as bremsstrahlung on the γ-spectrometer. It is therefore possible to perform a nondestructive analysis of some of the trace elements present in the oils. Other trace elements, where the induced activities are low or where the γ-spectrum is complicated by γ-photons from

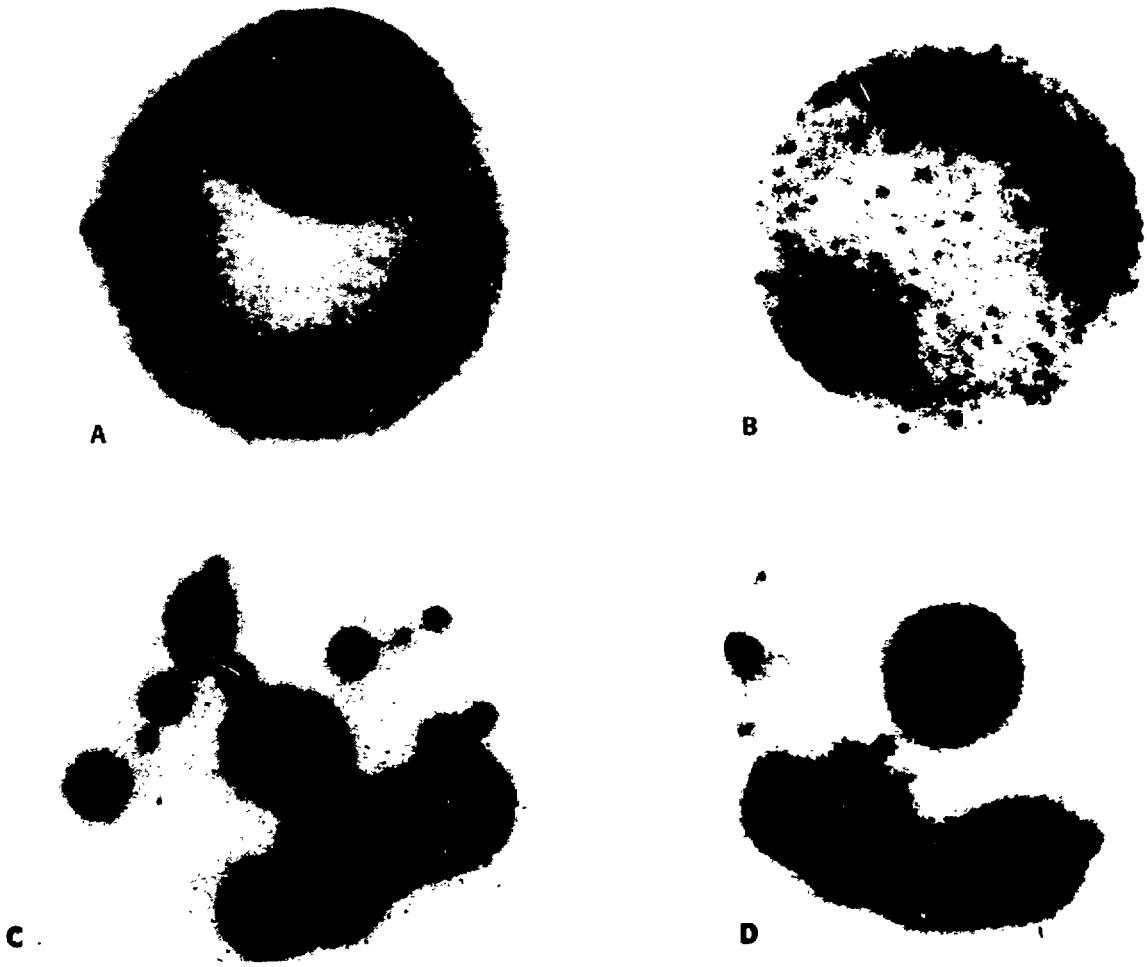


FIG. 2. Autoradiographs: A. Neutron activated refined fish oil; exposure time about 2 hr. B. Inactive fish oil where radioactive arsenic has been introduced from a water phase. C. Neutron activated refined soybean oil. The phospholipid content is low (25-50 ppm). D. Neutron activated refined peanut oil with a low phospholipid content (~25 ppm).

other activities, have to be separated by chemical methods before registration. This can be done either by wet or dry destruction of the oil or by different types of extraction methods. The extraction can be carried out by dissolving the oil after activation in a suitable solvent and extraction with water at an adjusted pH. The elements are then transferred into the water phase and subsequently separated by chemical methods. Saponification is an alternative method for bringing some of the trace elements into the water phase without destruction of the organic matter.

EXPERIMENTAL PROCEDURES

Materials

The oils analyzed in this investigation were either commercially available or produced in our laboratory.

Irradiation

About 1 ml of each oil was sealed off in quartz ampoules and was then ready for neutron activation. P.A. Chemicals (Merck, Darmstadt) of the elements to be analyzed (NaCl, NH_4Br, NaH_2PO_4, As_2O_3, $ZnCl_2$, $FeCl_3$, $MnCl_2$, $NicCl_2$ and $CuCl_2$) were sealed in the same kind of ampoules and irradiated together with the oil samples. Especially at irradiation times up to 24 hr, it is often advisable to dissolve the standard before sealing in the ampoules. The irradiation was performed with a neutron flux of $4 \cdot 10^{12}$ n/cm^2 sec in the nuclear reactor JEEP 2 Kjeller, Norway.

Separation and Registration

After activation the following procedures were used for preparing the different trace elements in the oils for registration. Both principles and detailed separation procedures for the elements to be analyzed have been discussed elsewhere (4) and only some of the main steps of the analysis are outlined here:

Saponification. During conventional saponification copper and nickel ammonium complexes were added as carrier. The carrier will exchange with the activated copper and nickel in the oil samples. After the saponification the nickel is precipitated with dimethylglyoxime and copper as copper sulfide.

Wet Destruction. The oils were treated with concentrated warm sulfuric acid, nitric acid and hydrogen peroxide. Carriers of the elements to be analyzed were added and conventional separation methods were applied after the destruction of the organic matter.

Extraction. The oils are dissolved in hexane and mixed with hydrochloric acid at a pH of about 2. Carriers of the elements to be analyzed are added to the water phase. The activated trace elements are extracted into the water phase and will exchange with the carriers; the different elements

TABLE III

Nondestructive Activation Analyses of Trace Elements in Vegetable Oils

Sample	(ppm) Mn	Zn	Cu	Na	Br
Crude oils	a	<0.05-1.1	<0.005-1.0	0.03-5	<0.01-4
Refined oils	<0.005-0.1	,	<0.005-0.3	<0.01-2	<0.01-4

[a]not determined.

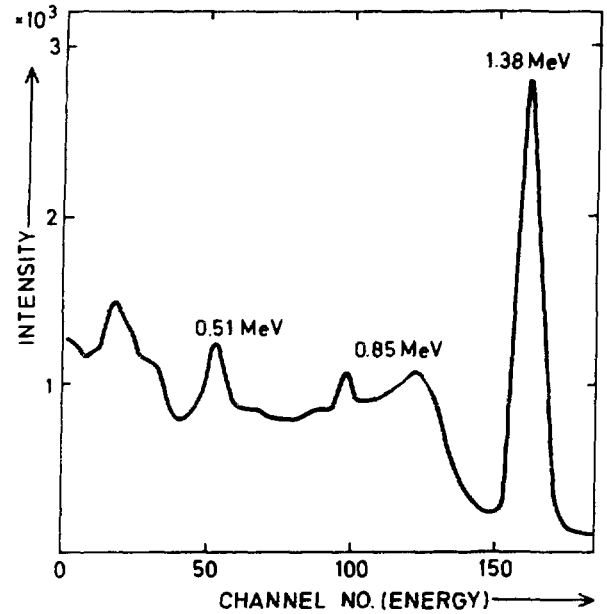


FIG. 3. Gamma spectrum of neutron activated linseed oil. The main peaks belong to Cu^{64} with 0.51 MeV, Mn^{56} with 0.85 MeV and Na^{24} with 1.38 MeV.

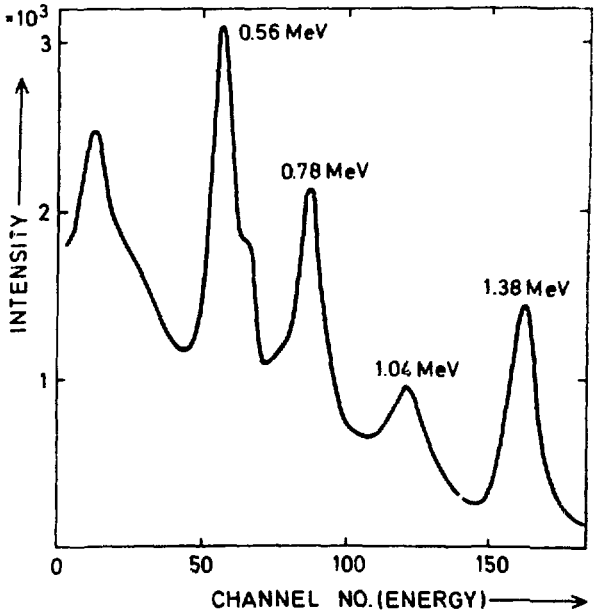
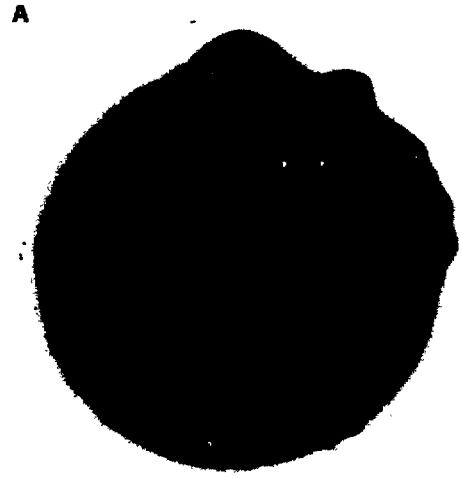


FIG. 4. Gamma spectrum of neutron activated coconut fat. The main photopeaks belong to Br^{82} with 0.56 MeV, 0.78 MeV, 1.04 MeV and Na^{24} with 1.38 MeV.

are then separated to the degree of purity that enable a recording with a γ-spectrometer.

Direct Registration. The oils are poured into inactive glass vials and are recorded directly with a γ-spectrometer.

Distribution Studies. The neutron activated oil is poured onto a thin plastic film and placed in contact with a photographic emulsion. An ordinary x-ray film can be used. After exposure the film is developed and handled as an ordinary x-ray film. The exposure is about 14 hr on a fine grain x-ray emulsion or 2 hr with a medium grained film. Gamma spectra of the oils are recorded after the exposure. The procedure should be repeated after a few days to see how the shortlived isotopes influence the distribution pattern on the film and correlate this with the γ-spectrum.



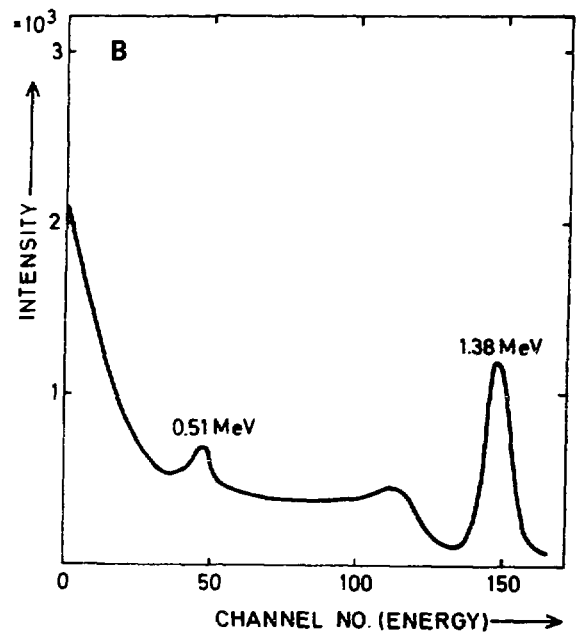


FIG. 5. Neutron activated linseed oil. A, Autoradiograph, exposure time about 2 hr. The background exposure is from P^{32} (phospholipid content ~2500 ppm). B, Gamma-spectrum recorded immediately after the exposure. Copper, Cu^{64} with 0.51 MeV and sodium Na^{24} with 1.38 MeV are the main γ-emitting isotopes. The bremsstrahlung from P^{32} is also recognized.

RESULTS AND DISCUSSION

Marine Oils

Crude oils manufactured from marine fishes (mackerel, herring, capelin and others) by conventional methods are relatively low in inorganics. Nondestructive analysis indicates that these oils contain usually less than 0.2 ppm sodium and less than 0.05 ppm copper. This is probably due to the way these oils are produced. The oil is in contact with relatively large amounts of water at 100 C followed by centrifugation. This insures an effective washing procedure and an efficient removal of the water from the oil.

Marine oils however contain some trace elements present as organic compounds (5,6). Among these arsenic and bromine have been studied in more detail. Figure 1 shows the γ-spectrum of capelin oil recorded about one day after activation and without any chemical treatment after the activation. The γ-spectrum consists mainly of the radioactive arsenic isotope As^{76} and the radioactive bromine isotope Br^{82}. When the oil is treated with alkali in the refining process the arsenic will follow the alkaline phase together with most of the phospholipids left in the oil. The bromine content will be reduced during the hydrogenation to about half of the original value. See Table I.

Due to the high content of bromine and arsenic a nondestructive analysis cannot be performed when the amount of inorganics in marine oils are low. In this case other methods have to be used to carry out an analysis. The contents of copper, nickel and iron have been analyzed in hydrogenated marine oils partly by the saponification and partly by the destruction procedure. Some of the results are shown in Table II. The two series of oil samples, No. 1 and No. 2 are from different productions.

The distribution of trace elements in a refined fish oil with a low phosphorus content is shown in Figure 2A. This oil however has a high background exposure and contains some inhomogeneities. The corresponding γ-spectrum indicates that sodium-24, arsenic-76 and bromine-82 are the main activities. As the sodium-24 with a half life of about 15 hr disappears, the inhomogeneities disappear. This indicates that the main radioactive isotopes left, bromine-82 and arsenic-76 with half-lives of 36 hr and 24 hr respectively, are homogeneously distributed and are therefore probably present as organic compounds soluble in the oil. These results were also confirmed when some experiments were carried out where radioactive arsenic was introduced into inactive oil from a water phase. Figure 2B shows an autoradiograph of radioactive arsenic introduced into a fish oil.

Vegetable Oils

There is no evidence so far that organic bound trace elements are present in vegetable oils. Nondestructive analysis of inorganics present in the oils with a high sensitivity for activation analysis is therefore possible. In vegetable oils one or more of the following trace elements can usually be analyzed without any chemical treatment: copper, manganese, zinc, sodium, chlorine, bromine and iron. Other trace elements have to be analyzed by radiochemical separation methods based on chemical treatment of the oils after the acvitation. Table III shows some results of nondestructive analysis of vegetable oils. Among the oils snalyzed are coconut fat, soybean oil, olive oil, peanut oil and linseed oil. Figure 3 and 4 show the γ-spectra of linseed oil and coconut fat. Copper, manganese and sodium are observed in the linseed oil whereas coconut fat contains mostly bromine and sodium. The content of inorganic ions in the different types of oils depends greatly on the possibility of contamination during the refining and hydrogenation steps and also on how the processed oils are stored afterwards. A simple washing procedure of the finished product may often reduce the level of impurities (parts per million) by a factor of 5-10.

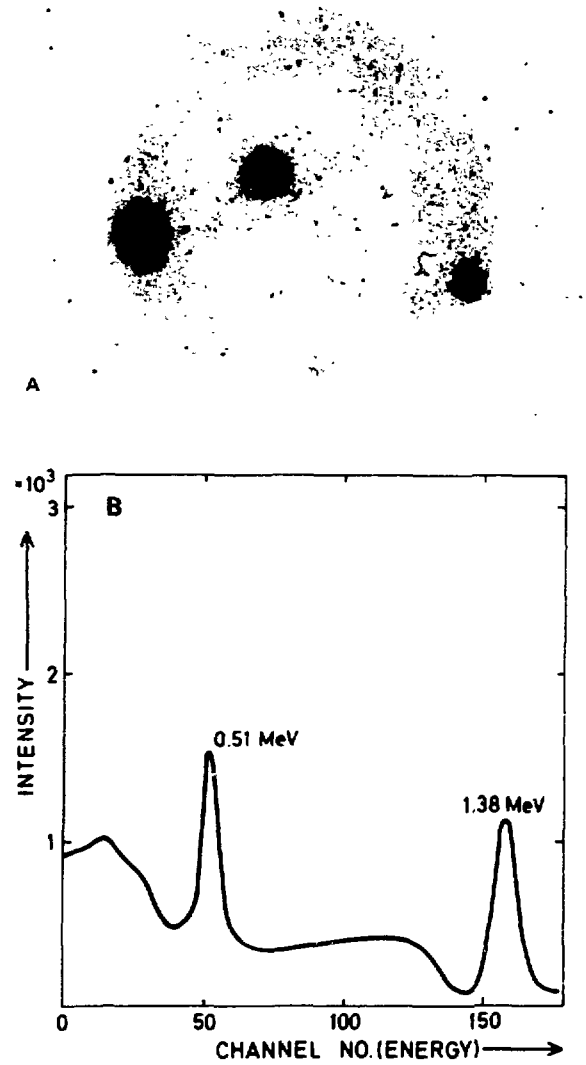


FIG. 6. Neutron activated olive oil. A, autoradiograph, exposure time about 2 hr (phospholipid content ~25 ppm). B, Gamma-spectrum recorded after the exposure. Cu^{64} and Na^{24} are the main radioactive isotopes present in the oil.

Figures 5 and 6 show two autoradiographs of activated linseed and olive oil together with corresponding γ-spectra. The linseed oil contained about 0.25% phospholipids and the olive oil about 0.003%. When the γ-spectra of the oils are compared with the corresponding autoradiographs it is evident that the inhomogeneous areas on the exposed film consist mainly of radioactive copper, sodium and bromine. The inhomogeneities on the autoradiographs disappear as the radioactive isotopes of copper, sodium and bromine disintegrate.

Although too few experiments have been performed, the results seem to indicate that oils relatively high in phospholipids have a more homogeneous distribution of trace elements than oils low in phospholipids. Figure 2C and 2D show two more autoradiographs of vegetable oils with a low phospholipid content. Oils with about the same amount of trace elements and with a high phospholipid content show an approximately homogeneous distribution of trace elements. These results are also confirmed by autoradiographing inactive oils with high and low phospholipid

content where radioactive tracers (Br^{82}, Cu^{64}, and Na^{24}) have been introduced from a water phase.

Besides the content of phospholipids, small amounts of water present in the oils may also be of importance in this connection, providing small areas with a higher water concentration where the trace elements probably can be concentrated.

When studying the effect of trace elements on the autoxidation of oils more emphasis should be concentrated on the distribution of these trace elements, and on how this distribution is influenced by the content of phospholipids and probably also of the content of small amounts of water in the oils.

ACKNOWLEDGMENT

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VIII

Analysis of Arsenic and Bromine in Marine and Terrestrial Oils

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ABSTRACT

Samples of marine and terrestrial oils of both plant and animal origin have been analyzed for arsenic and bromine content. Two oil samples (cod liver oil and oil extracted from mackerel fillets) were fractionated on silica gel columns and bromine was determined in the different fractions. The results obtained indicate that lipid soluble bromine and arseno organic compounds are characteristic components of marine animal and marine plant oils (seaweed). The results also show that the bromine is not localized in any particular compound or type of compounds. The bromine-containing compounds seem to be relatively stable, but the arseno-containing compounds are not. When oils containing arsenic and bromine were saponified, some of the arsenic and bromine compounds were found in the fatty acid fraction while others appeared in the water soluble fraction.

INTRODUCTION

It has been shown in previous work (1-3) that lipids extracted from various marine organisms contain arsenic and bromine as organic compounds. In analyses made on lipids from whale and fish, the contents of these elements were found to vary between 3-25 ppm for arsenic and 1-50 ppm for bromine. It has also been shown that arsenic is present as two or more lipid soluble arseno organic compounds in herring (4). Bromine and arsenic are likewise found in the lipid phase of fresh water fish (3,5). Bromine is also present in lipid extracts from fresh and salt water algae, 20-150 ppm and 10-15000 ppm respectively (3). Arsenic was not determined in these samples.

The results obtained so far indicate that the presence of arseno and bromine organic compounds is characteristic of oils produced from marine and fresh water fish. The purpose of this work was to analyze oils from other species of both terrestrial and marine origin in order to see if this assumption has a more general validity. In addition to analyzing, these oils–some in which both arsenic and bromine were present–were saponified so that the fatty acids could be isolated and analyzed separately.

It was also of interest to ascertain whether bromine was present as one or more specific bromine organic compound. Such a study was undertaken by fractionating some of the oil samples and analyzing the different fractions. By calculating the bromine as parts per million (μg Br/g oil), it should be possible to obtain an indication as to whether this element was present as one or a few discrete compounds, i.e., present in one or a few fractions, or whether it is more distributed in several or all of the different compounds in the oil.

EXPERIMENTAL PROCEDURE

Methods

The production and saponification of the oils were carried out using conventional methods. In some samples where little material was available and where the yield of oil was low, the saponification was carried out directly on the raw material.

Analyses for bromine and arsenic were made using neutron activation. Such analyses may be made without chemical treatment of the sample (oil and fatty acid) after the activation, providing that after activation they do not contain trace elements that will interfere with the detection of the induced radioactivity of the arsenic and bromine. Among elements which do interfer, sodium should be emphasized. The content of sodium and other inorganic ions may, however, be reduced by washing with distilled water. When little material is available, a solution of the material in a suitable solvent may be washed. Some solvents appear to have an affinity to inorganic ions, and may carry the ions into the oil phase where they are left behind when the solvent is evaporated. Chloroform seems to be such a solvent, and should be avoided (unpublished results obtained in the author's laboratory). Among the solvents which may be used are diethylether and toluene. By this method it is also possible to analyze small amounts of material (milligram amounts) without taking any special precautions. Transfer of the samples before and after the neutron activation should be accomplished with the aid of a suitable solvent. Further details concerning activation analyses of oils are discussed elsewhere (3,4,6).

The fractionation of the two oil samples was made on silica gel columns according to the directions given by Barron and Hanahan (7).

Materials

The raw materials used for the production of the oil samples were obtained from the local fish and meat market and also through the kind assistance of other laboratories.

TABLE I

Eluting Agent and Characterization of the Main Fractions From Chromatography of Fish Lipids on Silicic Acid

Fraction	Eluting agent	Amount, ml	Characterization of main components
A	Hexane (H)	150	Hydrocarbons
B	15% Benzene in H	400	Sterol esters
C	5% Diethylether in H	1330	Triglycerides and free fatty acids
D	15% Diethylether in H	950	Free sterol
F	30% Diethylether in H	600	Diglycerides
E	50% Diethylether in H	600	Diglycerides–monoglycerides
G	Diethylether (D)	600	Monoglycerides
H	3% Methanol in D	250	Phospholipids ?
I	5% Methanol in D	150	Phospholipids
J	80% Methanol in D	250	Phospholipids
K	Methanol	100	Phospholipids

TABLE II

Bromine and Arsenic in Oil and Fatty Acids Extracted From Marine Fishes and Invertebrates

Sample			Locality	Br, ppm		As, ppm	
				Oil	Fatty acid	Oil	Fatty acid
Capelin	*Mallotus villosus*	whole fish	Northern Norway	9.2	8.1	12.1	6.3
Herring	*Clupea harengus*	whole fish mature	Western Norway	5.8	2.2	13.8	9.2
Herring	*Clupea harengus*	whole fish	Skagerak	2.6	2.7	19.3	12.1
Mackerele	*Scomber scomber*	fillet	Southern Norway	2.8	3.1	8.2	4.1
Mackerel	*Scomber scomber*	liver	Southern Norway	16.5	8.5	13	6.2
Cod[a]	*Gadus morrhua*	liver	Western Norway	34	12	8.4	6.1
Cod[a]	*Gadus morrhua*	liver	Western Norway	36	9.2	9.9	7.2
Cod	*Gadus morrhua*	liver	Western Norway	26	6.8	10	6.0
Plaice	*Pleuronectes platessa*	fillet	Southern Norway	17.3	7.6	6.1	5.2
Clam	*Pecten maximum*	whole animal	Northern Norway	15	7.8	4.8	1.9
Squid	*Ommatostrephes sagittatus*	whole animal	Northern Norway	---	3.9	---	0.7
Starfish	*Asterias rubens*	whole animal	Oslo Fiord Norway	14.6	6.4	9.1	7.5
Shrimp	*Pandalus borealis*	whole animal	Oslo Fiord Norway	17	13.0	10.1	4.8
Lobster	*Homarus vulgaris*	whole animal	Southern Norway	50	≈3	4.7	≈3
Mussel	*Mytilus edulis*	whole animal	Western Norway	137	35	18	22
Snail	*Littorina littorea*	whole animal	Western Norway	294	82	84	32

[a]From the same locality.

Some of the vegetable raw oils were factory produced.

Treatment of the Samples

All samples were homogenized in a mechanical blender, and extracted with two volumes of chloroform-methanol 2:1. The extractions were carried out for 2-4 hr at 50 C on a waterbath, under reflux. The extract was then filtered, and the extraction was continued with a similar amount of solvent for another 2-4 hr. The two extracts were combined, the chloroform phase was removed in a separatory funnel, and finally removed under reduced pressure at 35-40 C. If a sufficient amount of oil was available, the oil phase was washed twice with distilled water. Usually it was necessary to add magnesium sulphate as a deemulsifying agent. The oil was then centrifuged. Where the oil yield was small, the oil was dissolved in toluene or diethylether and washed with water. After two washing processes the organic phase was separated by centrifuging and the solvent evaporated.

The saponification of the oils was performed in an alcoholic potassium hydroxide solution (2 N KOH in C_2H_5OH). The solution was subsequently made acidic with sulphuric acid and extracted with diethylether. The ether solution was then washed with water and the ether evaporated. Some seaweed samples with a low oil content were saponified directly. The fatty acids were separated as described for the other samples.

Fractionation of Oil

Oil produced from mackerel fillets and from cod liver were used for the fractionation experiments. The silica gel (Merck AG, Darmstadt, 0.2-0.5 mesh) used as solid phase was prepared in the following way: The gel was first dried at 110 C and then treated with the following solvents: diethylether, 15% benzene in *n*-hexane, and *n*-hexane. About 90 g of gel was added to a column with diameter 2.5 cm. Approximately 20 mg oil per gram of gel was used. The different eluting agents used and the amounts of each are given in Table I. The absorption of the eluate at 254 mμ was obtained with an Uvicord-Unit (LKB, Sweden). From the elution curves obtained, and information given by Barron and Hanahan (7), the eluate was devided into different fractions and the solvents were removed by evaporation.

Neutron Activation

The oil and fatty acid samples were neutron activated in quartz ampoules. The quartz ampoules were first washed with warm nitric acid and rinsed with distilled water. Standards (PA Chemicals: Merck AG, Darmstadt) of ammonium bromide and arsenic dissolved in hydrochloric acid (0.1 N) were neutron activated under the same conditions as the samples. The activation took place in the nuclear reactor JEEP 2, Kjeller, Norway, with a neutron flux of approximately 5×10^{12} n/cm^2 sec for 2 hr. The quartz ampoules were cooled to -196 C before opening in order to reduce the pressure from volatile compounds formed during the irradiation. The samples were then transferred to inactive glass vials. The standards were treated in the same way.

TABLE III

Bromine and Arsenic in Oil and Fatty Acids Extracted From Seaweed

Sample[a]	Br, ppm		As, ppm	
	Oil	Fatty acid	Oil	Fatty acid
Laminaria digitata	566	640	221	36
Laminaria saccharina	496	477	155	7.5
Laminaria hyperborea	368	385	197	16
Ascophyllum nodosum (1968)	61	27	7.8	5.2
Ascophyllum nodosum (1969)	56	25	49	21
Fucus vesiculosus	43	44	35	5.1
Fucus Serratus	40	39	27	6.1
Fucus spiralis	12	19	5.7	5.0
Pelvetia canaliculata	34	41	10.8	7.3

[a]The samples were collected at the west coast of Norway.

TABLE IV

Bromine in Oil Extracted From Birds, Terrestrial Mammals and Vegetables

Sample		Locality	Bromine, ppm
Hare	*Lepus timidus*	Inland Norway	<0.1
Reindeer	*Rangifer torandus*		0.12
Elk	*Alces alces*		<0.01
Cow	*Bos taurus*		<0.05
Wood-grouse	*Tetrao urogallus*		0.01
White-grouse	*Lagopus lagopus*		0.03
Lesser black-backed gull	*Larus fuscus*	West coast Norway	1.0
Common gull	*Larus canus*		0.6
Herring gull	*Larus argentatus*[a]		11.0
Herring gull	*Larus argentatus*[a]		13.2
Soybean oil			0.06
Olive oil[b]			0.03
Peanut oil			0.09
Linseed oil[b]			0.12
Coco fat			0.18

[a]Traces of arsenic present.
[b]Commercially produced raw oil.

Registration of the Samples

After neutron activation, measurement of the induced activity in the samples was made with the aid of a 2 x 2 in. sodium iodide detector coupled to a multichannel γ-spectrometer. The samples were not treated chemically before these measurements. Some of the samples contained traces of sodium and the registration of their activity was therefore postponed for 4-5 days to ensure that the activity from sodium -24 would not disturb the registration of the induced bromine and arsenic activity. The sensitivity for the analysis of bromine and arsenic when using this method is approximately 0.005-0.01 μg and 0.002-0.005 respectively. The sensitivity is reduced when the registration after activation is postponed, or if activities from other radioactive isotopes increase the "background" activity.

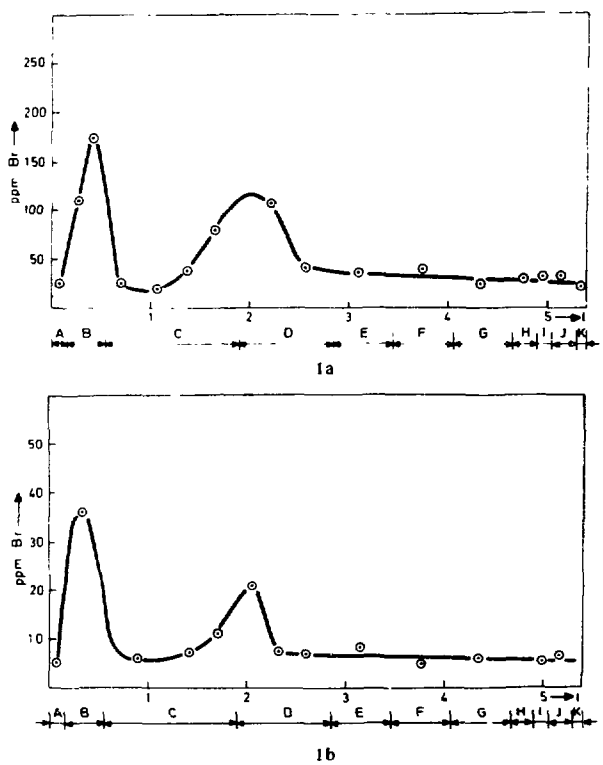

FIG. 1. The Bromine content in different fractions of cod liver oil (a) and oil extracted from mackerel fillet (b) fractionated in a silica gel column (see Table I for characterization of fractions).

RESULTS AND DISCUSSION

The results of the bromine and the arsenic analysis in the different oils and in the corresponding fatty acids are shown in the Tables II-IV. They confirm the earlier findings that lipid soluble arseno and bromine organic compounds are characteristic of oils of marine origin. This conclusion applies to lipids extracted from fish, different invertebrates, seaweed and algae. The amount of bromine in vegetable oils and in some oils extracted from terrestrial animals is significantly lower than in marine oils, and it is probably present as inorganic bromine which has not been removed from the oils by the washing process.

The only oil from organisms of terrestrial origin in which bromine was detected came from seabirds, and the bromine-organic components were here partly present in somewhat reduced amounts. Fish constitutes the main part of the diet of these birds and is most likely the source of the bromine-containing compounds found in them. The analytical results indicate both a gradual reduction in the content of bromine organic compounds through the feed chain from algae to fish and to seabirds, and that at least some of these components are quite stable. No arsenic was found in any of the terrestrial oils, except for some traces in the oil from seabirds.

When the arsenic content in marine oils is compared with the arsenic content in the corresponding fatty acids, the results show that at least two groups of arseno organic compounds are present in the marine oils. One type consists of an arsenic-containing acid which follows the fatty acids during the saponification process while the other type is converted to a water soluble compound during this process.

It is interesting to note that the lipid phase in seaweed also contains arseno organic compounds as do the lipids from the marine animals studied. The amount following the fatty acids in a saponification is, however, relatively lower than for the other marine oils studied. These compounds are probably formed from inorganic arsenic absorbed from the sea water, synthesizing first a water soluble arseno organic compound. Compared with the total amount of arsenic present in seaweed (30-110 ppm) (8,9), the amount of arsenic found in the oil represents a relatively small amount of the total arsenic present in the seaweed. The yield of extractable lipids here is usually from 3-6%. However it should be noted that the contents of arsenic particularly in oil extracted from some *laminaria* species exceeds that in any other marine raw material analyzed.

The analysis of bromine in the marine oils and in the

corresponding fatty acids shows that this element also is localized in the fatty acid fraction, but that the content is somewhat lower here than in the oils. This would indicate that the bromine organic compounds are not formed solely by an addition of bromine to double bonds in the fatty acids.

The results of the bromine analysis in the fractions from the two oils fractionated on a silica gel column (see Figs. 1a and b) give nearly the same picture of the bromine distribution in the different fractions, although the absolute amount of bromine is considerably higher in the cod liver oil than in the mackerel oil. Both diagrams show that the highest bromine concentration is located in fractions B and C-D. Fraction B (15% benzene in hexane) contains mainly sterolesters while the fractions C-D (5-15% diethylether in hexane) contains triglycerides, free fatty acids and sterols. It is also possible that the bromine may be present in other compounds which are eluted under these conditions, but which are present in smaller amounts and not found in this study. Although the results indicate that the bromine is randomly added to the main components of the oil, there may be one or more specific bromine organic compound located in fractions B and C-D.

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The Analysis of Arsenic in the Lipid Phase from Marine and Limnetic Algae

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The arsenic content in the lipid phase extracted from selected marine and limnetic algae has been analysed by use of neutron activation technique. The arsenic content varied from about 0.5 ppm to 5 ppm. The algae were cultivated in the laboratory using enriched cultures. The following types of algae were investigated: *Chlorella ovalis* Butcher, *Chlorella pyrenoidosa* Chick, *Oscillatoria rubescens* (D.C.), *Phaevdactylum tricornutum* Bohlin, *Sceletonema costatum* (Grev.) Cleve.

It has been shown earlier that the arsenic present in different marine organisms as well as in seaweed is in the form of lipid soluble and water soluble arseno organic compounds.[1–4] These compounds can probably be formed in two ways; it is possible that the various marine animals and plants themselves can synthesise compounds using inorganic arsenic which they absorb from their surroundings, or it may be that there is a well defined group or groups of micro organisms which is responsible for the synthesis of these compounds. In the latter case the arseno organic compounds could be transferred *via* the food chain to the more advanced organisms. A combination of the two alternatives may also be a possibility.

As far as we have been able to find out no quantitative data concerning the arsenic content of algae exist. Gautier [5] and others have demonstrated that arsenic is present in marine algae, but in all cases it proved to be difficult to obtain exact data due to small quantities available of algae material for the analysis. Neither is there any information available relating to the form in which the arsenic is present in these algae, although many have considered the hypothesis that the arsenic is present in the form of one or more arseno organic compounds. To clarify this situation it was of interest both to obtain quantitative data relating to the arsenic content in algae, and to obtain more pointers as to the form in which the arsenic is present.

The oil phases in the algae were selected for analysis since the risk of contamination by inorganic arsenic is far smaller for the oil phases than for the water phases. If the oil phase were contaminated with inorganic arsenic

or other inorganic ions, then it is a relatively simple process to remove this contamination. This can for example be achieved by washing the oil with distilled water, or if the amount of oil is small by dissolving the oil in an organic solvent as hexane and then washing this solution in distilled water.

The cultures used in this investigation were *Chlorella ovalis* Butcher, *Chlorella pyrenoidosa* Chick., *Oscillatoria rubescens* (D.C.), *Phaeodactylum tricornutum* Bohlin, *Sceletonema costatum* (Grev.) Cleve, and have been described previously.[6,7] The algae were cultivated in fresh or salt water media enriched with plant nutrients.[8] Arsenic was not added to the media. The only source of arsenic was therefore the arsenic present either in the water samples or as impurities in the components of the enriched solution. The concentration of arsenic in the culture media was of the order of 1 – 3 ppb.

EXPERIMENTAL

The algae were cultivated in a specially built "climate room" designed for the purpose. The details of this room and the conditions observed for the cultivation are described elsewhere.[6] Spherical flasks (2 l) were used to hold the cultures. The flasks stood on a "vibrating" table which was illuminated by fluorescent lamps (Philips TL 40 W/32). These gave an illumination of 6000 lux at the table surface. Every day, once the growth was well under way, one half of the algae solution was filtered off through a glass sinter filter. After the filtration the filter papers with the algae were stored in chloroform. Culture solution was then added to the flasks so that the volume of the solution where the growth occurred remained constant.

It took about 14 days of cultivation to produce enough algae material to allow a sufficiently sensitive analysis of the lipid phase obtained from the algae. The lipids were extracted from the algae specimens by adding methanol and water to the chloroform solution containing the filter with the filtered off algae. The resulting mixture was shaken for about 2 h, and then more water was added so that the chloroform phase became separated from the methanol – water phase. The chloroform phase was then separated, and evaporated. The lipids thus extracted were dissolved in hexane and the solution was then washed twice in distilled water.

The analysis of the oils was carried out using neutron activation. This method is very sensitive and well suited for the analysis of arsenic in oils as long as these do not contain other components which disturb the registration of the induced As-76 activity. The method has been described earlier[9] and only a few of the main points relating to the technique will be mentioned here. The oil specimens were irradiated in quartz ampoules. The oil was transferred to the ampoules by dissolving it in diethyl ether, and then evaporating the ether. The ampoules were then sealed and irradiated together with arsenic standard (As_2O_3 dissolved in 0.01 N HCl) for 2 h in a neutron flux of about 10^{13} n cm^{-2} sec^{-1}. After irradiation, the specimens were transferred to inactive glass vials. Also this transfer was performed by use of a suitable solvent.

The arsenic activity in the irradiated oil specimens was registered using a multichannel γ-spectrometer. The arsenic content in the specimens was estimated by comparing their spectra with the corresponding spectrum taken from the irradiated arsenic standard. For some of the specimens where a small amount of sodium was present it was necessary to wait 3 – 4 days before their γ-spectra could be registered. By this time most of the radioactive sodium isotope Na-24 had disintegrated. It should be mentioned that the bromine content of the oils extracted was small, so that the measurement of the As-76 activity was not disturbed by any activity from the radioactive bromine isotope Br-82 which is created when bromine is irradiated with thermal neutrons.

RESULTS AND COMMENTS

As can be seen from Table 1, the presence of arsenic has been demonstrated in all of the oil specimens extracted from algae. Since the algae were cultivated

Table 1. Yield of and arsenic content in oil extracted from algae cultivated in fresh and salt water.

	Alga	Water	Yield of oil (g)	As ppm
1.	*Sceletonema costatum*	salt water	0.050	1.3
2.	*Chlorella ovalis*	» »	0.032	0.7
3.	*Chlorella pyrenoidosa*	fresh water	0.019	0.5
4.	*Phaeodactylum tricornutum*	salt water	0.062	3.6
5.	*Phaeodactylum tricornutum*	fresh water	0.024	4.8
6.	*Oscillatoria rubescens* (Züricher See)	» »	0.030	0.5
7.	*Oscillatoria rubescens* (Steinsfjorden)	» »	0.016	0.4

under very favourable conditions in the laboratory with, among other things, a sufficient supply of culture solution, the values found for the arsenic contents must be regarded as qualitative and as giving an impression of the algal ability to synthesise arseno organic compounds under such conditions.

Since the arsenic content in the culture solutions probably varied, and was not exactly analysed each time, it is difficult to estimate any enrichment coefficient for arsenic in the algal oils. If the value of 1 – 3 ppb As is accepted (for the culture solutions) then the enrichment coefficient will lie in the region from 200 to 5000.

On the basis of the results obtained, it can be concluded that the arsenic present in lipids extracted from algae is organically bound in the same way as it is in oils extracted from more advanced marine organisms. It is therefore possible that the arsenic which is found in the algae is transferred *via* the food chain to other organisms, and that the algae form an important source for the arsenic which is present in the higher organisms.

If one considers the situation with regard to fish and other aquatic organisms, it seems reasonable to believe that the algae can also synthesise water soluble arseno organic compounds. This possibility is now being more closely investigated in our laboratory, by cultivation experiments where radioactive inorganic arsenic is added to the culture solution.

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X

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Location of Lipid-soluble Selenium in Marine Fish to the Lipoproteins

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It has been established that a lipid-soluble selenium compound is enriched in high molecular weight (m.w. ~5000) extracts from fish. The selenium compound is also enriched in oil extracted from fish by a mixture of non-polar and polar organic solvents (hexane–isopropanol) as compared to the oil extracted with non-polar solvents (hexane) only. When raw fish material is stored it gradually deteriorates and the selenium content in oil produced by boiling of the raw fish material, increases with time. The results indicate that at least part of the lipid-soluble selenium is bound as a lipoprotein.

1. Introduction

It has been shown that marine oils contain 0.1 to 2.0 parts/million of selenium[1] and that the selenium is present as a lipid-soluble organic selenium compound. Some of the oils analysed were produced industrially and some were isolated under controlled conditions in the laboratory either by treatment with hot water, or by extraction with chloroform–methanol mixtures. It was also shown that the lipid-soluble organic selenium compound has a polar character. Chromatographed on silicic acid, most of the lipid-soluble selenium compound was eluted in front of the bulk phospholipids by mixtures of chloroform and methanol.

When solutions produced from marine raw materials either by boiling or by enzyme hydrolysis are subjected to gel filtration, the selenium content is enriched in the high molecular weight fraction as compared with the original material.[2] The conclusion drawn is that the selenium very probably exists as compounds other than seleno amino acids (present in the protein phase analogous to the sulphur amino acids).

The purpose of this work was to study in more detail whether the lipid-soluble selenium compound shown to be present in marine oils is bound to the lipid phase in the high molecular weight fraction, i.e. whether it is part of a lipoprotein complex. Assuming that the lipid-soluble selenium compound is bound to proteins, it is also of interest to determine whether it may be enriched by means of selective extractions. The neutral oil may be extracted by a non-polar solvent, such as hexane. The lipids which

are bound to the proteins may then be extracted by a polar solvent, such as an alcohol, in addition to the hexane.

If the organic selenium compound is present in a lipoprotein complex, it is possible that the selenium content will be dependent upon the way in which the oil is isolated. When the oil is extracted from the raw material by chloroform–methanol, the lipid-soluble selenium compound will most likely be quantitatively extracted together with the neutral oil. On the other hand, if the oil is isolated by treatment of the raw material with water at different temperatures, the selenium organic compound may well behave like the phospholipids. Consequently, it will depend upon the conditions under which the isolation occurred and also upon the quality of the raw material used for extraction.

2. Experimental

2.1. Preparation of samples

Raw materials with different fat contents were selected for the analysis. Here cod fillet, cod liver and whole herring were used. Some samples of industrially produced glue water from herring and mackerel were also included.

The glue water is prepared by boiling homogenised raw material in glass apparatus for 20 min. Distilled water is added as the boiling proceeds. Oil produced in this process was centrifuged off and the glue water (the water phase) was then filtered and the filter washed once with distilled water. The glue water from cod fillet contained no visible traces of oil. The samples of glue water were concentrated to 4 to 5% dry matter, and stored at −20 C for subsequent molecular gel fractionation.

The fraction which was insoluble in water after the preparation of the glue water, i.e. the fish meal, was hydrolysed by incubation with a protease (Bioprase; Nagese and Co Ltd, Japan). This procedure has been described earlier.[2] The water-soluble part was filtered off and treated in the same manner as the glue water. Samples of homogenised fresh raw material from herring were similarly hydrolysed using the same enzyme. Because of the natural enzyme activity in this material, incubation time was held to 3 h: i.e. after considerably less time than for the material prepared by boiling with water.

The enzyme-hydrolysed samples and the glue water were subjected to molecular gel filtration on a dextran resin (Sephadex G25 medium, Pharmacia Fine Chemicals, Sweden). To ensure sufficient amounts of material in the fractions which were to be further analysed, 300 ml of solution holding 15 g of dry material was used in each fractionation experiment. In all the elutions a column of the type K 100/100 (Pharmacia Fine Chemicals Sweden) was used. As elution agent a weak aqueous ammonia solution with a pH value of about 9 was employed.

The absorption of the eluate at 254 nm was registered using a Uvicord spectrometer (LKB, Sweden). The various fractions were divided into a high molecular weight fraction, the protein fraction—(fraction 1), and intermediate molecular fraction, the peptide fraction—(fraction 2), and a low molecular fraction which mainly consisted of amino acids and salts—(fraction 3), see Figure 1.

The fractions were evaporated to a suitable volume and the lipids were extracted with chloroform–methanol (2:1) for about 2 h at room temperature under constant stirring. More water was added and the chloroform phase was separated off. For some of the fractions, the oil yield was small and it was difficult to continue the analysis without taking extra precautions. In such cases 1 ml of olive oil was added as a carrier. A previous analysis had shown that the olive oil contained no selenium. The mixture of olive oil and oil extracted from the gel-filtration fractions was then treated in the same way as the lipids extracted from the other fractions.

The extraction using hexane and thereafter hexane–isopropanol was applied to both raw material and to material where a part of the oil had been isolated by boiling with water. The conditions for these experiments are given in Table 2. Cod liver and herring were used in these experiments. For the extraction process, the ratio between the extracting medium and the raw material was 2 to 1. This mixture was shaken for about

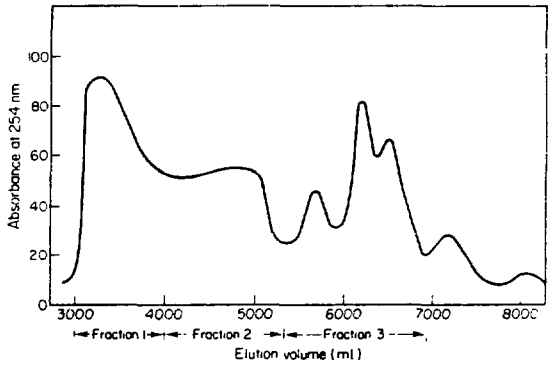

Figure 1. The u.v. absorbancy at 254 nm of the eluate from gel filtrated (Sephadex G25 medium) enzyme-hydrolysed cod liver residue. The protein, the peptide and the amino-acid fractions are indicated.

2 h and the hexane phase was separated off and the hexane evaporated. Prior to the determination of selenium the oils were washed twice with distilled water.

Preliminary experiments were performed on raw material which had been stored from 2 to 10 days at 4 to 8 °C (see Table 3). From these samples the oil was isolated by boiling with water.

All oil samples produced were analysed by neutron activation. The lipid fractions were transferred to quartz ampoules, sealed and irradiated for 24 h together with selenium standards in a neutron flux of approximately 1×10^{13} n/cm^2 s. After a "cooling off" period of about two weeks, the irradiated samples were transferred to inactive glass vials and their activity determined on a multichannel γ-spectrometer with a 2×2 in NaI crystal. A detailed description of the procedure has been given elsewhere.[3] For samples with a low phosphorus content it is usually possible to carry out the registration of selenium after a two week period without any prior chemical treatment. Figure 2 shows the γ-spectra of neutron-irradiated oil extracted from the high molecular weight fraction from enzyme-hydrolysed cod liver residue; Figure 3 shows the selenium standard. The spectra were recorded about 2 weeks after irradiation.

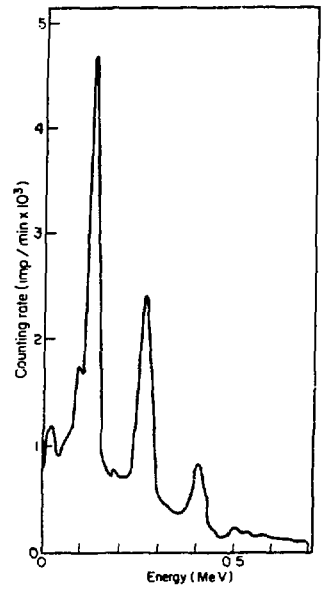

Figure 2. Gammaspectrum of neutron-activated oil extracted from the protein fraction of enzyme-hydrolysed cod liver residue.

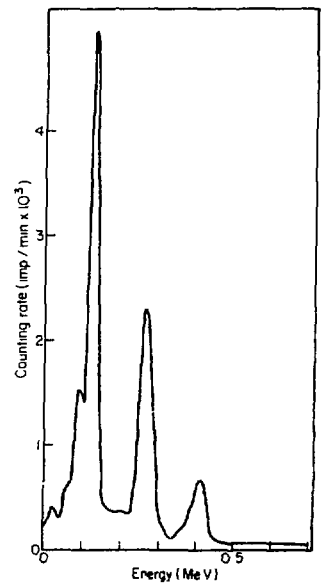

Figure 3. Gammaspectrum of the neutron-activated selenium standard.

TABLE 1. Yield and selenium content of oils extracted from fractions produced by molecular gel filtration of glue water and enzyme-hydrolysed presscake and fresh fish

Sample	Treatment	Fraction 1		Fraction 2		Fraction 3	
		Yield of oil (%)	Se	Yield of oil (%)	Se	Yield of oil (%)	Se
Mackerel	Factory prod. glue water (high quality)	0.8	15 parts/million	0.50	0.1 parts/million	0.3	3.0 parts/million
Herring	Factory prod. glue water (low quality)	5.4	0.41 parts/million	0.71	0.6 parts/million	0.2	0.1 parts/million
Cod liver	Laboratory prod. glue water	2.9	1.0 parts/million	0.48	3.0 parts/million	0.4	1.4 parts/million
Cod liver	Enzyme-treated presscake	0.2	33 parts/million	0.2	2.8 parts/million	1.1	1.2 parts/million
Cod fillet	Laboratory prod. glue water	—	0.025 μg	—	0.012 μg	—	0.04 μg
Cod fillet	Enzyme-treated presscake	—	0.040 μg	—	0.013 μg	—	0.03 μg
Herring	Laboratory prod. glue water	—	0.07 μg	—	0.008 μg	—	0.03 μg
Herring	Enzyme-treated presscake	—	0.11 μg	—	0.030 μg	—	0.02 μg
Herring	Enzyme-treated fresh fish	—	0.06 μg	—	0.07 μg	—	0.04 μg

TABLE 2 The selenium content (parts/million) in oils produced by successive water treatment, hexane and hexane–isopropanol extractions of cod liver and herring

Sample	Conditions	Water treatment		Hexane extraction		Hexane/isopropanol extraction	
		Yield of oil (g)	Se (parts/million)	Yield of oil (g)	Se (parts/million)	Yield of oil (g)	Se (parts/million)
Cod liver[a]	−20 °C	69	0.15	9	0.22	1.0	0.75
Cod liver	60 min 100 °C	74	0.22	7	0.20	1.4	0.65
Cod liver	4 h 100 °C	77	0.24	5	0.24	1.4	0.50
Herring[b]	+20 °C	—	—	15	0.065	40	0.16
Herring	4 h 60 °C	27	0.05	25	0.05	6	0.43
Herring	20 min 100 °C	1.0	0.06	45	0.09	13	0.21
Herring	1 h 100 °C	1.3	0.13	44	0.10	13	0.21
Herring	4 h 100 °C	17	0.075	27	0.09	14	0.064

[a] The cod liver contained ~40% oil.
[b] The herring contained ~5% oil.

3. Results and comments

Results presented in Table 1 indicate that lipid-soluble selenium is enriched in the high molecular weight fraction (fraction 1 in Figure 1), (m.w. 5000 or more) and hence is part of a lipoprotein complex. In particular this is evident in the samples of mackerel and cod liver glue water, of hydrolysed cod liver residue and in some of the samples of herring. Unfortunately, the amount of lipids in some of the samples prepared in the laboratory was too low to allow the yield to be determined. The selenium contents of the other samples do not give such definite results, although selenium is present in the oil isolated from the high molecular weight fraction in all samples.

The results of Tables 2 and 3 show that a certain amount of the selenium compound is present also in oil samples extracted with hexane or by treatment with water at 60 °C. This indicates that at least some of the selenium compound is either bound relatively weakly or exists free in the lipid phase. There is also the possibility that different selenium compounds are present and that more than one type of association, possibly to different proteins, may exist.

TABLE 3. The selenium content (parts/million) in marine oils from raw materials of different age

Sample	Storage time (days)	Se (parts/million)	Storage time (days)	Se (parts/million)	Storage time (days)	Se (parts/million)
Capelin	2	0.05	5	0.08	7	0.11
Herring	3	0.11	6	0.17	8	0.21
Mackerel	2	0.09	5	0.22	7	0.28

The results presented in Table 2 indicate that as the storage time increases and the raw fish samples gradually deteriorate, more of the selenium will follow the oil when this is isolated by boiling with water. The phospholipids behave in the same way. The content of phospholipids was measured by registration of the phosphorus isotope, ^{32}P, produced by the neutron activation.

The extraction experiments show that selenium is enriched in oil extracted by a mixture of hexane and isopropanol. The latter mixture is capable of splitting off the lipids in the lipoproteins. This effect may be observed particularly for samples where the majority of the neutral lipids were first removed by water treatment at temperatures of less than 100 °C. The results indicate that a certain amount of the selenium compound is bound in the lipid phase and that it is liberated in a way similar to the phospholipids.

For herring in particular, there seems to be an increase of the selenium content in the oil with boiling time up to about 1 h, then a decrease is observed (4 h). It may be that the heat treatment results in the transformation of the selenium compound into new substances having different solubility characteristics, or that the selenium compound itself could react with other components.

The evidence is that the lipid-soluble selenium compound occurs in the high molecular fractions produced from the different solutions as part of a lipoprotein complex. This indicates that the compound can probably be localised to the cell membrane and should be of interest in connection with the theory which proposes that selenium has a function related to the protection of the cell membrane.[4]

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The Presence of Volatile, Nonpolar Bromo Organic Compounds Synthesized by Marine Organisms

XI

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ABSTRACT

The presence of volatile, nonpolar bromine-containing compounds in marine organisms is demonstrated. These compounds represent, especially in tissue containing a high fat content, ca. 0.1-1.0% of the total amount of bromo organic compounds present in marine oils. In tissue with a low fat content, a higher concentration of bromo organic compounds is found. It is concluded that these compounds are probably synthesized in one or more stages in the marine food chain. These compounds may follow and disturb the analyses when isolating and determining chlorinated hydrocarbons originating from industrial and other sources of pollution.

INTRODUCTION

It has been shown that marine oils contain lipid soluble bromo organic compounds (1-3). The content of organic bound bromine varies between 3 and 50 ppm. When samples of marine oils are fractionated on a silica gel column using mixtures of chloroform and methanol as eluting agents, bromine is found in all the groups of different components fractionated. It does not seem that the bromine is localized to any particular group of compounds. It is concluded that marine organisms are able to synthesize lipid soluble bromo organic compounds (3).

In connection with contaminants that are released from industrial and other sources of pollution, extensive studies involving the analysis and characterization, especially of compounds consisting of chlorinated aliphatic and aromatic hydrocarbons, have been initiated. These compounds are in the main lipid soluble and when present in the marine environment they tend to be stored and also enriched in the lipid phase in marine organisms. Although the effect of many of these compounds is not known, one must assume that they will be harmful when present even in small quantities. This applies particularly to those organs such as fish liver that, in addition to being a storage place for fat, also have a high enzyme activity.

Most of these compounds may be enriched by steam distillation using a nonpolar solvent such as cyclohexane to collect the distilled compounds.

The presence of halogenated compounds has been demonstrated, mainly by gas chromatography (GC), where an EC-detector (electron capture) has been used. By connecting the GC with a mass spectrometer (MS) it is possible to identify the various components. In order to carry out such an identification ca. 10 ng or more of each compound is required. Using a gas chromatograph with an EC-detector only, it is not possible to distinguish between chlorinated, brominated, iodinated or other EC-sensitive organic compounds. Among the latter some esters, ketones, nitro compounds and thioles should be mentioned. An identification of the different compounds depends here on available standards.

Relating to the pollution aspect, it should be of interest to determine whether there exist volatile compounds among the lipid soluble bromo organic compounds, which marine organisms are able to synthesize themselves. They may then be detected under the same conditions applying to the detection of the chlorinated hydrocarbons from pollution sources.

Such a hypothesis could be confirmed if one were able to demonstrate the presence of bromo organic compounds among the volatile organic compounds that can be steam distilled from marine organisms. Following such a distillation the absolute quantity of bromine present can be determined by neutron activation of the cyclohexane phase.

TABLE I

The Bromine Content (ppb in the Lipid Phase) in the Volatile, Nonpolar Fraction of Marine Organisms

Organism	Sample		Locality	Yield of oil, %	Br μg/kg oil, ppb 1 Dist.	2 Dist.
Cod liver oil	*Gadus morhua*	Oil	Northern Norway, 1924	100	4	3
Cod liver oil	*Gadus morhua*	Oil	Northern Norway, 1940	100	5	2
Cod liver oil	*Gadus morhua*	Oil	Lofoten Norway, 1960	100	4	2
Cod liver oil	*Gadus morhua*	Oil	Lofoten Norway, 1969	100	5	2
Cod	*Gadus morhua*	Filet	Western Norway, 1971	0.4	63	68
Cod	*Gadus morhua*	Filet	Western Norway, 1972	0.4	50	50
Cod	*Gadus morhua*	Liver	Western Norway, 1972	55	3	1
Cod	*Gadus morhua*	Filet	Lofoten Norway, 1972	0.4	130	44
Cod	*Gadus morhua*	Liver	Lofoten Norway, 1972	61	2	1
Mackerel	*Scomber scomber*	Filet	Southern Norway, 1969	20	8	4
Halibut	*Hippoglossus hippoglossus*	Filet	Helgeland Norway, 1971	9.3	6	4
Halibut	*Hippoglossus hippoglossus*	Filet	East of Greenland, 1971	10.5	10	8
Capelin	*Mallotus villosus*	Whole fish	Northern Norway, 1969	9.2	25	7
Capelin	*Mallotus villosus*	Whole fish	Northern Norway, 1972	9.3	5	<0.2
Herring	*Clupea harengus*	Whole fish	Langesund Fiord, 1971	8.8	7	2
Shrimp	*Pandalus borealis*	Whole fish	Oslo Fiord, 1969	0.4	75	82
Mussel	*Mytilus edulis*	Whole animal	Trondheim Fiord, 1971	16[a]	0.9	<0.3
Seaweed	*Laminaria hyperborea*	Whole plant	Western Norway, 1971	3.2	557	358
Seaweed	*Ascophyllum nodosum*	Whole plant	Western Norway, 1971	3.1	592	227

[a]Dried material.

This analytical method is significant and has a high sensitivity for bromine and the other halogens. When the amount of each halogenated compound is too low to make an identification with a coupled GC-MS the method for determining if the compounds giving response on the EC-detector also contain halogens will be of special interest.

Some preliminary GC and GC-MS analyses were carried out on cyclohexanedistillate from seaweed *(Laminaria hyperborea)*, shrimp and cod liver oil (1940). More than 50 compounds were detected with EC-detector, but no one present in sufficient amount for MS analyses contained bromine, supporting the hypothesis that the bromine is distributed on many volatile compounds.

EXPERIMENTAL PROCEDURES

Various samples of fish, seaweed, shrimp and shellfish as well as a series of cod liver oils were included in the experiments. One of the oil samples was produced in 1924, the other in 1940, 1960 and 1969, respectively. Table I should be consulted for further details on the samples.

The cod liver oil, 100-200 ml, was mixed with distilled water in the ratio of 1:5 in a spherical 2 liter flask, the latter being connected to a conventional distillation apparatus. Five milliliters cyclohexane was added and the solution was then heated to boiling. The cyclohexane together with ca. 50 ml of water was distilled over. The solution was cooled to below 50 C, a further 5 ml of cyclohexane was added and the distillation was repeated a second time, now collecting 5-10 ml of water. The function of the last added cyclohexane is essentially that of flushing out remnants of the volatile nonpolar compounds in the apparatus into the receiving flask. Each sample was distilled once more following the same procedure, and the two distillates were kept separate in the subsequent work.

The extraction of volatile organic compounds from the fish samples and the other raw materials was performed by the same distillation procedure as that used for cod liver oil. The samples were homogenized, and distilled water was added to give a ratio of one part of raw material to two parts of water.

After distillation the cyclohexane phase was collected, and 25 ml distilled water was added and redistilled. This is to remove any remaining inorganic ions, in particular bromide ions, which could have contaminated the distillate and disturbed the subsequent analysis. The total amount of lipids in these samples was determined by chloroform-methanol extraction.

The bromine content of the cyclohexane solution was finally determined by neutron activation analysis. One milliliter of the cyclohexane solution was transferred to a quartz ampoule previously treated with concentrated nitric acid and washed in distilled water. The ampoule was sealed, and together with a bromine standard irradiated with a neutron flux of ca. 5×10^{12} n/cm^2 sec in a nuclear reactor for 2 hr. Following irradiation the samples were transferred to nonactive glass vials and their activity measured using a multichannel γ-spectrometer without any prior chemical treatment.

RESULTS AND DISCUSSION

All the neutron-activated cyclohexane solutions gave nearly pure bromine spectra when their activity was measured ca. 1 day after irradiation. If these solutions also contain corresponding chloro- and iodine-organic compounds, then the chlorine isotope Cl-35 with a half life of 37.3 min and the iodine isotope I-128 with a half life of 25 min will be formed when they are subjected to neutron irradiation. These radioactive isotopes can be detected using a γ-spectrometer, if the measurements are carried out immediately after the irradiation, i.e., within the time of a few half lives. In a few samples only traces of the radioactive sodium isotope Na-24 were detected. Contamination due to the presence of inorganic ions can therefore be regarded as negligible.

The results of the bromine determinations in the two distillations together with the content of lipids extractable with chloroform-methanol are given in Table I. The amount of volatile bromo organic compounds is given as μg Br/kg extractable lipids (ppb). The amount of bromine found in the distillate from fish raw material represent ca. 0.1-1% of the total bromine content of the marine oils. This is especially the case for cod liver oil and the fat fish. In cod fillet there seems to be a certain accumulation of the bromine-containing compounds compared with the liver. Also the other samples with a low content of lipids have a higher concentration of these compounds. When evaluating the considerable higher bromine content in the two distillates produced from brown algae, it should be borne in mind that the content of bromine found in the lipids extracted from seaweed is higher here than in marine fish (3).

The results obtained from the analysis of cod liver oil should be regarded as minimum values, as some of the volatile compounds may have evaporated during the production of the oil.

The presence of bromo organic volatile compounds in all the samples analyzed, especially in the samples from 1924 and 1940, indicate that the marine organisms are able to synthesize this type of compound. The presence of bromo organic compounds originating from pollution sources together with chlorinated hydrocarbons cannot be disregarded, but the contribution of such compounds is probably negligible compared with compounds originating from marine organisms, especially in relatively uncontaminated areas. Based on earlier results where it has been demonstrated that no one or no group of soluble bromo organic compounds dominate in the lipid phase in marine organisms, it is also probable that several volatile bromo organic compounds are present. This is also demonstrated by comparing the content of the bromo organic compounds found in the first distillate with that in the second (Table I). In most of the samples analyzed there is a certain reduction of the bromo organic compounds from the first to the second distillate, indicating the presence of both volatile and less volatile compounds.

One of the conclusions that may be drawn from these findings is that most of these bromine-containing compounds will probably pass the usual methods of sample clean-up employed in analysis for chlorinated hydrocarbons of man-made origin in the environment, and they will be detected by electron capture gas chromatography. The possibility of their being mistakenly identified as pollutants with long biological half lives therefore exists. Furthermore the ubiquitous occurrence of these brominated hydrocarbons among all classes of organic compounds indicates that their mechanism(s) of formation is not very specific, and that chlorine may be incorporated as well.

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XII

The Analysis of Organically Bound Elements (As, Se, Br) and Phosphorus in Raw, Refined, Bleached and Hydrogenated Marine Oils Produced from Fish of Different Quality[1]

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ABSTRACT

Marine oils have been produced in pilot plant by boiling, pressing and separation of the press liquor from raw material (mackerel and herring) of different levels of spoilage. The difference in quality is obtained by varying the period of storage. Some of the oil samples have been refined and hydrogenated. In samples taken both from the raw oils and from oils at the different steps in the processing, organic bound arsenic, selenium, bromine and phosphorus are analyzed. When the raw material deteriorates during storage, an increase in the selenium and phosphorus content in the oils produced from these materials is observed, whereas the bromine and the arsenic content is nearly constant. During the refining the arsenic and phosphorus disappear almost completely from the oils, whereas the selenium content is reduced to about two-thirds and the bromine content is nearly unaffected. In the hydrogenation step the selenium disappears relatively fast and the bromine more slowly.

INTRODUCTION

It is well known that, in catalytic hydrogenation of oils, the marine oils will cause a considerably faster inactivation of the catalyst than the oils of vegetable origin. Sulphur compounds are the most frequently occurring of these catalyst contaminants (1,2). However it is possible that other compounds present in marine oils could affect the catalyst. Bromine, arsenic and selenium, which are all present in the form of fat soluble compounds in these oils, can here be mentioned as potential contaminants. It has furthermore been observed that the poisoning of the catalyst in hydrogenation of marine oils becomes more pronounced as the quality of the raw material from which

[1]Presented at the ISF XIth World Congress, Gøteborg, Sweden, June 1972.

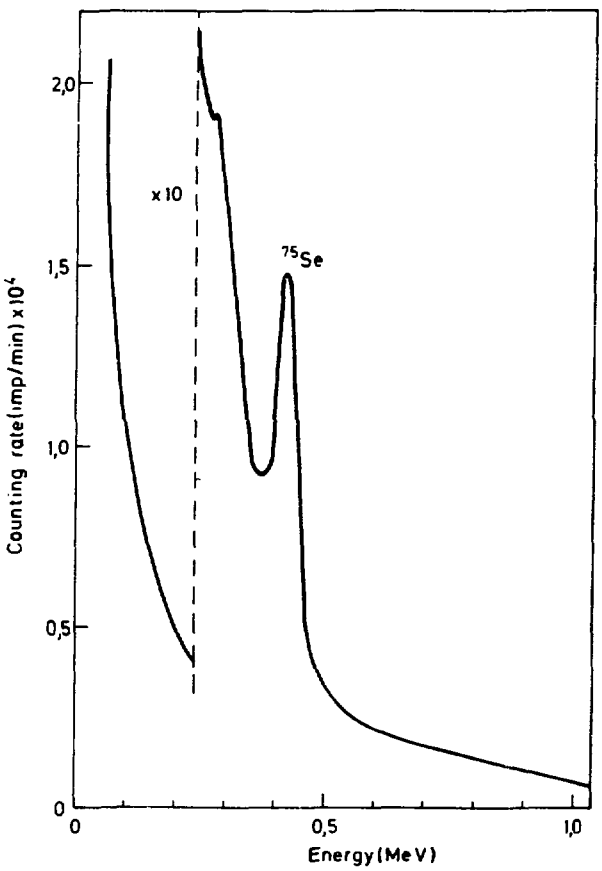

FIG. 1. γ Spectrum of neutron-activated crude oil sample registered 1.5 months after inactivation, showing the bremsstrahlung spectrum from ^{32}P and the sum peak of ^{75}Se at 0.4 MeV.

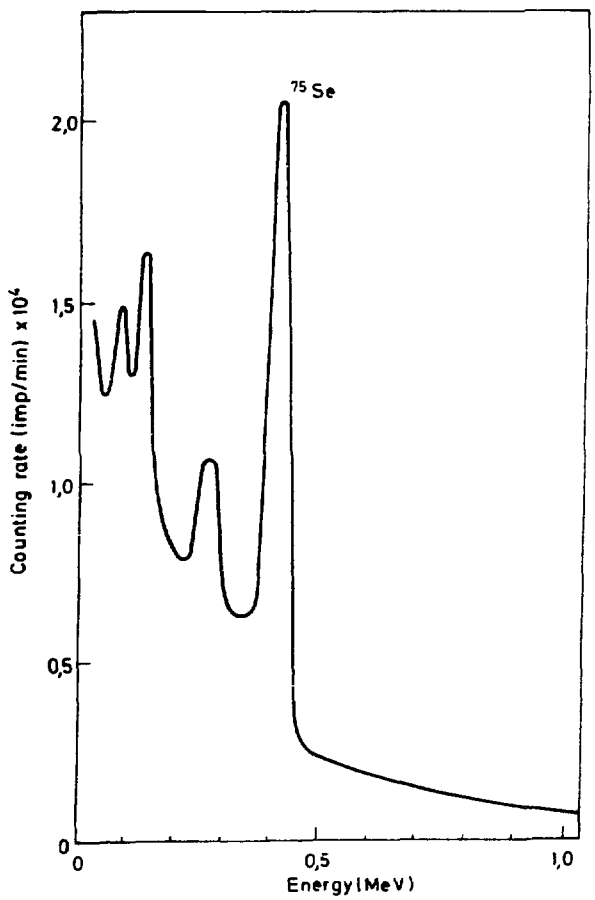

FIG. 2. γ Spectrum of a neutron-activated bleached oil sample registered 1.5 months after activation. Only the γ spectrum of ^{75}Se can now be seen.

TABLE I

Trace Elements in Oil Produced from Fish of Different Quality

Sample	Locality and date of catch	Storage, days	TVN,[a] mg N/100 g	FFA in the oil, %	Se, ppm	Br, ppm	P, ppm	As, ppm
Herring	West coast of Norway 6/2/70	0	13	0.6	0.09	4.3	<1	6.5
		6	88	2.3	0.14	4.0	72	6.6
		13	156	6.7	0.28	4.1	66	7.6
Herring	Shetland 13/10/70	0	13	0.2	0.02	4.4	6	5.3
		2	22	0.6	0.04	5.0	12	5.4
		5	83	9.8	0.22	4.3	111	5.3
		8	133	16.4	0.26	3.8	230	5.3
Mackerel	North Sea 24/10/70	0	6	0.2	0.05	3.1	<2	5.2
		3	35	1.3	0.06	2.5	8	5.2
		7	91	11.5	0.11	3.3	99	5.2
		10	134	16.5	0.11	2.9	106	4.9
Mackerel	West coast of Norway 8/9/70	0	18	0.3	0.05	3.7	8	4.6
		6	87	1.9	0.19	3.7	12	5.5
		12	141	3.8	0.21	3.2	20	4.9
Capelin	North coast of Norway	0	31	2.5	0.09	3.6	4	9.1
		...[b]	116	10.4	0.12	3.4	141	7.8
		...[b]	198	10.9	0.10	3.6	137	8.5

[a]TVN = Total volatile nitrogen; FFA = free fatty acids.
[b]Low quality raw material.

the oils is produced decreases. This effect can be correlated with an increase of the sulphur content in the oil (2).

The presence of selenium-containing compounds in marine oils was demonstrated in our laboratory 1-2 years ago (3). No information exists to indicate whether the content of these compounds increases as the raw material deteriorates. Concerning the bromine, it has been shown that bromine-containing compounds are present as both neutral and polar lipid soluble compounds, and it is suggested that the bromine seems not to be associated to one or more specific groups of lipids (4). It is therefore less probable that the bromine content of the oil will be affected by the quality of the raw material from which the oil is produced.

The variation of the content of arsenic in oils produced from raw material in which the quality ranges from good to very bad is not known. However it has previously been shown that most of the arsenic is removed during the alkaline-refining process (5) and will probably not have any inactivating effect on the catalyst.

The purpose of this investigation was in the first instance to study in more detail how the selenium, arsenic, phosphorus, i.e., the phospholipids, and the bromine contents varied in oils produced from raw fish materials of differing qualities, and thereafter to follow these elements in the oils during the alkaline-refining, the bleaching and the hydrogenation processes.

Neutron activation was used as an analytical method for determining the elements to be analyzed. Even though the precision of this method is not as great as other methods based on spectrophotometry for phosphorus per se, it is quite adequate for the large changes measured in the present work. Furthermore it is advantageous to be able to measure phosphorus along with selenium, arsenic and bromine on the same samples, especially when the determination is performed nondestructively.

EXPERIMENTAL PROCEDURES

Herring and mackerel were used in these experiments. In addition some industrially produced capelin oils of varying quality were analyzed. The oils were produced from raw materials which had been stored for varying lengths of time. The intervals at which samples were taken from storage were chosen so that the quality of the fish changed from good to very bad. The storage took place under anaerobic conditions, and the temperature was kept at a level corresponding to air temperature normally encountered during corresponding industrial storage. The storage period varied from 0-13 days. The condition of the fish material was characterized by analyzing the total volatile nitrogen (TVN) content in the raw material, and the free fatty acid (FFA) content in the oils produced. (TVN and FFA were determined at the Norwegian Herring Oil and Meal Industry Research Institute.) A few chosen series of oils were then alkaline-refined, bleached and hydrogenated. The hydrogenation was carried out under standard conditions in a laboratory scale using nickel catalyst (1). Oil samples were taken at given intervals. Further information concerning the treatment and the characterization of the oils are given elsewhere (1).

Neutron activation was used for the analysis of bromine, selenium, arsenic and phosphorus. Only a brief description of the analtyical technique is given here. Samples of ca. 0.5 g were weighed and sealed into quartz ampoules. The samples, together with bromine, selenium, arsenic and phosphorus standards, were irradiated with thermal neutrons at a flux of ca. 5×10^{12} n/cm^2 sec for 20 hr in the nuclear reactor JEEP 2 at Kjeller, Norway. When the irradiation process was completed, the oils were transferred to inactive glass vials, and their activities counted on a 400 channel γ spectrometer with a 3 x 3 in. NaI well-type crystal. The induced activity in bromine (^{82}Br) and in arsenic (^{76}As) with half lives of ca. 34 hr and 26 hr, respectively, can be measured 1-7 days after irradiation. The radioactive sodium isotope ^{24}Na was detected in some of the samples, and in these cases the counting of the bromine and the arsenic activity was postponed until the interference from ^{24}Na compton became negligible.

When the radioactive bromine and arsenic isotopes in the oils had disintegrated, the radioactive selenium isotope ^{75}Se with a half life of 120 days could be registered. The neutron activated phosphorus, ^{32}P emits only β rays when decaying and can be determined by measuring the "bremsstrahlung." After subtracting the background activity and the contributions from the other radioactive isotopes, in particular ^{75}Se, the phosphorus was analyzed by summing up a known number of channels in the γ spectrum obtained. Figures 1-2 show examples of the γ spectra of neutron-activated crude and bleached oil showing the presence of phosphorus and selenium, and selenium, respectively. The spectra were obtained 1.5 months after the irradiation.

RESULTS AND DISCUSSION

The results of the selenium, arsenic, bromine and phosphorus determinations, carried out on samples of raw

TABLE II

Selenium and Bromine Content in Samples of Oil Taken at Different Stages in the Refining and Hydrogenation Process

	Mackerel						Herring					
	Stored 0 days		Stored 6 days		Stored 13 days		Stored 0 days		Stored 6 days		Stored 12 days	
Sampling stages	Se, ppm	Br, ppm	Se, ppm	Br, ppm	Se, ppm	Br, ppm	Se, ppm	Br, ppm	Se, ppm	Br, ppm	Se, ppm	Br, ppm
Crude oil	0.09	4.5	0.15	4.2	0.28	3.9	0.05	3.7	0.19	3.7	0.21	3.2
Alkaline-refined oil	0.05	4.7	0.09	4.0	0.24	3.6	0.04	3.3	0.14	3.6	0.16	3.3
Bleached oil	0.05	3.7	0.09	3.9	0.19	3.3	0.04	3.0	0.12	3.3	0.15	3.6
Hydrogenated oil, 10 min	<0.02	0.6	<0.02	0.7	<0.02	0.7	<0.02	0.6	<0.02	0.7	0.02	1.2
Hydrogenated oil, 25 min	<0.02	0.4	<0.02	0.4	<0.02	0.6	<0.02	0.3	<0.02	0.3	<0.02	0.9
Hydrogenated oil, 60 min	<0.02	0.3	<0.02	0.4	<0.02	0.6	<0.02	0.2	<0.02	0.5	<0.02	0.8
Hydrogenated oil, 120 min	<0.02	0.3	<0.02	0.2	<0.02	0.4	<0.02	0.2	<0.02	0.2	<0.02	0.5

oil produced from the same raw material which had been stored at different lengths of time, are shown in Table I. Even though relatively few samples have been analyzed in each series, the results indicate that a significant increase in the amount of extractable selenolipids occurs, during this period of storage. The results also indicate that the main release of these selenolipids from the tissue and into the extractable oil phase takes place after 4-6 days storage. The extraction of phospholipids seems to follow the same pattern. From a relatively low level of 2-10 ppm phosphorus in the beginning of the storage period (0-4 days), the content increases with a factor of 5-10 during the next 5-6 days. In the case of bromine, the results show that the content of this element is approximately constant in oils produced from the same raw material. This is in accordance with earlier results which show that brominated compounds are distributed among both polar and nonpolar lipids (5). Even the content of arseno compounds does not seem to be dependent upon the quality of the raw material from which the oil is produced, in spite of the fact that the arseno organic compounds must be characterized as typical polar lipids. No absorption of these compounds to the tissue seems to take place in the same way as with the seleno and phospholipids.

The results of the bromine and selenium analyses of the oil samples taken at different stages during the processing of the oil are presented in Table II. They also confirmed that the phospholipids and the arseno organic compounds are washed almost quantitatively out during the alkaline treatment. Neither bromine nor selenium are removed during the alkaline-refining and the bleaching of the oil. Here the selenium content is only reduced to about one-third. In the case of bromine, the different refining steps seem to have little or no effect.

During the hydrogenation process a significant fall in both the selenium and bromine contents is observed. This is most pronounced for the selenium, which disappears almost completely already after a hydrogenation period of 10 min. The bromine disappears more slowly. However due to a considerable higher content, the bromine compounds may have a more pronounced effect on the catalyst as an inactivating agent.

On the basis of these results one can conclude that both bromine and selenium organic compounds present in marine oils seem to have an inactivating effect on the catalyst during the hydrogenation of the oils. The bromine content of the oils will in the main be independent of the quality of the raw material from which the oil is produced, while the content of the selenium will increase as the raw material deteriorates. Since the presence of corresponding selenium and bromine organic compounds in vegetable oils has not been detected, the effect of these compounds will be a characteristic of marine oils when they are hydrogenated.

ACKNOWLEDGMENT

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XIII

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THE ABSORPTION AND METABOLISM OF ARSENIC IN FISH

by

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INTRODUCTION

It has been shown that fish contain arsenic both in the form of lipid soluble and water soluble arseno organic compounds (1—4). Experiments on the growth of single celled algae in cultures where radioactive arsenic has been added to the culture solution, showed that these algae were able to synthesise both lipid soluble and water soluble arseno organic compounds (5). It was considered that these organisms would be the main source of the arseno organic compounds demonstrated in the marine food chain; in organisms such as fish, shrimps and other invertebrates. However, if arsenic occurs in compounds which are essential to fish and other marine organisms, the possibility that they themselves are able to synthesise these compounds have to be considered. A better understanding of this problem would be of interest from both a biological point of view, and in connection with arsenic containing pollution and waste.

In order to study the absorption of inorganic arsenic by fish, two series of experiments based upon the use of inorganic radioactive arsenic were carried out, one with radioactive arsenic added to the feed and the other with the arsenic mixed into the water.

In the feeding experiments, two types of feed were used. One consisted of terrestrial feed compounds, and contained about 0.3 ppm arsenic, mostly present in the form of inorganic arsenic. The other was based on a marine feed composition containing about 15 ppm of arsenic in the form of arseno organic compounds. The aim of the experiment was to establish accumulation, respectively depletion of arsenic in the fish. If differences in the arsenic content between the two groups could be observed, a second experimental period with inorganic radioactive arsenic added to the feeds should make it possible to establish whether the absorption of radioactive (i.e. inorganic) arsenic is influenced by the level of arsenic in the fish. Two new groups of fish were started as controls on the same two diets a couple of days before radioactive arsenic was added to the two feed compositions during a feeding period of 14 days. In all four groups, both in the lipid and the non lipid phase, the content of synthesised radioactive arseno organic compounds were determined both during the feeding period and in the subsequent period where the pattern of depletion was investigated.

The arseno organic compounds produced by the fish were studied using molecular gel filtration of aqueous solutions produced from the fish, and further by thin layer chromatography of these fractions enriched in radioactive arsenic. In order to get information on the distribution of the radioactive arsenic in the fish in the depletion period, when the content of inorganic arsenic in stomach has been reduced to a negligible level, autoradiographic studies were carried out.

EXPERIMENTAL

Absorption of arsenic.

Rainbow trout (*Salmo gairdneri*) were used in all the experiments. The experimental conditions have been described earlier (6). Four groups with 35—40 fish in each group were used. The feed made up from terrestrial components consisted mainly of minced meat. About 2% carboxy methylcellulose (CMC) was used to hold the mixture together. The marine feed consisted of enzyme hydrolysed powdered defatted cod liver mixed with minced coalfish. CMC was also here used to hold the mixture together. The radioactive arsenic isotope As-74 with $T_{\frac{1}{2}}$ = 17,7 d (As-74, AJS, 1P, Amersham, England) was sprayed on to the feed mixture and mixed well in before the CMC was added.

The activity of the feed was checked by measuring the specific activity (activity/g of feed) for 10 samples. The variation between these samples was less than 15%. The feed were given in small lumps which were eaten before they began to disintegrate. During most of the period when the two feed types were used, the fish in all groups exhibited a good appetite and normal growth. Only at the end of the period some stagnation in the growth was observed.

The groups fed terrestrial feed is called T1, and the groups fed marine feed is called M1. The two parallel groups started after about two months are correspondingly called T2 and M2.

Samples of fish were taken during the whole experimental periods, and stored at —20 °C prior to analysis.

In the experiments where the absorption of inorganic arsenic from the water was studied, the radioactive arsenic was added to the water using dosing pump (LKB, Sweden) operating at a speed of about 60 ml/hour. The radioactive solution was injected into the main water supply tube through an injection needle. The flow rate of the water through the aquarium during these experiments was 4 l water/min.

The analysis.

The fish selected during the feeding period with radioactive feed and likewise from the depletion period, were weighed and the radioactivity (counts/min, g fish) measured using a scintillation detector (3×3" NaI crystal). The fish were ground subsequently and homogenised in a blendor, and the lipids extracted using a chloroform/methanol mixture (v/v, 2/1) as extracting solvent. The extraction was carried out at room temperature under constant stirring for 2 hours. Some more water was then added and the chloroform phase was separated off. The chloroform-lipid phase was washed twice with distilled water, and the chloroform removed by evaporation.

The yield of lipids was determined by weighing. The radioactivity of the arsenic present as lipid soluble arsenic containing compounds was measured using a scintillation detector (2×2" NaI well type crystal).

The lipid phase extracted from some of the fish samples were analysed in order to obtain information as to the possible synthesis of lipid soluble arseno organic compounds, by observing the possible appearance of radioactivity in the polar fraction, as previously reported for fish lipids (2). The lipids were redissolved in chloroform, and transferred to a column (height: 15 cm, internal diameter: 1 cm) loaded with activated silica gel (Kieselgel 0.2—0.5 mm f. chrom, E. Merck A.G., Darmstadt, W. Germany). After washing with chloroform in order to remove neutral lipids still present in the solution the elution was carried out with respectively 10, 20 and 30% methanol in chloroform. The radioactivity was measured for each of these fractions.

After the extraction of the lipids from the homogenised raw fish, distilled water was added to the non lipid phase and the mixture boiled for 20 min. in an Erlenmeyer flask, whereupon the aqueous solution (the glue water) was filtered and the filter with the undissolved material washed once with distilled water. The washings were combined with the filtrate. All the samples of glue water produced from fish which had received radioactive feed and also fish taken up to 10 days after the feeding with radioactive arsenic was stopped, were eluted on an ion exchange resin (Dow 2 × 8, 200—400 mesh). The pH of the solution transferred to the column was 5—6, and distilled deionized water was used as eluting agent. The radioactivity in the aqueous solution was measured using the same counting equipment as used for the lipids. The ion exchange step is necessary in order to remove radioactive inorganic arsenic still present in the fish. The removal of inorganic radioactive arsenic was also studied by adding separately 6.6 N HCl to the glue water and to the solid phase. The mixture was heated to about 100 °C. The inorganic arsenic would during this procedure, evaporate as arsenic trichloride (7).

Molecular gel filtration of selected samples of glue water was carried out on a dextrane resin (Sephadex G-25 fine, Pharmacia fine Chemicals, Sweden). Before the collection of the fractions the eluate was recorded on an Uvicord spectrophotometer (LKB, Sweden) measuring the absorbance at 254 nm. A more detailed description of the gel filtration procedure used in this study is described elsewhere (3). After measuring each fraction, the most radioactive fraction produced by the gel filtration was analysed further by thin layer chromotagraphy. A system with cellulose substrate (1 mm thickness) and a developing solvent consisting of chloroform/methanol/ammonia in the ratio 2:2:1 was used (8).

Autoradiography.

The distribution of the synthesised arseno organic compounds was studied by use of autoradiographic technique. Selected samples of fish taken out after the period when the use of radioactive arsenic added to the feed had been discontinued, i.e. in the depletion period, were used in a study of how the radioactive arsenic was distributed in the fish. Samples of fish stored at —20 °C were cut up into sections, perpendicular to the backbone. The fish should during this operation be kept at a temperature of +4 °C or less. The sections were polished using fine grain emerypaper and placed in contact with a photographic emulsion (Ilford Industry G X-ray film). A thin layer of cellophane was placed between the fish sections and the photographic emulsion to prevent any of the compounds in the fish sample coming in contact with the film emulsion and thereby giving spurious darkening of the film. During the exposure the samples were placed in light tight boxes and kept at —20 °C. After an exposure period of about 2 1/2 months, the film was developed in the same way as ordinary X-ray film.

Activation analysis.

Glue water produced of fish samples taken from the four groups were evaporated to dryness at 105 °C. Samples of 100—200 mg were taken out and sealed in quartz ampoules, neutron activated together with an arsenic standard and analysed for arsenic using a method slightly modified which has been previously described (4). After the neutron irradiation, arsenic carrier was added and the organic matter was decomposed using sulphuric acid and hydrogen peroxide. The arsenic was then precipitated as arsenic sulphide and the γ-spectrum of As-76 recorded by a multichannel gamma ray spectrometer. It should here be noted that the radioactive arsenic isotope formed by neutron activation, As-76 with a half life of 26 h, has different nuclear properties from the As-74 isotope used in the feeding experiments.

RESULTS AND COMMENTS

Figure 1 shows how the radioactive arsenic mixed into the feed is absorbed by the fish from the four groups during the period in which they received radioactive arsenic added to the feed, and in the subsequent period when the addition of radioactive arsenic in the feed is

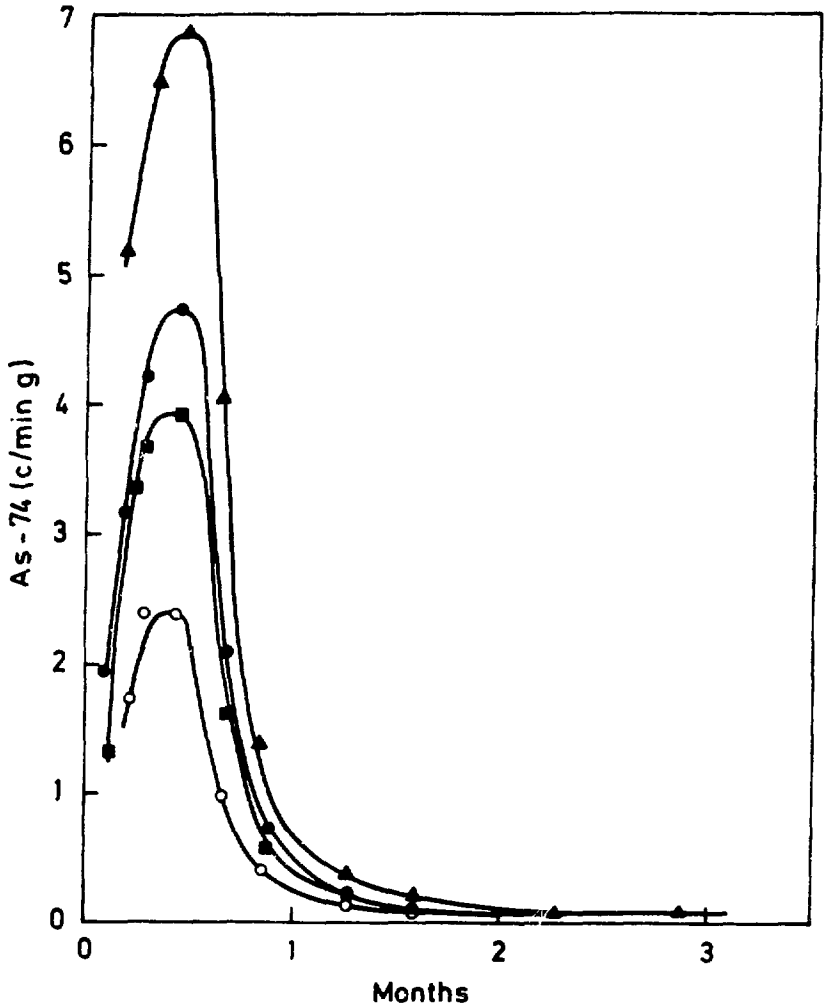


Fig. 1. The content of As-74 in fish taken from the four groups T1 (▲—▲), M1 (○—○), T2 (●—●) and M2 (■—■) in the period when feeding with As-74 was added to the feed and in the subsequent depletion period.

discontinued. In the beginning of the depletion period a steep decrease in the activity is observed. After 6—10 days the curves level out. At this time the contents of radioactive inorganic arsenic in the fish is negligible at least in the oil and in the aqueous extracts (glue water) produced by boiling fish samples. This is demonstrated for the glue water by ion exchanging and measuring the activity, i.e. arsenite-arsenate absorbed to the ion exchange resin. The same result is obtained for the oil when washing the oil and counting the oil between each washing procedures. The results of the HCl treatment of the solid phase of the same fish show, however, that there may be some inorganic arsenic or organic arsenic compounds decomposed to inorganic arsenic by HCl, located in the meal phase as the activity decreases 10—30% during this treatment. The results here are however rather scattered and may also be caused by individual variations between the different fish samples.

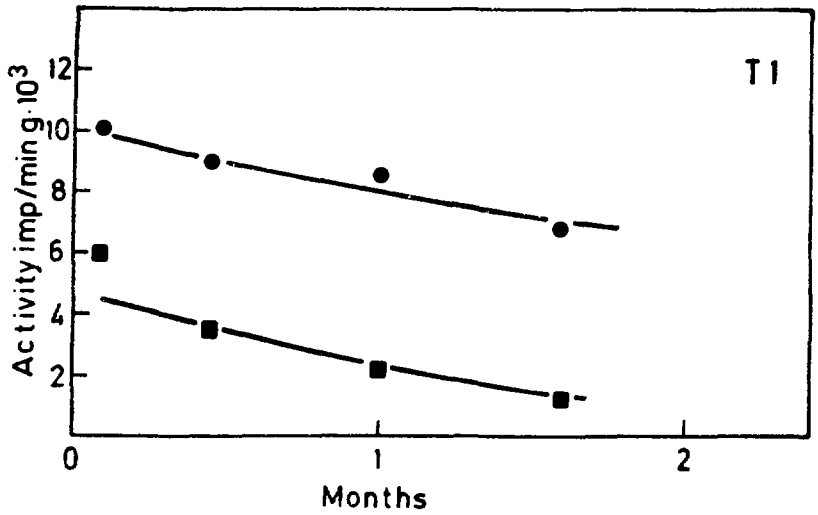
The results presented in Fig. 1 show further that the absorption of inorganic arsenic is influenced by the quantity of arseno organic compounds present in the feed. After a 2 months period of feeding prior to the addition of radioactive arsenic with the two different feeds, there is a significant difference in the absorption of inorganic (radioactive) arsenic in the two groups of fish. In the two groups starting with the two different feed compositions just before the radioactive arsenic was added to the feed, only a small difference in the uptake of arsenic is observed. The results indicate here that the uptake of radioactive inorganic arsenic mixed into the feed is dependent of the content of arsenic present in the fish, as discussed later, only a small fraction of this arsenic is converted by the fish to arseno organic compounds.

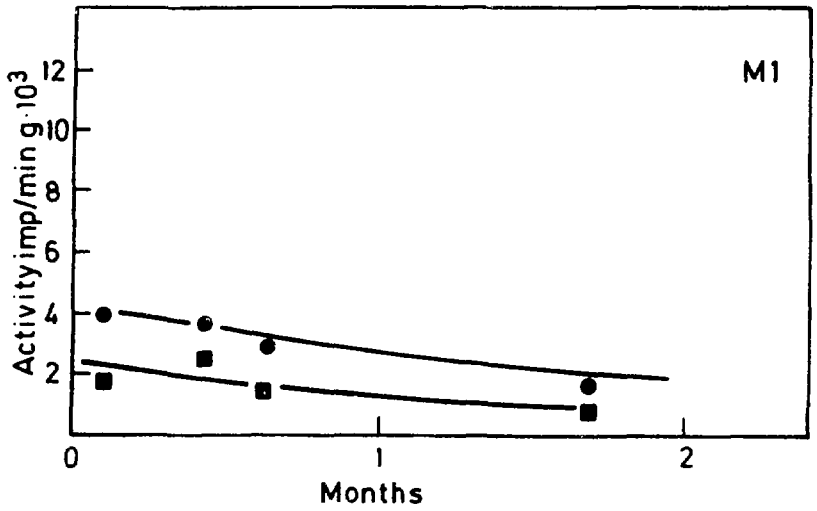
In Fig. 2a—d are shown the radioactivity of lipid soluble and water soluble arseno organic compounds present in the fish after the radioactive feed has been discontinued. The curves indicate that the lipid soluble arseno organic compounds have about the same biological half life, about 40—50 d, as the water soluble ones. However, taking into consideration that there may be considerable variations between the fish samples it is difficult to draw any definite conclusions concerning this matter.

In the two groups, MI and TI, fed on marine and terrestrial feed which started 2 months before the addition of radioactive arsenic, there is a significant difference especially in the amount of radioactive lipid soluble arseno organic compounds. In the two other groups M2 and T2, only a small change in the level of radioactive water soluble arseno organic compounds is observed.

When comparing the arsenic activity from the arseno organic compounds present in the lipid and the non lipid phase of the fish samples

with the total amount absorbed, see Fig. 1 and 2a—d, it is observed tha- only a small fraction of the inorganic arsenic is converted to arseno organic compounds. The amount of inorganic arsenic in the terrestrial feed composition is less than 0.3 ppm and in the marine feed probably in the area of 1 ppm. As only a small fraction of this is absorbed and converted to arseno organic compounds in the fish, the results demonstrate





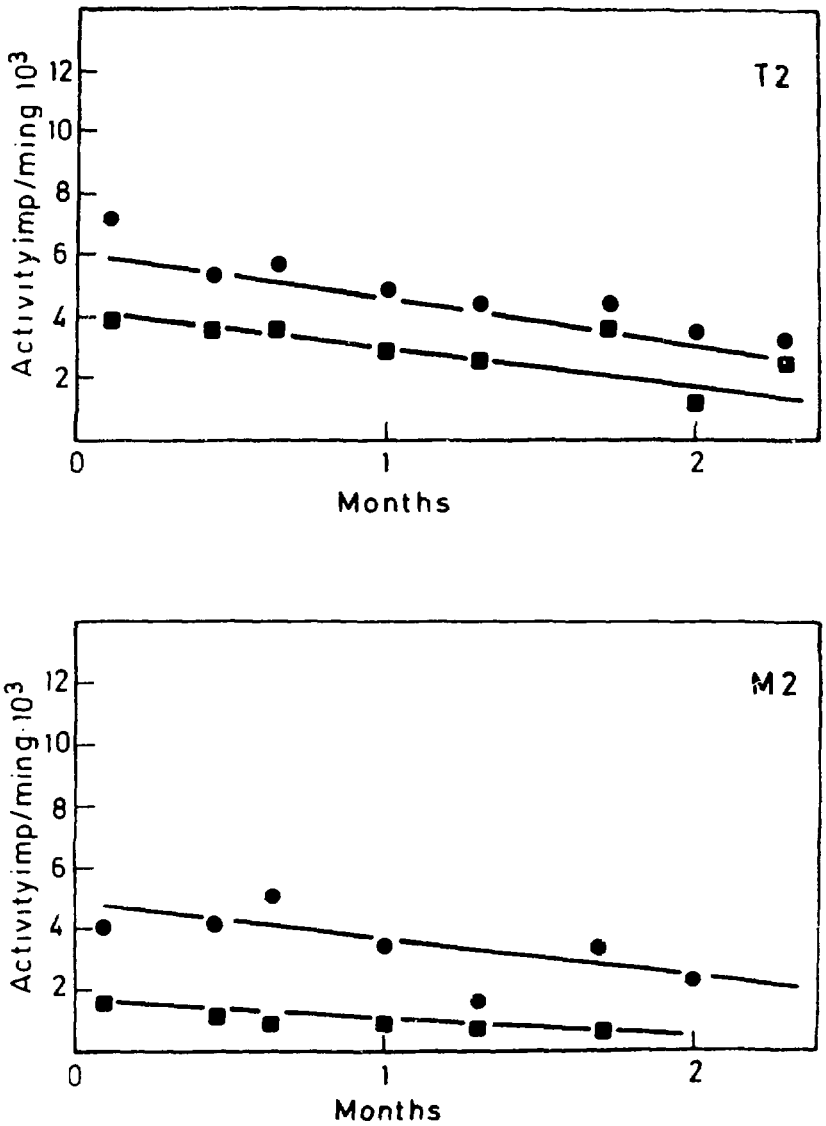


Fig. 2a—d. The content of radioactive (imp As-74/g matter) lipid soluble (● — ●) and water soluble (■ — ■) arseno organic compounds in fish taken from the four groups. Zero time represents the date when the addition of As-74 to the feed is discontinued.

that this source of arsenic represents a quite inessential contribution to the total quantity of arsenic found in fish. The arsenic present in the fish is therefore probably supplied from other marine organisms at lower stages in the marine food chain. This is also demonstrated by the results presented in Fig. 3. Here the results show that the two different feed compositions had a pronounced effect on the level of arsenic in the fish. Although there is some scattering in the determinations, the results show that after 1—2 months the groups fed on the marine feed have a considerably higher level of arsenic in the glue water than the other groups of fish fed on the terrestrial feed. Starting with about 20 ppm arsenic in dehydrated glue water in all groups, the M groups increase to 30—50 ppm while the arsenic content in the T groups decrease to about 10 ppm. These results suggest that the arseno organic compounds from lower stages in the marine food chain represent probably the main sources of arsenic present in fish.

The results of the molecular gel filtration experiments carried out on glue water extracts are presented in Fig. 4, where the absorbance at 254 nm of the eluate and the radioactivity registered (counts/min g dry material) for fractions obtained during the gel filtration are shown. They confirm that the water soluble arseno organic compounds synthe-

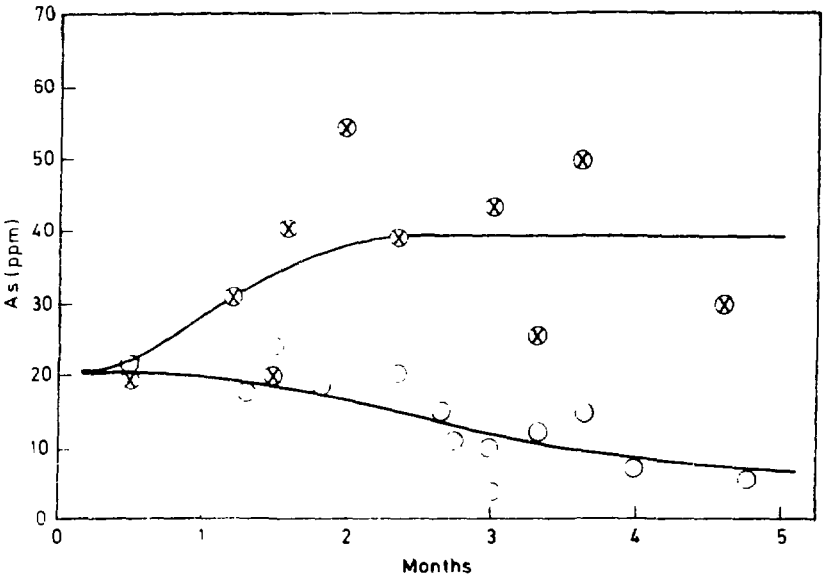


Fig. 3. The Absolute amount of arsenic in glue water produced from fish fed on marine (○ — ○) and terrestrial feed (× — ×).

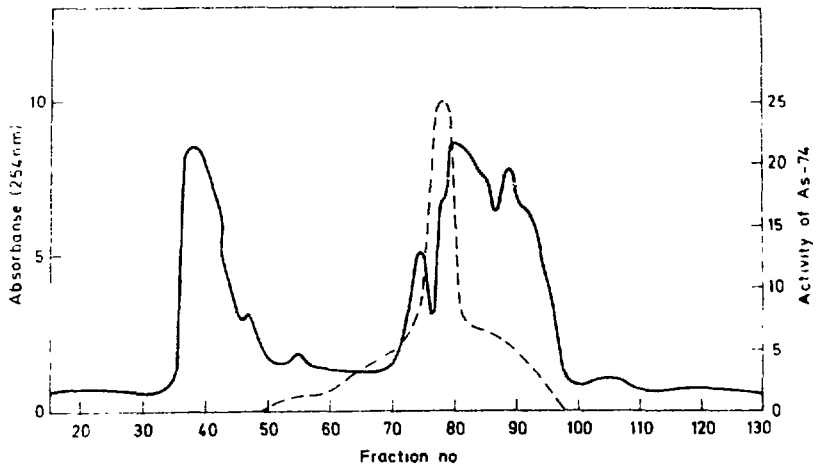


Fig. 4. The absorbance at 254 nm (———) and the radioactivity from As-74 (- - - - -) of molecular gel filtrated glue water. The glue water was produced from fish taken 6 days after the depletion started.

sised by the fish, are eluted in the same fractions as have been established for the main amount of organic bound arsenic in fish (3). Autoradiography applied on thin layer chromatograms of the molecular gel filtrated fractions with highest specific activity produced from fish taken in the depletion period, shows one radioactive arseno organic compound with a Rf value of about 0.30. Due to the low specific activity of the arseno organic compounds present in the glue water, only one radioactive arseno organic compound was detected. The Rf value, 0.30, corresponds to the results obtained when water soluble radioactive arseno organic compounds synthesised in single celled algae were analysed in the same way (5). Both the results from the molecular gel filtration and the thin layer chromatography indicate that the main water soluble arseno organic compound which is synthesised by the fish, is also synthesised by algae and probably by other marine organisms.

The results of the fractionation studies where the neutral part of the lipids was separated from the polar fractions on a silicagel column show that the arseno organic lipids synthesised by the fish follow the polar fractions. This is in agreement with previous results where the total amount of arseno lipids in fish were studied (2). Most of the radioactive arseno lipids was detected in the eluates using 20 and 30% methanol in chloroform as eluting agent.

Fig. 5—7 show how the radioactive arseno organic compounds are distributed in some selected sections of the fish. These compounds seem

especially to be enriched in the eyes, the throat and the gills, the pylorus organ and in some less distinct area. Due to a relatively rough technique used for preparing the samples and also the use of a fast X-ray-emulsion, a rather low resolution on the autoradiographs was obtained. Besides the organs already mentioned also the liver and the kidney exhibit a strong accumulation of radioactive arsenic immediately after the feeding of radioactive arsenic was discontinued. But this arsenic disappears faster from these organs in the depletion period than from the other organs. This may indicate that the synthesis or part of the synthesis of the arseno organic compounds occurs in the liver. No significant accumulation was observed in the fish muscle and in the skin. The strong concentration of arseno organic compounds in the eyes and in the throat and gills, that is in the most pronounced mucus membrane regions show that these compounds may have a bacteriostatic effect and is used by the fish to protect these area against microorganisms.

Fig. 8 shows the absorption of inorganic radioactive arsenic by fish when the arsenic was added to the water. Ordinarily the fish samples

Fig. 5. Autoradiograph of a section cut perpendicular on the back bone of the fish and through the eyes. The fish was taken 12 days after the depletion period started.

Fig. 6. Autoradiograph of a fish section prepared in the same way as in Fig. 5, but from a different area in the head of the fish. The fish taken 12 days after the depletion started.

were kept in inactive water for a period of 5 min. before they were taken out and the absorbed activity recorded. Some corresponding values for fish washed in inactive water for 1 day are also presented. These last results show that at least 25—30% of the absorbed activity is washed out during this period.

In fish samples selected from this latter series of experiments and which were treated in the same way as previously described, the presence of neither lipid soluble or water soluble arseno organic compounds was detected. Even though it was diffcult to achieve a sufficiently high specific activity in the water without using excessively large quantities of radioactive arsenic, it should nevertheless have been possible to detect radioactive arsenic in the oil phase if the synthesis of lipid soluble arseno organic compounds occurred in the same way as when arsenic was added to the feed. The results show also that some inorganic arsenic can be washed out, indicating at least to some degree a passive absorption. The activity of the water was in average 50 imp/min ml. Comparing this value with the amount absorbed in the fish ca. 0.4 imp/min g, it is evident that the absorption of arsenic is a rather inefficient or slow process.

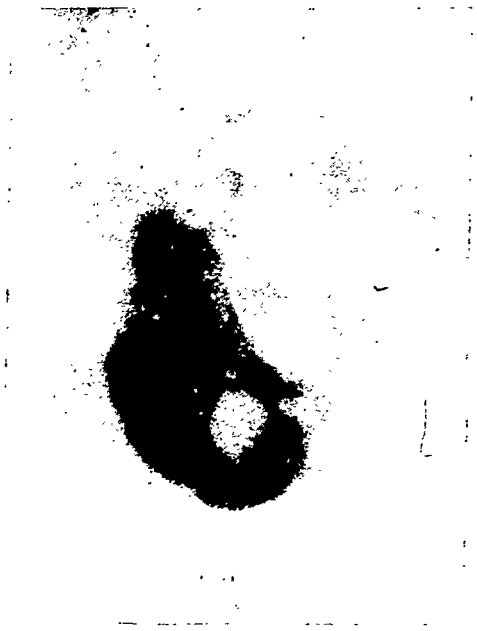


Fig. 7. Autoradiograph of a fish section prepared as in Fig. 5 taken from the stomach and through the pylorus organ. The fish was taken 12 days after the depletion started.

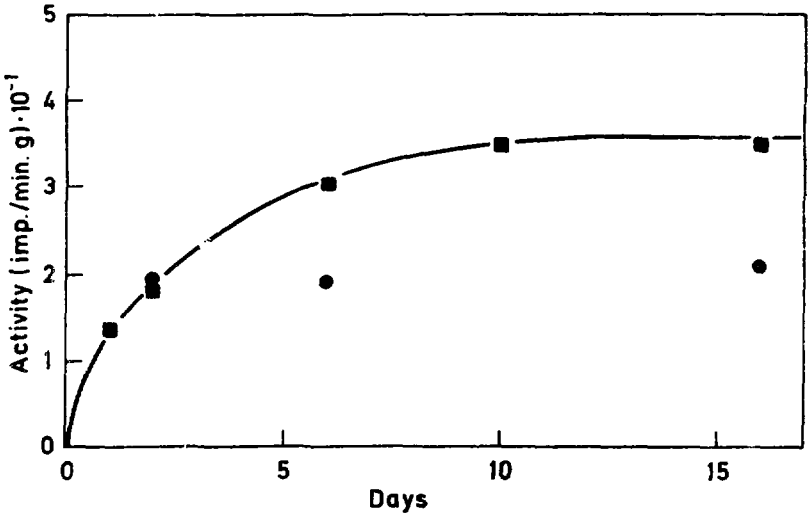


Fig. 8. The absorption by the fish of As-74 (imp As-74/g fish) added as inorganic arsenic to the water. The fish were kept in inactive water 5 min (■—■) and 1 day (———) before counting.

CONCLUSION

On the basis of the results obtained during these experiments it can be concluded that fish are able to synthesise both fat soluble and water soluble arseno organic compounds from the inorganic arsenic to be found in the feed eaten by the fish. However, this inorganic arsenic plays an insignificant role as source for the organic bound arsenic found in fish. The major part is supplied as already synthesised arseno organic compounds, from lower stages in the marine food chain. An accumulation of arseno organic compounds in specific organs shows that the compounds may possibly have a significance for fish. It is proposed that this may be as a bacteriostatic agent which acts to protect those regions of fish which are especially vulnerable to attack from microorganisms.

Inorganic arsenic present in the water will also be absorbed by the fish, but neither water soluble nor lipid soluble arseno organic compounds could be detected by the available methods used in this investigation.

ACKNOWLEDGEMENT

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SUMMARY

The absorption and metabolism of inorganic arsenic in rainbow trout (*Salmo gairdneri*) has been investigated by means of radioactive tracers. The results obtained show that the fish will absorb inorganic arsenic when this is mixed into the feed, and synthesise both lipid soluble and water soluble arseno organic compounds. The absorption of inorganic arsenic seems to depend upon the quantity of arsenic present in the fish. The absorption of inorganic arsenic decreases as the concentration of arsenic in the fish increases. When radioactive arsenic (As-74) is added to the water, absorption seems to take place through the skin and gills. This absorption, as far as can be determined by the methods used in this investigation, does not result in the formation of arseno organic compounds. When As-74 is mixed in the feed and thus absorbed through the digestive track, autoradiographic investigations indicate that arseno organic compounds are synthesised and accumulated in the eyes, the throat and gills and certain of the internal organs.

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Trace Metal Contents of Fish Meal and of the Lipid Phase Extracted from Fish Meal

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In fish meal produced industrially and in the laboratory from mackerel (*Scomber scomber*), herring (*Clupea harengus*), Norway pout (*Boreogadus esmarkii*) and capelin (*Mallotus villosus*), the following trace elements have been determined: cadmium, zinc, lead, copper, iron and cobalt, and, in the lipid phase extracted from the meals: selenium, arsenic, bromine, zinc, iron and copper. In addition phosphorus was determined. The results show that the level of zinc, lead and iron is higher in the industrially produced meals compared to those produced in the laboratory. Assuming that the zinc to cadmium ratio occurring naturally in seawater is about 100 to one, then the results indicate that zinc seems to be enriched relative to cadmium in the fish meal. Besides the elements arsenic, bromine, selenium and phosphorus which are present in the lipid phase as organic compounds, zinc, iron and cobalt are also present. These are probably complexed bound by the phospholipids. The selenium detected in the lipid phase (0.3 to 3 parts/million) shows that the organic lipid-soluble selenium compounds do not decompose during the production of the fish meal.

1. Introduction

There has in recent years been a noticeable increase in the interest shown in both the analysis of trace elements and their physiological significance, the latter especially in connection with feed for domestic animals. Here it is important to give the animals a balanced mixture of trace elements in their feed composition to ensure a good growth and development. It is also important to control the presence of any potentially harmful elements. Among these the toxic heavy metals should be mentioned.

There are relatively few new data available on the content of many of the important trace elements to be found both in fish and in feed produced from fish.[1–3] This applies to both unwanted, i.e. toxic, and "essential" trace elements. Moreover, little is known about the chemical state in which these elements occur, whether they are present as part of organic molecules, complexed bound in the lipid or non-lipid phase or as inorganic ions. It should also be noted that the level of trace elements may be increased above that occurring in the original raw material during the manufacturing process.

Selenium belongs to the most interesting trace elements present in marine raw material. It is one of the essential elements and is also highly toxic. Fish should be recognised as an important natural source of selenium. As has been shown previously,[4,5] selenium is here present both in the meal remaining after the extraction of the

lipids and in the lipid fraction obtained from marine raw materials. It was of particular interest in connection with the present work to see whether the seleno organic compounds existed in the lipid phase after the production of the meal.

Besides selenium the element cadmium should be mentioned. Considerable interest has been focused on this element due to its high toxicity.[6,7]

The aim of this investigation was first to get more information about the content of some of the more important trace elements present in the lipid and the non-lipid phase in industrially produced fish meal. By comparing these values with corresponding values from laboratory produced meals eventual contamination during the industrial production may be evaluated.

2. Experimental

2.1. Sample collection

Samples of industrially produced fish meal from herring, mackerel, Norway pout and capelin were collected. Special efforts were made to obtain meal samples from different manufacturers and to ensure that the samples were produced from fish from different localities and as far as possible caught at different times of the year. Two samples of meal produced from anchovies were also analysed. The sample of capelin, mackerel and herring used for the laboratory meal production was obtained through kind assistance from SSF.[a]

2.2. Sample preparation

The industrially produced meals were dried at 105 C until their weight remained constant. They were then ready for ashing. For the laboratory production of fish meal, about 2 kg of whole fish (capelin, herring and mackerel) was used. These were first homogenised in a Waring blendor and then boiled for approx. 20 min in a glass apparatus. Some distilled water was added before the boiling. After the boiling the samples were filtered and the oil was separated from the aqueous phase (the glue water) by centrifuging the filtrate. The glue water was then added to the meal phase (presscake) which was dried to constant weight under conditions corresponding to those applying for the industrially produced meals.

The meal samples were ashed in a muffle oven at 450 C for approx. 12 h. The ashes were afterwards dissolved in 5 ml of 2 N-HCl. About $\frac{1}{2}$ g of ash was used. The solution was then diluted to 25 ml.

The lipids present in the industrially produced meals were extracted using a chloroform/methanol mixture (v/v, 2:1). About 4 parts of water were added to 1 part of meal before the extraction and the ratio between the meal-water phase and the extracting solution was 1 to 2. The liquid was then filtered and the insoluble solid phase washed once with chloroform/methanol (v/v, 2:1). This solution which was added to the extraction solution containing the bulk of lipids. The chloroform phase was then separated off and the chloroform evaporated. The methanol–water phase was mixed

[a] Norwegian Herring Oil and Meal Industry Research Institute.

together with the solid phase. This mixture was then dried to constant weight. The oil phase was washed twice with distilled water. The total amount of lipids was extracted from the homogenised raw material used for the production of meal in the laboratory. The lipids were extracted, separated and washed as the lipids extracted from the industrially produced meal.

2.3. Analysis

The analysis of the trace elements, selenium, arsenic, bromine, zinc, iron and cobalt, in the lipids extracted from the meal, was performed by use of neutron activation. The phosphorus content of the lipid phase was also analysed by this method. For the analysis, samples of from 0.5 to 1 ml were used. The selenium present in defatted meal was analysed by the same method, using samples of about 0.5 g. The oil and meal samples to be analysed were sealed in quartz ampoules and irradiated in a nuclear reactor at a flux of $5 \cdot 10^{12}$ nm/cm^2 s for 18 h together with standards of the elements to be analysed. The method has been described in more detail elsewhere.[8] After irradiation the samples were transferred to inactive glass vials and registered, without any prior chemical treatment, using a 3 · 3″ sodium iodide well type crystal detector coupled to a multichannel gamma ray spectrometer. The registration of the activity of bromine and arsenic took place 3 to 5 days after the irradiation while that of selenium, zinc and cobalt was carried out about 1.5 months after the irradiation. The radioactive phosphorus isotope P-32 is a pure β-emitter and was here analysed by means of the "bremstrahlung" spectrum. The sum peak of 1.17 MeV and 1.33 MeV at 2.50 MeV was used for the analyses of cobalt.

The iron content of the lipid phase was analysed by atomic absorption spectrophotometry (Perkin–Elmer Model 303). The samples were dissolved in xylene and diluted to 15% before the measurements.

Because of the relatively high zinc content in the meal, the solutions produced by the hydrochloric acid treatment of the ashed meal had to be further diluted by a factor of 50 to 100. These solutions could then be measured directly. A deuterium background corrector (Perkin–Elmer) was used to avoid scattering effects. The detection of cadmium, lead and copper was achieved by first extracting them into an isobutyl ketone phase using ammoniumpyrrolidinedithiocarbamate as a complexing agent. This solution was then used for the analysis. Standards were prepared in the same way. The use of such a method increases the overall sensitivity and at the same time enables one to avoid the disturbance arising from salts to be found in the dissolved ash.

The iron content in the meal was analysed using X-ray fluorescence (Philips, 1540). The dried meal was carefully pulverised in an agate mortar, 10% wax (Hoechst) was added as a binder and tablets were pressed and analysed without any further treatment. Standards with known amounts of iron added were prepared in the same way.

In order to check the ashing process and the analysis of cadmium in the meal samples, ten selected samples were analysed by neutron activation. Here the radioactive cadmium was isolated radiochemically before registering the activity on a multichannel gamma ray spectrometer.

Some of the industrially produced fishmeals have been analysed for iron and zinc before.[2] These results are marked with an asterisk in this study.

3. Results and comments

The results of the analyses are given in Tables 1 and 2. When comparing the trace element contents in the industrially produced and the laboratory produced meals it is demonstrated that for iron and zinc in particular and partly also for cadmium and lead, the values found for the industrially produced meals are higher. This is probably due to contamination occurring in the production process. Differences in uptake of these elements at the various localities may, however, also play a part in this connection.

Selenium was detected in the lipid phase extracted from both the industrially and the laboratory produced meals. This indicates that the lipid-soluble seleno organic compounds observed in marine oils are relatively stable and that they do not decompose during the industrial production of fish meal. Most of the selenium present in fish meal is localised in the protein phase, where at least some of it is probably present in the form of seleno amino acids. It is, however, important to be aware that a certain amount of the selenium is present in the lipid phase. In this latter case these selenium compounds will certainly have other absorption properties than the selenium present as seleno amino acids in the proteins, when the fish meal is used as a component in domestic animal feed compositions. The results indicate further that the selenium content in mackerel, especially in the lipid phase, seems to be higher than in the other species of fish studied.

The results of the cadmium analyses show a relatively large variation. On the basis of the higher zinc content observed in the industrially produced meal, a corresponding increase in the cadmium content should be postulated since these elements normally will follow each other.

Few data on the natural cadmium content of seafish are available. The results obtained in the present work indicate, however, the same level of cadmium as reported elsewhere.[6] The concentration in mackerel meal seems to be somewhat higher than in meals produced from other fish. If the ratio of zinc to cadmium occurring in seawater is taken to be 100 to 1,[9] then it appears that this ratio has increased noticeably in most of the fish material analysed where values of 100 to 1000 are observed. In the case of the industrially produced fish meals, it must be assumed that any zinc–cadmium contamination which may occur during the production process will mask and reduce the zinc–cadmium ratio originally present in the untreated raw fish material. It should therefore be concluded that based on the results obtained in this investigation the zinc–cadmium ratio in marine fish seems to increase compared with the values observed in seawater.

No significant increase of lead or copper seems to occur during the industrial production process. Only a few of the meals gave values which could be due to accidental contamination occurring during the production process. Otherwise the values obtained agree with the values reported earlier.[1,6,10]

The relatively large variation in the phosphorus content of the lipid samples extracted from the fish meals is interesting and suggests that a high percentage of the phospholipids decompose either during the storage of the raw material prior to the production or during the production process itself, or during the storage of the meals after production is completed.

TABLE 1. Trace element (parts/million) in laboratory produced fishmeal and in the lipid phase of the meal

Sample	Locality	Date of collection	Yield of lipids	Lipids					Meal						
				As	Br	Se	Zn	Fe	P	Cd	Zn	Pb	Cu	Fe	Se[a]
Herring	Shetland	17 August 1971	13.6	3.1	15.7	0.72	3.97	4.7	82	0.05	62	0.48	4.60	55	1.37
Herring	Shetland	18 July 1971	24.0	4.5	3.7	0.59	1.69	0.62	108	0.15	45	0.32	3.40	64	1.37
Mackerel	Shetland	2 August 1971	32.8	4.3	6.1	1.2	1.84	5.7	99	0.29	38	0.23	3.50	62	1.46
Mackerel	Shetland	19 August 1971	25.3	5.5	5.6	1.3	1.52	3.0	93	0.51	59	0.47	4.24	62	2.38
Capelin	Finnmark	17 February 1971	27.7	5.2	3.3	0.29	0.54	1.02	547	0.16	58	0.52	4.90	53	0.47
Capelin	Finnmark	2 October 1971	30.0	5.5	5.1	0.39	1.95	2.1	267	0.28	48	0.35	4.95	43	0.68

[a] Defatted meal.

TABLE 2. Trace element content (parts/million) in factory produced fishmeal and in the lipid phase of the meal

Sample	Locality	Date of collection		Yield of oil	Lipids						Meal						
					As	Br	Se	Zn	Fe	Co	P	Cd	Zn	Pb	Cu	Fe	Se[b]
Capelin	Båtsfiord Finnmark	April	1968	8.1	23.2	8.2	0.34	1.4	2.6	0.023	4361	0.08	88	1.1	4.7	111[a]	1.6
Capelin	Norheimsfeltet	March	1968	6.3	19.0	9.0	0.26	2.7	15	< 0.02	7609	0.08	114	1.7	5.6	164[a]	1.3
Capelin	Syltefiord Finnm.	February	1968	4.2	9.2	13.7	0.6	20	36	0.21	2191	0.12	96	1.3	4.8	757[a]	1.2
Capelin	Syltefiord Finnm.	March	1968	10.0	7.3	9.2	0.3	3.9	13.3	0.045	5496	0.15	146	1.3	5.7	440	1.2
Capelin	Tromsø		1970	11.1	5.9	10.8	0.3	4.6	5.3	< 0.02	6006	0.17	150	2.0	3.4	300	0.6
Capelin	Jøvik Troms		1970	9.2	—[c]	—[c]	0.07	1.1	2.8	0.05	8700	0.12	139	1.1	2.6	86	1.0
Capelin	Båtsfiord		1971	12.2	6.9	8.6	0.22	4.0	5.6	0.029	4265	0.18	92	3.6	3.3	160	0.96
Mackerel	North Sea	May	1968	8.3	10.2	16.0	2.9	25	33	< 0.02	4880	0.18	100	3.8	5.2	375	2.8
Mackerel	North Sea	November	1968	5.3	10.1	11.2	3.3	42	67	0.09	7391	0.22	79	2.6	4.3	251[a]	3.9
Mackerel	North Sea	September	1968	6.1	12.2	15.5	2.3	54	47	0.10	2818	0.86	142	1.4	7.1	176[a]	4.2
Mackerel	North Sea	June	1968	6.4	15.0	9.3	1.8	49	48	< 0.02	2305	0.29	76	2.7	5.8	196[a]	2.5
Mackerel	North Sea		1971	2.2	14.0	21.7	2.6	6.2	5.4	0.08	22 310	0.13	94	1.3	4.8	208	3.6
Summer																	
herring	Varanger Finnmark	July	1968	9.7	7.4	11.5	0.9	15.6	29	0.08	3600	0.30	138	1.4	4.9	328	1.9
herring	Persfiord Finnm.	May	1968	6.5	4.8	10.2	1.2	9.3	31	< 0.02	6783	0.13	183	1.3	4.3	248[a]	2.1
herring	Østbanken Finnm.	May	1968	3.3	15.8	11.7	1.1	17	18	< 0.02	6433	0.41	118	1.0	7.3	160[a]	2.1
herring	Jøvik Troms	September	1968	4.6	12.0	9.7	1.0	6.0	8.7	0.06	9266	0.02	90	1.0	6.8	189[a]	1.6
North-Sea																	
herring	Shetland	August	1968	6.2	12.9	12.0	0.9	12.4	14.5	0.038	5042	0.13	93	0.8	7.5	288[a]	2.1
herring	Shetland	June	1967	7.9	7.5	14.4	2.3	21	36	0.20	7520	0.44	147	1.8	13.8	270	4.2
herring	Shetland	June	1968	3.8	16.3	8.1	0.6	15	18	< 0.02	6733	0.18	124	1.4	7.8	167[a]	1.5
herring	North Sea	June	1968	7.6	9.4	18.5	2.6	22	36	< 0.02	4304	0.28	102	1.1	2.9	276[a]	1.7
Winter	Sula, Skolmen,																
herring	Slettingen	February	1967	9.6	4.6	10.0	1.1	8.2	24	0.05	3345	0.34	97	1.3	3.7	155	1.8
herring	Frøyabanken	March	1968	5.8	13.3	8.7	0.5	2.5	7	0.03	6935	0.24	98	2.6	9.5	258[a]	1.9
herring	Frøyabanken	February	1968	7.7	20.2	12.5	0.8	4.0	7	0.027	10 888	0.17	87	0.7	6.9	174[a]	2.0
herring	Frøyabanken	February	1968	8.5	8.8	12.6	1.7	13	15	0.08	5628	0.19	104	2.3	2.3	140[a]	2.1
Norway pout	North Sea	June	1968	5.2	7.0	10.3	0.42	2.1	2.8	0.027	1600	0.17	82	7.5	8.3	757[a]	1.8
Norway pout	North Sea	March	1968	5.2	8.7	9.7	0.46	1.4	4.4	0.025	2936	0.12	65	2.1	4.9	340[a]	1.5
Norway pout	North Sea	June	1968	6.3	11.8	12.5	1.0	3.0	4.6	0.035	4963	0.34	57	1.5	10.4	190	2.3
Anchoveta	Peru		1969	5.6	7.1	17.1	0.8	15	47	0.15	6470	0.96	639	1.3	11.3	222	1.0
Anchoveta	South Africa		1969	4.6	10.7	12.2	0.6	13	63	0.12	7304	0.46	69	1.6	6.7	295	1.2

[a] Values from[2].
[b] Defatted meal.
[c] Not determined.

The content of organically-bound arsenic and bromine is higher in the lipids extracted from the industrially produced meal than in the laboratory produced oil and also compared with earlier results.[11,12] It should here be noted that laboratory produced oil was extracted with chloroform methanol from the whole fish. An eventual accumulation of these compounds in the lipids extracted from the meal e.g. due to absorption, will only have a small effect on the concentration of the arsenic- and bromine-containing compounds in the oil when all oil is extracted in one operation. The lipids present in the industrially produced meal (5 to 10%) contribute only 5 to 20% of the total amount of lipids in the fish.

In addition to the organically bound trace elements, arsenic, bromine and selenium: zinc, iron and cobalt have also been analysed in the lipid phase produced from the meals by extraction. The content of both iron and zinc is relatively high, especially in the lipid samples extracted from mackerel meal and suggests clearly a complexing agent present in the lipid phase. Earlier results[4] suggest that the phospholipids in the lipid phase may be responsible for complexing inorganic ions.

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The Synthesis of Fat and Water Soluble Arseno Organic Compounds in Marine and Limnetic Algae

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Two green algae (*Chlorella ovalis* and *Chlorella pyrenoidosa*), one blue green (*Oscillatoria rubescence*) and two diatoms (*Phaeodactylum tricornutum* and *Skeletonema costatum*) were cultivated in fresh and/or salt water media containing radioactive arsenic ions. The incorporation of arsenic into various lipid and water soluble fractions was studied by chromatographic separation and radioactivity measurements. All the algae were found to synthesise both lipid and water soluble arseno organic compounds. Acid treatment converted different arsenolipids into a water soluble product which seemed to be identical to an arseno organic compound isolated from fat free algal material under mild conditions.

Growth of the two algae, *Phaeodactylum tricornutum* and *Chlorella ovalis*, was not influenced by the presence in the medium of 10 – 30 ppm and 1 – 3 ppm of arsenic salts having a 1:1 ratio of tri to pentavalent arsenic ions. Enrichment of arsenic (as arseno organic compounds) in the algae corresponded to 200 – 3000 times the concentration in the medium. Algae seem to be an important source of the arseno organic compounds found in higher marine organisms.

Earlier work has shown that there is a significant difference in the level of arsenic in marine plants and animals as compared with that found in terrestrial plants and animals.[1,2] It has also been established that in marine animals the arsenic both in the lipid and the non lipid phase is present as arseno organic compounds.[3–5] Other results indicate that at least lipid soluble arseno organic compounds are present in marine algae.[6] Arsenic has not been detected in the lipid phase in either plants or animals of terrestrial origin, indicating that these do not synthesise arseno organic compounds corresponding to those found in marine organisms.[3]

Little is known about the structure and chemistry of the arseno organic compounds present in marine organisms. Earlier results indicate that they are biologically very stable, as they are not broken down to inorganic arsenic when they are taken in orally by mammals.[7] These results also imply that the arseno organic compounds which are found in marine samples are less toxic than inorganic arsenic, especially arsenite.

The presence of both lipid soluble and water soluble arseno organic compounds in fish and other marine animals suggest [6] that marine algae may serve as a source of the arseno organic compounds found in organisms higher in the food chain. Previous results indicate that fish (*Salmo gardneri*) is able to synthesise lipid and water soluble arseno organic compounds from arsenite/arsenate mixed into the feed. The amount of arseno organic compounds present in the fish from this source is, however, shown to be small compared to that derived from lower stages in the marine food chain.[8]

In view of these findings it is of interest to study the uptake of arsenic in algae and to characterise the lipid soluble (and eventually the water soluble) arseno organic compounds synthesised by the algae in more detail. An investigation of this type may be carried out under laboratory conditions by adding radioactive arsenic to algal cultures and subsequently analysing for the radioactive compounds synthesised.

EXPERIMENTAL

Growth experiments. The algae were grown in 2 l spherical flasks at the Norwegian Institute for Water Research as previously described [6,9] in nutrient enriched media [10] based on uncontaminated sea water for *Chlorella ovalis* Butcher, *Phaeodactylum tricornutum* Bohlin and *Skeletonema costatum* (Grev.) Cleve. The green algae *Chlorella pyrenoidosa* Chick as well as *Phaeodactylum tricornutum* and *Oscillatoria rubescens* (D. C.) were grown in a fresh water medium based on distilled water with nutrients added. When the growth of the algae was well under way (approx. one week), half of the algal solution was filtered off each day, and new culture medium was added from the stock solution. The filters were stored in chloroform prior to analysis.

For each culture experiment 10 l of medium were prepared and between 0.01 and 0.1 mCi of arsenic tracer (As-74, AJS.1, Amersham) was added. At the time of addition the radioactive arsenic tracer consisted of a mixture of arsenite and arsenate (3/2).

In a second series of experiments *Chlorella ovalis* and *Phaeodactylum tricornutum* were grown with inactive arsenite/arsenate (1/1) added to the radioactive arsenic in the following amounts: 3, 30, 3×10^2, 10^3, 3×10^3, 10^4, and 3×10^4 μg/l.

To compare arsenite and arsenate in the absorption process, two identical cultures of the alga *Phaeodactylum tricornutum* were started. To one culture was added 30 ppb As^{3+} together with 0.04 mCi As^{3+} tracer, and to the other 30 ppb As^{5+} with the corresponding amount of As^{5+} tracer. Half a liter of the culture was filtered after 1 d, 2 d, 4 d, and 8 d, respectively. The amount of As^{3+} and As^{5+} in the filtered medium, after the experiments were finished, was determined by molecular gel filtration.* Samples were stored at -20°C prior to analysis.

Separation of organic and inorganic arsenic. Lipids were extracted from the algal material with chloroform/methanol/water (4/2/1). Following separation from the aqueous-methanolic phase, the chloroform phase was washed twice with distilled water to which inactive arsenite-arsenate had been added in order to dilute any inorganic radioactive arsenic ions present in the chloroform. The algal material was subsequently boiled in water for 20 min to produce an aqueous extract. The pH of this was adjusted to 7 – 8 by NH_3, and the solution was run through a column of anion exchange resin (Dowex 2 × 8, 200 – 400 mesh) previously equilibrated with 0.2 N HCl and washed with water. Inorganic arsenic ions are adsorbed on the resin under these conditions, while organic bound arsenic is eluted.[5]

Inorganic radioactive arsenic was also removed as volatile $AsCl_3$ by distilling it off from a solution adjusted to 6.6 N HCl. During this treatment the As^{5+} present will be reduced to As^{3+}. $AsCl_3$ will distil at 100°C; to prevent decomposition of arseno organic

* The two valencies of arsenic will be separated on a 1 m column loaded with Sephadex G 25 Fine (Pharmacia Fine Chemicals, Sweden), using 0.05 M NH_3 as eluting agent (unpublished results).

compounds, the temperature should be kept at 100 – 105°C, particularly towards the end of the process. The degree of separation obtained was estimated on thin layer chromatography by autoradiography.

All measurements of radioactivity were performed with a 2 × 2″ NaI "welltype" scintillation detector.

Thin layer chromatography (TLC). Adsorbent layers of about 1 mm thickness were used for the analyses performed by TLC, in order to obtain sufficient amounts of the synthesised radioactive arsenic compounds to allow the autoradiographic measurements. The use of 1 mm adsorbent layers resulted, however, in a reduction of both the separation and reproducibility usually obtained with TLC.

A portion from the aqueous extracts produced by boiling of defatted algal material was chromatographed in a system of chloroform/methanol/ammonia (2/2/1) (system 1) with cellulose powder (MN 300, Macherey, Nagel & Co., Düsen, GFR) as adsorbent.[11] The plate dimension was 20 by 20 cm. Some fractions from separation of the water extracts by molecular gel-filtration were characterised in a two-dimensional TLC procedure, in which the solvent system described above was used in the first direction, and methanol/water/pyridine (10/5/1) in the second (system 2).

The lipids extracted from the algal material were investigated using two different techniques. A sample of 10 – 20 mg of oil was dissolved in chloroform and polar lipids separated from the non-polar ones on a silica gel column (SiO_2, 0.2 – 0.5 mm, for chromatography, E. Merck AG, Darmstadt, West Germany). The non-polar lipids were eluted with chloroform, and the polar lipids subsequently with 90 % methanol in chloroform. As more than 98 % of the radioactivity followed the polar fraction, the latter was characterised further by "Kieselgel" (7731, E. Merck AG, Darmstadt, West Germany) using chloroform/methanol/water (65/25/4) for the development [12] (system 3).

The algal lipids were also analysed directly by TLC in the system used for the water extract (system 1, described above). The lipid soluble arseno organic compounds followed the solvent front. They were detected by autoradiography, scraped out and refluxed in hydrochloric acid (6.6 N) for 2 h. The hydrolysate was concentrated by evaporation and the residue extracted with ethanol. Samples of the ethanol solution were subsequently analysed by TLC on system 1. All plates were sprayed with ninhydrin reagent in order to detect amino acids or other compounds containing amino groups.

To see whether inorganic arsenic ions could be complexed by algal material and move in the TLC systems employed, radioactive arsenite-arsenate was added to inactive aqueous algal extracts and subjected to analysis by TLC in system 1.

Molecular gel-filtration. Aqueous algal extracts were subjected to molecular gel filtration on a dextrane resin (Sephadex G 25 Fine, Pharmacia Fine Chemicals, Sweden). A column with a diameter of 15 mm and a height of 25 cm (K 15/25, Pharmacia Fine Chemicals, Sweden) was used, and absorbance at 254 nm in the eluate monitered by a Uvicord spectrophotometer (LKB, Sweden). A 0.005 N (pH 7 – 8) ammonia solution was used as eluting agent. The eluate was collected in 10 ml fractions and the activity of each was measured. The fraction with the highest radioactivity was analysed by TLC in system 1. Aliquots from each fraction were tested for ninhydrin-positive compounds by application to filter paper, drying and spraying with ninhydrin reagent in the conventional manner.

Autoradiography. Radioactive arsenic compounds separated on TLC were detected by autoradiography. The TLC plates were dried thoroughly and placed in contact with sensitive X-ray emulsion (Ilford Industry G film). Exposure time was 1 – 14 days, depending on the amount of activity present. The film was subsequently developed as ordinary X-ray emulsion.

RESULTS AND DISCUSSION

TLC analyses of the extracts from the defatted algae all show a basic spot with an R_F value of about 0.30 (A, Figs. 1 and 2). The thickness of the adsorbent layer and poor reproducibility in preparing the plates resulted in considerable variations of this R_F value. This spot (A) is present in all the aqueous extracts produced from different algal species investigated. Despite the

Fig. 1. Autoradiograph of TLC plate of aqueous extracts produced from *Phaeodactylum tricornutum* (1) and *Chlorella ovalis* (2). The exposed area (dark) shows the presence of radioactive arseno organic compounds. The main compound is marked A. More detail is shown on the original film. Analytical condition: Cellulose substrate (1 mm); developing solvent, chloroform/methanol/ammonia, 2/2/1 (system 1).

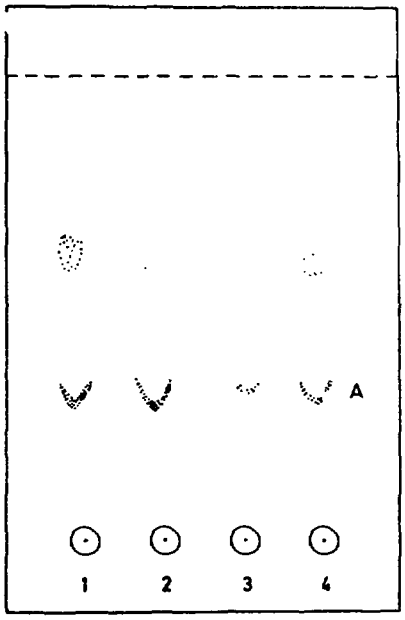

Fig. 2. Reproduction of an autoradiograph of TLC plate of aqueous extracts from *Phaeodactylum tricornutum* cultured in salt water (1) and in fresh water (2), and *Chlorella ovalis* in salt water (3) and *Chlorella pyrenoidosa* in fresh water (4). The main radioactive arseno organic compound present is compound A. Analytical conditions as described in Fig. 1.

lack of a rigid proof, the spot is supposedly caused by a single compound, namely compound A. Its presence is independent of the means used to remove the inorganic radioactive arsenic ions. There are also some weaker and more diffusely exposed areas corresponding to compounds with higher and lower R_F values. This is particularly so for the chromatograms where the aqueous extracts were treated by the ion exchange process. The amount of these compounds, in particular those with an R_F value greater than 0.30, are reduced in extracts subjected to treatment by 6.6 N hydrochloric acid. Compounds with R_F values less than 0.30, which are more polar than A, are present in all the water extracts.

A slight exposure at the spots of application on TLC plates is seen mainly for the extracts treated with hydrochloric acid. This should be compared

with results obtained when radioactive arsenite-arsenate was added to an inactive algal solution, demonstrating that the inorganic arsenic ions are stationary or moving only slightly under the TLC conditions used. Stationary radioactive arsenic compounds in the aqueous algal extract thus probably consist of arsenite-arsenate or other types of arsenic ions.

The ion exchange process is the preferred method for removal of inorganic radioactive arsenic from the aqueous algal extracts. The method is less drastic than the treatment with hydrochloric acid and seems not to cause changes in the chemical status of the arsenic. Some of the arseno organic compounds may, however, be irreversibly absorbed to the ion exchange resin. Changes, or decomposition of arseno organic compounds, may also take place in the preparation of the aqueous extracts, particularly during the boiling step. Formation of compound A is the most likely reaction.

TLC of the aqueous extracts from the algal clones of *Phaeodactylum tricornutum*, cultured both in fresh and salt water, and the two species, *Chlorella ovalis* and *Chlorella pyrenoidosa*, cultured in salt and fresh water, respectively, indicates that the concentration of compounds A is somewhat higher in the algae grown in salt water (Fig. 2).

The radioactivity in the non-polar and in the polar fractions of the algal oil, after these had been separated on the silica gel column, shows that nearly all the radioactivity (> 98 %), *i.e.* all the lipid soluble arseno organic compounds synthesised by the algae, follow the polar fraction. These results are in accordance with previous results reported for the lipid soluble arseno organic compounds present in fish oils.[13,14]

TLC-separation of the polar lipids made visible by autoradiography (Fig. 3) indicates the presence of lipid soluble arseno organic compounds different from those detected in the aqueous extracts. Moreover, the pattern of arsenic containing compounds varies with the various algae studied (*Chlor-*

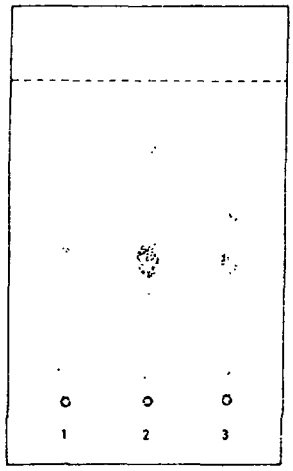

Fig. 3. Reproduction of an autoradiograph of TLC plate of the polar lipids from *Sceletonema costatum* (1). *Phaeodactylum tricornutum* (2) *and Chlorella ovalis* (3). Analytical conditions: Kieselgel G substrate (1 mm); chloroform/methanol/water, 65/25/4 (system 3).

ella ovalis, *Oscillatoria rubescens*, and *Phaeodactylum tricornutum*). A similar difference has been reported for lipid soluble arseno organic compounds in invertebrates.[15] This observation could be of significance, but new lipid soluble arseno organic compounds may be created as a result of the experimental conditions when extracting and fractionating the algal lipids.

TLC of algal lipids in system 1 shows the lipid soluble arseno organic compounds to move with the solvent front, as do the other algal lipids. In some of the samples, however, a well defined spot with an R_F value of *ca.* 0.30 was found. Presumably this is traces of compound A found in the aqueous extract.

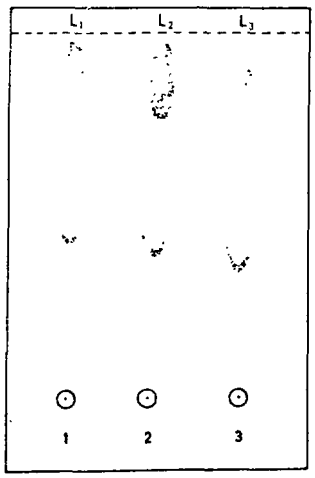

Fig. 4a. Reproduction of autoradiographs of TLC plates of the total amount of lipids extracted from *Oscillatoria rubescens* (1), *Phaeodactylum tricornutum* (2), and *Chlorella ovalis* (3). The lipids (L^{1-3}) will have R_F values close to 1 in system 1.

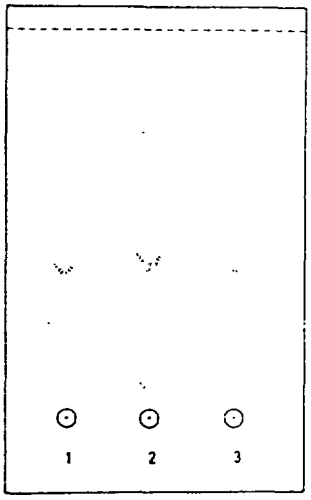

Fig. 4b. The lipids (L^{1-3}) shown in Fig. 4a were scraped out, treated with HCl and extracted with ethanol (see text). These extracts were analysed under the same conditions as described in Fig. 4a (see text).

Scraping out the lipid soluble compounds as detected by autoradiography and refluxing with hydrochloric acid (6.6 N) again produced a well defined spot at R_F 0.30 on repeated TLC-separation in system 1. Apparently compound A from the aqueous extract is also produced from the lipid soluble compounds on treatment with hydrochloric acid. A positive ninhydrin reaction is always associated with this compound. This fact becomes especially relevant when the positive reaction was obtained with compound A prepared from lipid soluble arseno organic compounds isolated by TLC, since this operation will remove most foreign ninhydrin positive material. Two-dimensional TLC of

the most radioactive fraction from the molecular gel-filtration gave coincidence between radioactivity and ninhydrin reaction.

Figs. 5 a-b show how the production of lipid and water soluble arseno organic compounds varies, as the concentration of inorganic arsenic ions is

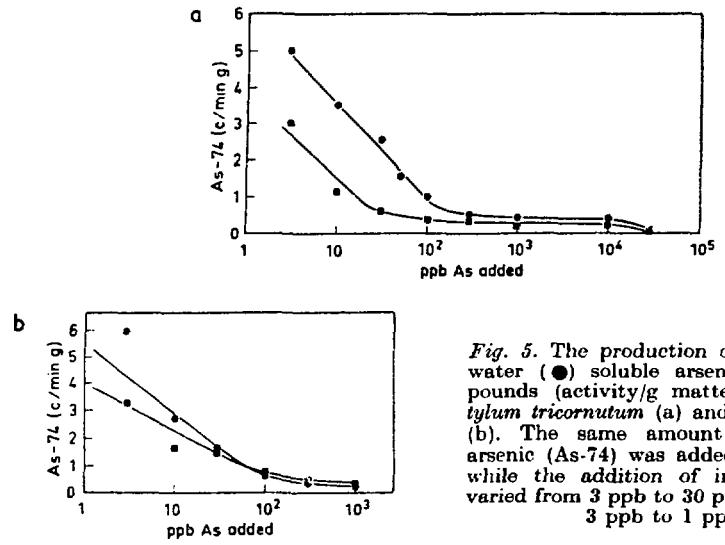

Fig. 5. The production of lipid (■) and water (●) soluble arseno organic compounds (activity/g matter) in *Phaeodactylum tricornutum* (a) and *Chlorella ovalis* (b). The same amount of radioactive arsenic (As-74) was added in each batch while the addition of inorganic arsenic varied from 3 ppb to 30 ppm (a) and from 3 ppb to 1 ppm (b).

increased in the medium under otherwise identical conditions. The ordinate is activity (counts/min) divided by weight of lipids and of dehydrated aqueous extracts, respectively. Both *Phaeodactylum tricornutum* and *Chlorella ovalis* show the same behaviour, which indicates two different patterns of uptake. One applies to media containing up to about 100 ppb of inactive arsenic ions added, and represent an active absorption of the arsenic. The percentage amount of arsenic converted to arseno organic compounds depends on the concentration of arsenic ions in the medium. The other applies to concentrations of arsenic from 100 ppb up to 10 – 30 ppm As for *Phaeodactylum tricornutum* and at least to 1 ppm As for *Chlorella ovalis*, and shows that an approximately constant fraction of the arsenic in the medium is absorbed. In the latter case an equilibrium between the arsenic absorbed and the arsenic present in solution seems to be established up to the concentration where the toxic effect of the arsenic begins to limit the alga's growth (and finally causes its death at around 30 ppm arsenic added (*Phaeodactylum tricornutum*)).

The results of the experiments where arsenite and arsenate were added independently and with radioactive arsenic tracer of the same valency show that more lipid soluble arseno organic compounds are synthesised by the culture with As^{5+} added. This is especially so in the first samples taken after one day. Gel filtration of the media shows that the arsenic in the two solutions

of As^{5+} and As^{3+}, respectively, will tend to reach an equilibrium between As^{3+} and As^{5+}. Although preliminary and not conclusive, the results indicate that the arsenate is used or preferred for the synthesis of arseno organic compounds in the algae.

Some results for the accumulation of arsenic in the algae compared to the concentration of arsenic in the culture media were obtained. The measurement of the radioactivity of the lipid and aqueous phase (counts As/g), produced from the various algae, was compared with corresponding measurements of the radioactivity of the medium. These results are shown in Table 1. They indicate

Table 1. Accumulation of arsenic [a] in algae as arseno organic compounds in the lipid phase and in the aqueous phase, respectively.

	Culturing media			
	Salt water		Fresh water	
	Lipid phase	Aqueous phase	Lipid phase	Aqueous phase
Phaeodactylum tricornutum	2900	2000	2800	1800
Chlorella ovalis	1600	1300		
Chlorella pyrenoidosa			400	190
Oscillatoria rubescens			540	240
Skeletonema costatum	1100	710		

[a] The calculation is based on the ratio between organic bound As-74 in the lipid phase and inorganic As-74 in the medium, and correspondingly in the aqueous phase and in the medium.

an accumulation factor of 250 – 3000 in the algae. It should be noted that the arsenic is somewhat more enriched in the lipid phase. From Figs. 5 a – b it must also be assumed that the accumulation is dependent upon the amount of arsenic present in the culture medium. Here the degree of accumulation will decrease with increasing arsenic concentration in the culture solution up to about 100 ppb (*Phaeodlactylum tricornutum* and *Chlorella ovalis*),

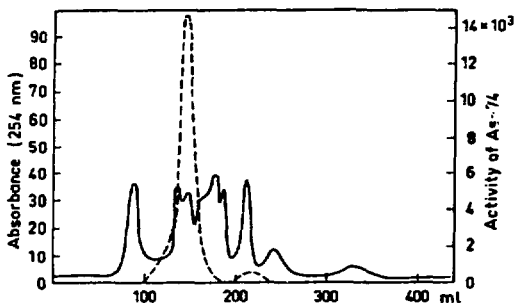

Fig. 6. UV absorbance (–) at 254 nm of molecular gel-filtrated aqueous extract from *Phaeodactylum tricornutum*. The radioactivity (---) of the eluate was measured in fractions of 10 ml.

and then be constant until at least a concentration of about 1 – 10 ppm arsenic in the solution is reached. The accumulation in this region is a factor 15 – 20 below the values found with no inactive arsenic added to the medium.

The results of the molecular gel filtration analysis of the water extracts are presented in Fig. 6. They show that the radioactive arseno organic compounds are eluted in two regions, one of smaller and one of larger molecular weight. The larger lies in the region where the majority of the water soluble arseno organic compounds from fish glue water are eluted. [4] This indicate that the main water soluble organic arsenic compounds in algae and fish are of similar molecular weight. By testing the fractions with ninhydrin reagent it is shown that these compounds are eluted before the amino acids. The two-dimensional TLC analyses of this fraction and subsequent autoradiography of the plate showed that no other radioactive arseno organic compounds could be detected and furthermore that the fraction shows a positive ninhydrin reaction.

CONCLUSIONS

On the basis of the results obtained in this work it is concluded that unicellular algae of limnetic as well as of marine origin are able to synthesise both fat soluble and water soluble arseno organic compounds from inorganic arsenic ions. The lipid soluble compounds are relatively unstable and may by a suitable treatment be converted to a water soluble arseno organic compound which cannot be distinguished from that which is most abundant in aqueous extracts. This main compound is present in all the algal species studied regardless of whether they are cultivated in salt or in fresh water. The results suggest that the arseno organic compounds present in the algae may be one important source for corresponding compounds found in marine organisms at higher stages in the marine food chain.

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THE PRESENCE OF LIPID-SOLUBLE SELENIUM COMPOUNDS IN MARINE OILS

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SUMMARY

A new group of seleno-organic compounds present in marine organisms has been demonstrated. They are lipid-soluble and the main part seems to have a polar character. In the samples analysed the content of selenium is between 0.02 and 4.6 ppm.

INTRODUCTION

Interest in the element selenium and its role in biological systems has steadily grown since 1957 when Schwarz[1] showed that certain selenium compounds could replace vitamin E in curing liver necrosis provoked by vitamin E deficiency in rats. Since then other deficiency diseases in animals due to lack of selenium have been described[2].

The existence of various organic selenium compounds which can be synthesized from inorganic selenium salts, in particular by plants[3,4] and microorganisms[5], is known. It has been shown that some of these compounds, *e.g.* selenomethionine and selenocysteine, are produced by a metabolic pathway analogous to that applying to the corresponding sulphur amino acids (methionine and cysteine). Among unidentified and important compounds are those which were first described by Schwarz[6]. It has been established that the active principle consists of two selenium compounds. It appears to have a higher therapeutic effect and to be less toxic than selenite or any other organic selenium compound investigated, and found to have a therapeutic effect. Various seleno-organic compounds have been synthesized and their biological potential tested. Some of these compounds were shown to be better than sodium selenite[7]. None of the organic selenium compounds which have been discovered in plants and animal organisms have been typically lipid-soluble. Among biotopes marine animals appear to contain more selenium than corresponding samples of terrestrial origin. In fish and other marine organisms selenium contents of approx. 1–4 ppm have been registered[8].

Recent data[9,10] indicate that the selenium in fish is partly present in the lipid phase as organic compounds. This suggests a new group of seleno-organic compounds that are synthesized either by the fish, or by a lower stage in the food chain. The purpose of this investigation is to study these compounds in further detail, particu-

larly to see if they have a polar character and whether there may be contamination from inorganic selenium, *i.e.* selenite. Furthermore, it was of interest to extend the range of samples investigated.

EXPERIMENTAL

Industrially produced marine oils were selected for this investigation along with a series of oils produced in our laboratory. The latter were extracted from homogenized raw material by chloroform–methanol (2 : 1, v/v), or by boiling with water for 20 min in a glass apparatus. After the boiling the oil was separated by centrifugation. To remove inorganic ions all oil samples, including those from industrial sources, were washed twice with distilled water. Previous results[11] have shown that this procedure is very efficient in removing inorganic ions from oils.

To check whether selenite may be sequestered by the oil, the following experiments were carried out. 1 μg of selenite with radioactive selenite (^{75}Se) added, was dissolved in 10 ml of distilled water and mixed with 10 ml of various oil samples produced both by boiling with water, and by extraction with chloroform–methanol. After shaking for 1 h at approx. 40 °C the oil phase was separated from the aqueous phase by centrifugation. The radioactivity in samples from both the oil and the water phase was measured with a NaI well-type crystal.

Experiments were further carried out to determine whether the lipid-soluble organic selenium compound had a polar character. Oil samples (approx. 3 g) produced by boiling were dissolved in 25 ml of chloroform and mixed with activated silica gel

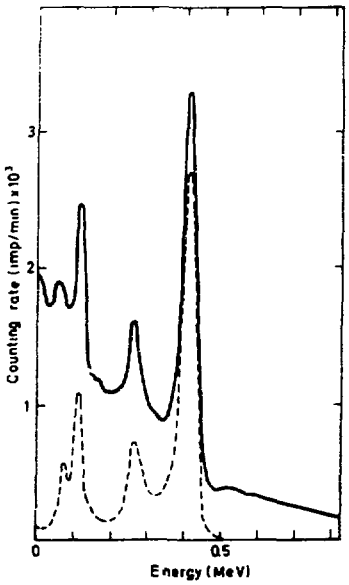

Fig. 1. γ-spectrum of neutron-activated oil extracted from flounder (——) and of the selenium standard (- - -).

(0.2–0.5 mesh, Merck AG, Darmstadt). After shaking for 1 h, the chloroform phase with the neutral lipids was decanted. The silica gel was transferred to a column (height 17 cm, inner diameter 2 cm) and eluted with chloroform until no oil could be detected in the eluate. The polar lipids remaining adsorbed to the silica gel were eluted with 250 ml of a 10 % mixture of methanol in chloroform, and followed by 250 ml of a 50 : 50 mixture of the same solvents. The eluates were collected, and the solvent evaporated.

The analysis of the oil specimens was carried out by neutron activation, as described previously[11]. The samples with selenium standards were irradiated at a neutron flux of $1 \cdot 10^{13}$ $n/cm^2 \cdot s$ for 20 h. Following irradiation, the samples were transferred to inactive glass vials and the induced selenium activity (^{75}Se) in the oils was measured directly after allowing short-lived radioactive isotopes to disintegrate. When activity from ^{76}As and ^{82}Br predominates, it is necessary to wait about 2 weeks before the selenium activity can be measured. With phospholipids present in the oil the isotope ^{32}P with a half life of 14.3 days is produced. It is then necessary to postpone the measurements, depending on the phosphorous content in the sample. This occurs particularly with oils which are produced by chloroform–methanol extraction. Only small amounts of phosphorous were detected in the fractions eluted with 10 % methanol in chloroform. Fig. 1 shows γ-spectra of an oil extracted from flounder and of the selenium standard, respectively, recorded on a 3 inch × 3 inch NaI well-type crystal[12]. The measurement was made 14 days after neutron activation.

RESULTS AND COMMENTS

Results obtained when radioactive selenite was mixed into the oil from an aqueous solution, with subsequent determination of the radioactive selenium present in the oil and aqueous phase, show that less than 1 % selenite is present in the oil phase after separation of the two phases. This experiment and the general experience with the washing procedure demonstrate that the selenium in the marine oils is present as lipid-soluble selenium compounds. This is confirmed by results which indicate that at least some of the lipid-soluble selenium compounds are located to the lipoproteins[9] and by the fact that the selenium in marine oils is not removed by washing with dilute alkali (0.1 M NaOH). Arsenic and phosphorous (as organic and inorganic compounds) are both removed by this procedure[10].

The results of the selenium analysis are given in Table I for both the industrially and the laboratory produced oils. The content of selenium varies from 0.02 to 4.6 ppm. Although relatively few samples have been analysed, the results indicate a higher selenium content in samples produced by extraction with chloroform–methanol. This is in agreement with results published previously[9], where a connection between these compounds and the lipoproteins is established. The great variation in the content of selenium in different oils should be noted. As lipids make up only a small part of most organisms, this large variation contributes little to the total selenium content in the organism.

Although some data on the sulphur content in marine oils are available (10–600 ppm*), it is far below the amounts present in non-lipid protein-rich samples.

* Preliminary results from X-ray fluorescence spectrometry at the author's laboratory.

TABLE I

THE SELENIUM CONTENT (ppm) IN MARINE OILS

Sample		*Method of production*		*Se (ppm)*
Herring (immature)	*Clupea harengus*	Laboratory produced	Boiled with water	0.26
Mackerel (1972)	*Scomber scomber*	Laboratory produced	Boiled with water	0.14
Herring (mature)	*Clupea harengus*	Industrially produced		0.31
Mackerel	*Scomber scomber*	Industrially produced		0.29
Capelin	*Mallotus villosus*	Industrially produced		0.07
Cod liver	*Gadus morrhua*	Industrially produced		0.20
Flounder	*Pleuronectes platessa*	Laboratory produced	Extracted with chloroform/methanol	1.0
Mackerel (1970)	*Scomber scomber*	Laboratory produced	Extracted with chloroform/methanol	0.73
Mackerel liver (1970)	*Scomber scomber*	Laboratory produced	Extracted with chloroform/methanol	1 3
Mackerel (1972)	*Scomber scomber*	Laboratory produced	Extracted with chloroform/methanol	0.60
Herring	*Clupea harengus*	Laboratory produced	Extracted with chloroform/methanol	0.18
Cod liver	*Gadus morrhua*	Laboratory produced	Extracted with chloroform/methanol	0.25
Cod fillet	*Gadus morrhua*	Laboratory produced	Extracted with chloroform/methanol	0.27
Tunny	*Thunnus Thynnus*	Laboratory produced	Extracted with chloroform/methanol	4.6
Halibut	*Hippoglossus hippoglossus*	Laboratory produced	Extracted with chloroform/methanol	0.04
Shrimp	*Pandalus borealis*	Laboratory produced	Extracted with chloroform/methanol	0.12
Mussel	*Mytilus edulis*	Laboratory produced	Extracted with chloroform/methanol	2.0
Squid	*Ommastrephes sagittatus*	Laboratory produced	Extracted with chloroform/methanol	0.02

TABLE II

THE SELENIUM CONTENT IN FRACTIONS OF MARINE OILS SEPARATED ON SILICA GEL COLUMN

Sample	*Eluted with chloroform*	*The selenium content (ppm)*	
		Eluted with 10% methanol in chloroform	*Eluted with 50% methanol in chloroform*
Mackerel oil	0.05	3.2	0.9
Herring oil	0.14	2.3	1.1
Capelin oil	0.08	1.3	0.3
Cod liver oil	0.04	3.9	1.2

Here values of 7000 ppm have been reported[13]. Most of the selenium in the latter samples is probably present as seleno-amino acids and are synthesized along the same routes as the sulphur-amino acids. This suggests thus that the lipid-soluble seleno-organic compounds are either heavily enriched in the lipid phase compared with the analogous sulphur compounds, or that such lipid-soluble sulphur compounds are not formed at all.

Similarly, in Table II the results from the fractionation on silica gel are given. These show that selenium is enriched in the fractions eluted with 10% and 50% of methanol in chloroform. As compounds eluted under these conditions are generally recognized to have a polar character, these selenium compounds are polar in nature. Furthermore, varying degrees of polarity are exhibited, showing that several lipid-soluble selenium compounds must be present.

The consequence of these findings is at present difficult to evaluate. They may, however, throw some new light on the presumed connection between vitamin E and selenium in their metabolic action and on the role of selenium in living organisms in general.

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Separation and Analysis of Organic-bound and Inorganic Arsenic in Marine Organisms

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A method for the separation of stable arseno-organic compounds from inorganic arsenic and the subsequent determination of both forms of arsenic is described. The separation is based on volatilisation of inorganic arsenic as arsenic trichloride by addition of hydrochloric acid to 6.6 N and subsequent removal by distillation at 100 C. Remaining arsenic is bound in arseno-organic compounds that are stable under these conditions.

Total arsenic in the samples analysed is reduced by 0.5 to 3 parts million by this treatment. Determination of arsenic was performed by neutron activation and X-ray fluorescence.

1. Introduction

It has been shown that arsenic is present in marine organisms as both fat-soluble and water-soluble arseno-organic compounds.[1–5] The arsenic content of such samples varies from about 2 to approx. 150 parts/million. Feeding experiments on rats, in which the effect of inorganic arsenic has been compared to that of arsenic present in marine raw materials such as shrimps, indicates that arsenic from marine raw materials is metabolised along lines quite different from those of inorganic arsenic.[6] It has furthermore been shown that only a small amount of the inorganic arsenic present in the feed given to fish (rainbow trout) is converted to arseno-organic compounds and that only very little of the inorganic arsenic absorbed from the water is converted to arseno-organic compounds.[7]

There seems to be general agreement that most of the arsenic present in marine organisms is organically bound and less toxic than inorganic arsenic. To the author's knowledge no definite data are, however, available to indicate in what forms arsenic exists, i.e. whether it is present as stable organic compounds, as inorganic arsenic or as organic compounds easily broken down when taken in by living organisms. It should also be mentioned that release of arsenic with increasing amounts of inorganic arsenic, mostly industrial waste, may upset the normal pattern of absorption of arsenic by plants and animals.

A more thorough investigation of these problems requires an analytical method which enables one to separate and analyse individually the arseno-organic compounds and the inorganic arsenic, respectively, present in marine organisms. In previous studies both lipid- and water-soluble arseno-organic compounds have been analysed[1,4] without, however, determining the amount of inorganic arsenic in the same sample. The aim of this study was to develop a method for separating stable organic-bound from inorganic arsenic in marine organisms, allowing the content of arsenic to be

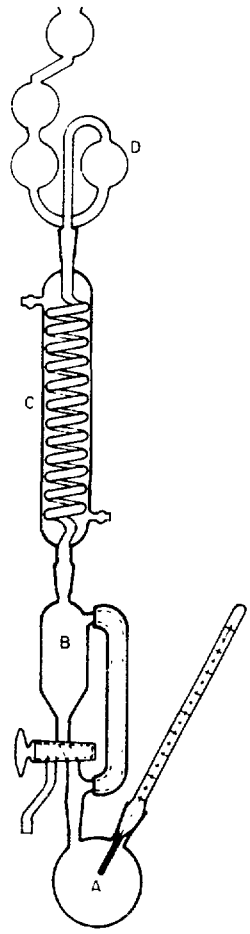

Figure 1. Combined wet combustion-distillation apparatus. A, distillation flask; B, receiver; C, reflux condenser; D, splash head.

determined in each of the two fractions. Furthermore the total amount of arsenic, i.e. the sum of organic bound and inorganic arsenic, should be controlled in the samples to be analysed. Neutron activation and X-ray fluorescence were selected as analytical methods.

It has been shown that the majority of the arseno-organic compounds present in marine raw materials are stable. They will for instance tolerate treatment with 6.6 N-hydrochloric acid at 100 C without or with only negligible decomposition.[8] Under such treatment trivalent inorganic arsenic forms the volatile compound arsenic trichloride with a boiling point of approx. 130 °C. Inorganic arsenic might therefore be removed by distilling it as arsenic trichloride under conditions where the stable arseno-organic compounds are not volatile. For the separation and determination of organic-

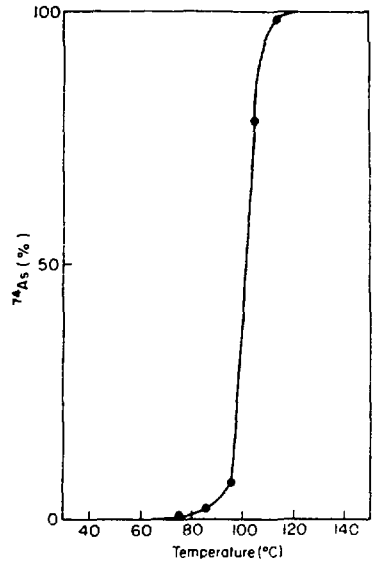

Figure 2. Distillation of radioactive inorganic arsenic as $AsCl_3$, in different temperature intervals.

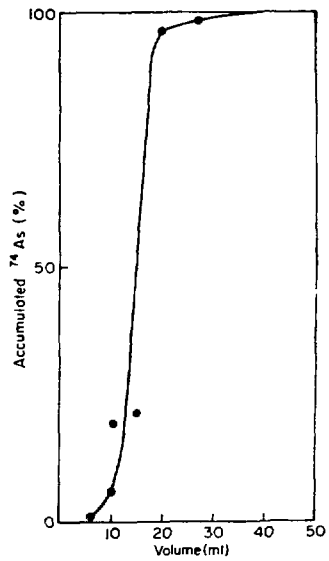

Figure 3. Distillation of radioactive $AsCl_3$ at 100 to 110 C from a 50-ml solution adjusted to 6.6 N in HCl.

bound and inorganic arsenic to be based upon this technique, more information concerning the parameters and quantitative aspects of the distillation must be determined.

2. Preliminary studies

For the distillation experiments an apparatus as shown in Figure 1 was used. This consists of a refluxing and a distillation section and has been described in detail before.[9-11] As sample material for these experiments fish meal and aqueous fish extracts (glue water) were used. Both were produced in the laboratory from fresh homogenised fish. Radioactive pentavalent arsenic (~0.1 μCi) as sodium arsenate (AJS.1, Amersham, England) was added in 10 ml of distilled water. The measurement of radioactive arsenic in the different fractions was carried out using a NaJ well-type scintillation detector. The sample was boiled for 20 min and filtered. Meal and glue water were subsequently evaporated to dryness and dried at 110 °C. Aliquots (3 to 4 g) of this material were transferred to the distillation flask A (Figure 1) and 50 ml of 6.6 N-hydrochloric acid was added. The sample was heated to, and maintained at, temperatures of 70 to 80, 80 to 90, 90 to 100 and 100 to 110 °C, respectively. For each temperature range the distillation was continued for one hour or until 90 to 95% of the solution was transferred to the receiver B. Before each distillation Fe^{2+} (approx 1 g) was added to ensure reduction of all pentavalent arsenic to the trivalent state.

The results are shown in Figure 2, and indicate that the arsenic starts to distil at a temperature of 100 to 110 °C. The results for the meal and the glue water were the same. A second experiment was carried out to investigate the course of the arsenic trichloride distillation at 100 to 110 °C. 50 ml of 6.6 N-hydrochloric acid was added to samples as previously described together with radioactive inorganic pentavalent arsenic. The mixture was heated to the boiling point. The distillation was started after about 20 min of refluxing and fractions of 5 ml were collected. Radioactivity of each fraction was measured. The results of these experiments are shown in Figure 3. It can be seen that by the time about 20 ml, i.e. ~40%, of the hydrochloric acid has distilled over, more than 95% of the radioactive arsenic tracer has been distilled over as well. When adding a chloride containing salt instead of hydrochloric acid or omitting the reducing agent after dissolving the sample, the yield of distilled arsenic is reduced to 40 or 50%.

3. Experimental

3.1. Materials

When selecting samples for the analysis it was considered important to use both processed and fresh raw material and with both a low and a high content of arsenic. The samples were obtained from localities along most of the Norwegian coast and included fresh fish, fish extracts, fish meal (commercially produced), seaweed, mussel and shrimps. They were stored at −20 °C prior to analysis.

3.2. Separation and analysis of arsenic

Samples of about 2 g of dry material were thoroughly ground to a fine powder, weighed and transferred to the distillation flask A (see Figure 1). Distillation of inorganic arsenic was performed as described under the preliminary studies, except that the amount of

hydrochloric acid was reduced to 25 ml and the distillation procedure was repeated once. In cases where the sample existed as an aqueous solution, the distillation was started after about 10 min of refluxing. Dry samples were boiled for about 20 min under the same conditions. When 2/3 of the solution was distilled into the receiver B, this was drained off and a further 25-ml portion of 6.6 N-hydrochloric acid was added to flask A. The distillation process was repeated once. The cooling section and receiver were then rinsed with distilled water: this water and the two distillates were combined and the solution was made up to 100 ml with distilled water. In some samples the two distillates were analysed separately as a check on the distillation.

The organic-bound arsenic was determined by decomposing the undistilled residue which remained in the flask A, after removing the remaining hydrochloric acid by continued distillation. Decomposition was achieved by addition of 5 ml of concentrated sulphuric acid and heating until white fumes of sulphur trioxide were observed. When the mixture had become homogeneous it was cooled and hydrogen peroxide (30%) was carefully added until the dark colour from carbon in the solution disappeared. Inorganic arsenic was subsequently isolated by distillation as above. Hydrobromic acid may also be used as a reducing agent.[10]

The analysis for arsenic in the distilled fractions was carried out by neutron activation. Aliquots (1 ml) of the distillate were sealed into quartz ampoules. These were exposed to a neutron flux of $5 \cdot 10^{12}$ neutrons cm^{-2} s for 2 h together with an arsenic standard. Isolation and registration of the induced radioactive arsenic isotope, ^{76}As, with a half life of 26 h was carried out as previously described.[12]

The total amount of arsenic in the sample was determined both by neutron activation and by X-ray fluorescence. For neutron activation analysis the same procedure as described for undistilled fractions was followed. About 0.2 to 0.5 g of sample (dry weight) was used and the dissolution of the sample after activation was again performed with sulphuric acid and hydrogen peroxide and distillation of arsenic as arsenic trichloride.

Arsenic is one of the elements most suitable for analysis by X-ray fluorescence. The limit of sensitivity for the analysis of this element in biological material is in the region of 2 to 5 parts/million (Philips, model 1540), depending somewhat upon the matrix and upon which other elements are present in the sample. In the preparation of the dry samples for analysis, these were first homogenised and then pressed into tablet form. 10% wax (Hoechst) was added as a binding agent. Solutions were used directly without any pretreatment. Arsenilic acid was used as a standard and gave a linear response for arsenic in the relevant range of concentrations, i.e. from about 5 to about 150 parts/million.

4. Results and comments

The preliminary studies show that the arsenic is quantitatively distilled under the conditions used. This is also confirmed when determining arsenic in repeated distillates from the same sample. Here less than 5% of arsenic was found in the second distillate.

The results of the analyses are presented in Table 1. They show reasonable agreement between the two methods. Besides the direct determination of organic-bound and inorganic arsenic, as shown in the Table, the amount of stable organic-bound arsenic

TABLE 1 Organic bound and inorganic[a] arsenic in marine samples

Sample	Locality	Arsenic (parts/million dry weight) Neutron activation Inorganic	Organic	Total	X-ray fluorescence
Shrimp *Pandalus borealis*	Skagerak	2.5	29.5	30	37
Shrimp *Pandalus borealis*	Oslofiord	1.8	36.5	42	39
Mussel *Mytilus edulis*	Western Norway	1.5	12.4	14	12
Mackerel *Scomber scombrus*	Southern Norway	1.8	4.0	4.7	6
Mackerel *Scomber scombrus*	Southern Norway	1.1	8.9	9.2	5
Haddock *Melanogrammus aeglefinus*	Møre	0.9	12.0	10.8	17
Cod *Gadus morhua*	Møre	1.3	23.0	--	20
Capelin *Mallotus villosus*	Northern Norway	1.3	10.9	6.1	7
Tunny *Thunnus thynnus*	West of Slotterøy	1.2	8.4	—	5
Coalfish *Pollachius virens*	Trøndelag	1.0	7.8	7.2	9
Herring *Clupea harengus*	Western Norway	1.7	3.4	4.2	5
Herring *Clupea harengus*	Western Norway	1.0	5.2	—	7
Herring *Clupea harengus*	Northern Norway	1.3	5.7	—	4
Defatted enzyme hydrolysed cod liver *Gadus morhua*	Lofoten	1.4	53.6	52.6	59
Defatted enzyme hydrolysed cod liver *Gadus morhua*	Northern Norway	3.2	17.9	23	—
Seaweed *Laminaria hyperborea*	Trondheimfiord	0.9	139	142	—
Extract of herring *Clupea harengus*	Western Norway	0.8	28.4	27	29
Extract of coalfish *Pollachius virens*	Western Norway	0.7	35.6	37	37
Herring meal[b] *Clupea harengus*	Western Norway	1.0	5.2	6.5	5
Herring meal *Clupea harengus*	North Sea	1.3	5.7	6.9	4

—, Not determined.
[a] Includes also organic bound arsenic degradeable by 6.6 N-HCl.
[b] Factory produced.

can also be estimated by subtracting the arsenic which can be removed by distillation with hydrochloric acid from the values obtained by the neutron activation or X-ray fluorescence analysis for the total amount of arsenic.

The results indicate a constant level of inorganic arsenic or arseno-organic compounds degradeable to inorganic arsenic by the hydrochloric acid treatment. For all the samples analysed these values were between 0.5 and 3 parts/million. This level seems to be independent of the absolute amount of arsenic present in the marine samples, for fresh raw material, for aqueous extracts produced from this material and for dehydrated material such as fish meal. The hydrochloric acid treatment as described here is a rather drastic procedure so that the figures quoted presumably represent the maximum amount of inorganic and chemically degradeable arseno-organic compounds present in marine organisms. This may also include arseno-organic intermediates from the synthesis of the more stable arseno-organic compounds.

Acknowledgement

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Analysis of Trace Elements, Phosphorus and Sulphur, in the Lipid and the Non-lipid Phase of Halibut (*Hippoglossus hippoglossus*) and Tunny (*Thunnus thynnus*)

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The following elements have been found in the lipid phase of samples of halibut (*Hippoglossus hippoglossus*) and tunny (*Thunnus thynnus*): arsenic, bromine, selenium, zinc, iron, phosphorus and sulphur, and in the non-lipid phase: cadmium, lead, copper, selenium, zinc, iron, arsenic, mercury and sulphur.

Of the results the following should be emphasised: a surprisingly high content of sulphur was observed in the lipid phase suggesting a new group of lipids. The results indicate, furthermore, a rather constant selenium to sulphur ratio in defatted meal while the corresponding ratio in the lipid phase is more variable. No accumulation of cadmium compared with zinc seems to take place.

1. Introduction

There is today considerable interest in the toxic heavy metals in fish and other marine organisms, mostly in connection with environmental problems.[1–5] Besides this aspect of the field, it should be recognised that a number of the trace elements are of importance to the growth and development of living organisms. Increased knowledge of the level and distribution, particularly of the significant trace elements in the marine organisms, is therefore desirable from this point of view.

The level of trace elements in marine organisms may, in addition to variations caused by human endeavour, vary from one location to another depending on natural sources of these elements. The age of the organisms will also be significant with regard to accumulation of these elements, especially for organisms at a high level in the food cycle.

The purpose of this work was both to study the effect which some of the natural conditions have upon the level of trace elements, by selecting samples of a non-stationary fish and of a stationary one caught at different localities, and to get more information about some of the more important trace elements present in fish. Halibut (*Hippoglossus hippoglossus*) was chosen as representative for a relatively stationary species of fish. Old individuals of tunny (*Thunnus thynnus*) were selected as examples of non-stationary fish.

Among aspects of interest which warrants closer examination, is the relation between the levels of selenium and sulphur (both in the lipid and the non-lipid phase). Relatively large selenium contents, about 1 to 3 parts/million, are reported in fish.[6–8] Lipid-soluble selenium compounds have also been found[9, 10] If the latter are synthesised in a manner analogous to that of the sulphur compounds, which have been shown for the sulphur

and seleno amino acids in plants,[11] the selenium–sulphur ratio should be expected to be constant both in the meal and in the lipid phase. Furthermore one may obtain more evidence for the possible connection between methyl-mercury and selenium which has been reported previously for tunny.[12]

Likewise information may be obtained as to whether cadmium is enriched, relative to zinc after passing through several stages in the marine food cycle.

2. Experimental

The samples of tunny were selected from old individuals. They were all caught off the Western coast of Norway and weighed from 200 to 280 kg. All tunny samples consisted of dark muscle tissue. Samples of young and old halibut were obtained from four different localities, namely Danmarksstredet, the Faeroe Islands, East of Greenland and the Skjoldbanken. The weights of these fish are given in Table 1. All samples were frozen and stored at −20 °C prior to analysis.

The received samples were all treated along the following lines. They were homogenised in a Waring blendor and extracted with an equal amount of hexane per homogenised sample for 2 hours at room temperature and under constant stirring. The two phases were separated by centrifugation and the extraction was repeated twice. The same extraction procedure was then repeated using hexane/isopronanol (1 : 1). The two extracts were handled separately in the following processing. The hexane phase was separated from the isopropanol/water mixture by addition of more water and the latter mixture was transferred back to the extracted tissue. The hexane was evaporated and the lipids washed twice with distilled water.

The defatted samples were boiled in a glass apparatus for 20 minutes. Some distilled water was added prior to boiling. The samples were filtered and washed twice with distilled water. Both the insoluble phase (the meal) and the water-soluble part (the aqueous phase) were dried at 90 to 100 °C for 6 h and weighed. Samples (2 to 10 g depending on the ash content) were ashed at 450 °C for 8 h. The ash was dissolved in 2 N-hydrochloric acid and in conc. hydrochloric acid, respectively. The solutions were diluted with distilled water to 25 ml.

The determination of zinc, iron, selenium, copper, lead and cadmium in the meal and in the dehydrated aqueous phase was carried out as reported elsewhere,[13] using atomic absorption spectrophotometry and X-ray fluorescence techniques for zinc, cadmium, lead, copper, arsenic and iron and neutron activation for the analysis of selenium. The zinc content was also analysed by the latter technique. Mercury was determined together with the selenium in the meal by non-destructive activation analysis (Figures 1 and 2). In the aqueous phase the mercury was analysed in some samples by flameless atomic absorption spectrophotometry. During the ashing process the iron may form oxides which are not easily dissolved in 2 N-hydrochloric acid. Hence the solution produced by treating the ash with conc. hydrochloric acid was analysed for iron.

In the two lipid phases from each sample of fish, arsenic, bromine, selenium, zinc and phosphorus were determined by neutron activation,[13] while iron was determined by atomic absorption spectrophotometry[13] and sulphur by X-ray fluorescence. In the latter case dimethylsulphoxide was used as a standard and refined olive oil as matrix

TABLE 1. Yield of lipids, meal and dehydrated aqueous phase from tunny (*Thunnus thynnus*) and halibut (*Hippoglossus hippoglossus*) muscle

No.	Sample	Weight (kg)	Locality	Date of collection	Total lipids (%)	Meal[a] Yield (%)	Meal[a] Ash (%)	Aqueous phase[a] Yield (%)	Aqueous phase[a] Ash (%)
1	Tunny	280	West of Telavåg	2.8.1971	0.5	31.6	2.8	2.0	16.7
2	Tunny	265	West of Telavåg	3.8.1971	1.6	20.4	2.5	4.0	17.0
3	Tunny	193	West of Bremnes	14.8.1971	0.7	23.0	6.0	3.7	16.5
4	Tunny	267	West of Slotterøy	18.8.1971	42.8	11.0	3.2	1.5	12.3
5	Tunny	280	West of Slotterøy	18.8.1971	10.1	22.2	3.9	3.9	16.6
6	Tunny	237	West of Bremnes	19.8.1971	22.3	18.0	7.5	2.1	16.7
7	Tunny	250	West of Bremnes	12.8.1971	6.6	20.4	3.5	2.8	13.0
8	Halibut	78.5	Denmark Strait	1.4.1971	5.7	17.4	6.4	3.4	19.7
9	Halibut	19.5	Denmark Strait	1.4.1971	4.7	13.5	4.9	2.7	19.3
10	Halibut	39	Faeroe Islands	1.7.1971	12.5	11.5	5.6	4.7	27.1
11	Halibut	19	Faeroe Islands	1.7.1971	7.4	19.0	5.8	2.1	14.2
12	Halibut	76	East of Greenland	1.4.1971	9.1	14.8	2.3	2.5	29.5
13	Halibut	28.5	East of Greenland	1.4.1971	10.4	17.8	3.9	2.5	17.7
14	Halibut	47	Skjoldbanken, Helgeland	6.9.1961	9.3	16.0	9.1	3.4	13.2
15	Halibut	18	Skjoldbanken, Helgeland	6.9.1971	8.3	15.4	4.8	2.7	27.3

[a] Defatted, dehydrated.

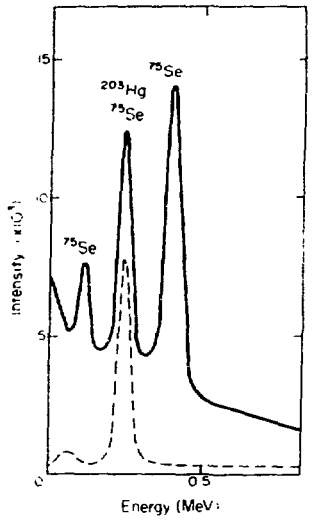

Figure 1. Gamma-spectra of neutron activated tunny meal (— -) produced from sample 5 and a mercury standard (- -) recorded ~3 months after activation.

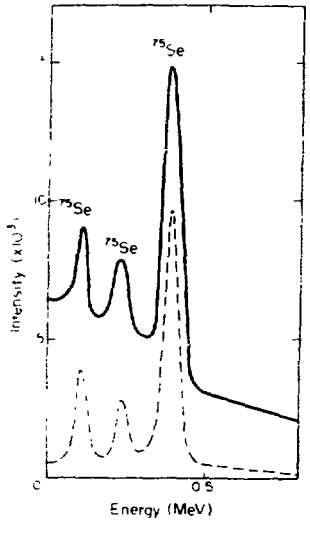

Figure 2. Gamma-spectra of neutron activated dehydrated aqueous phase (— -) produced from sample 5 and selenium standard (- - - -) recorded ~3 months after activation.

for the standard for the determination of sulphur in the lipid phase and sodium sulphate in distilled water as standard for the determination of sulphur in the aqueous phase

3. Results and comments

The yield of defatted, soluble and insoluble materials after dehydration at 90 to 100 °C, together with ash contents and the yield of total lipids, are given in Table 1. These values are rather constant for the halibut samples, demonstrating relatively similar living conditions both for young and old individuals. For the tunny a high variation is observed. This is especially the case for the content of lipids, which varied from approximately 1 to 2 % up to more than 40 %. This also influences the yield of defatted insoluble and soluble material in these samples. When the yield of dry material from the aqueous phase and the meal is considered in view of the fat content of some of the tunny, particularly samples 2 and 6, these values are more in accordance with the rest.

The results of the trace element analysis of the meal and the dehydrated aqueous phase are given in Table 2. They show that the tunny which cover a great area and occupy positions near the top of the marine food chain show large variations in the content of the different trace elements analysed. This again reflects a considerable variation in their type and amount of prey and also that the different localities may give a different exposure to trace elements. These variations are probably natural in origin, rather than caused by pollution. The halibut samples taken from the various localities did not show such a great difference in the content of trace elements analysed for. Nor could any particular difference between young and old individuals be observed.

The content of the heavy metals lead, copper, zinc and cadmium seems on average to be within the same range as formerly reported.[12, 13] The values for iron found in halibut, are, however, remarkably low.

Special concern has been focused on cadmium to see if this element is enriched in the food chain as compared to zinc. It has been shown previously[13] that the cadmium : zinc ratio in fish is lower than that reported for seawater, namely 1 : 100.[14] The results obtained in this study are in agreement with these findings, see Table 4. It should be noted, though, that due to uncertainties connected with the cadmium and zinc determinations in seawater it is, at present, difficult to draw any definite conclusion on this matter. On the assumption that the cadmium : zinc ratio in seawater lies between 1 and $2 \quad 10^{-3}$, the results indicate some enrichment of zinc compared to cadmium.

The fractionation between the meal and the aqueous phase of the defatted samples show that the copper content is higher in the aqueous phase for both tunny and halibut and that the content of zinc and cadmium is higher in the meal. This is in accordance with previously reported results.[7]

The selenium content of the different samples varies from a level which should be characterised as normal for marine fish, i.e. 1.5 to 3.5 parts/million and up to very high levels for some samples (tunny samples 2 and 7). The values found here lie far beyond what is normally regarded as the toxic level for selenium in mammals. Since the sulphur content in all meals is rather constant, the results indicate that there should exist selenium compounds which are created independently of the sulphur compounds, i.e. not as analogous to the sulphur amino acids, see Table 4. Peculiar to these high

TABLE 2. The contents of trace elements and sulphur (parts million) in the meal and dehydrated aqueous phase of tunny and halibut

Sample no.	Meal									Dehydrated aqueous phase								
	Cd	Pb	Cu	Se	Zn	Fe	As	Hg	S	Cd	Pb	Cu	Se	Zn	Fe	As	Hg	S
1	0.05	0.30	0.41	2.8	18	52	4	5.1	5800	0.07	0.61	5.0	1.8	22	310	7.3	0.53	550
2	0.14	0.26	2.9	10.3	42	190	6	8.8	7200	0.07	0.88	2.7	62	30	166	21.1	0.55	1500
3	0.10	0.32	0.25	2.7	27	370	5	3.7	7000	0.05	0.58	4.4	10.3	26	290	21.8	0.55	5440
4	0.27	1.7	0.76	3.3	189	75	4	3.7	8300	0.05	0.59	3.6	3.3	77	110	6.7	0.18	900
5	0.08	0.17	0.08	2.6	23	35	<3	3.0	7400	0.09	1.13	1.6	5.0	36	120	7.6	0.19	550
6	0.16	1.2	1.4	3.8	76	90	5	5.9	8900	0.12	0.90	13	1.5	12	—	—	—	—
7	0.35	0.47	5.3	33	94	340	7	6.8	5700	0.10	0.69	13	70	25	540	23	0.86	3800
8	0.02	0.45	1.8	2.1	29	<15	5	1.3	8100	0.01	0.72	1.7	1.1	6.0	60	93	0.10	1280
9	0.16	1.26	2.2	2.5	35	25	<3	7.0	8000	0.03	0.59	1.1	1.1	4.2	<20	37	0.06	1570
10	0.06	1.9	0.57	2.5	64	15	5	5.7	9800	0.08	0.39	8.4	1.8	6.6	18.0	171	—	560
11	0.07	0.34	1.2	1.4	29	15	10	1.2	8400	0.04	1.71	3.5	2.2	10.9	<20	235	—	1100
12	0.05	0.29	0.40	2.0	25	15	<3	3.7	8700	0.05	0.38	2.2	3.4	5.9	13.5	5.4	—	1170
13	0.03	0.33	0.90	1.6	20	14	8	0.9	9300	0.04	0.43	2.6	2.1	3.7	11.6	186	0.06	850
14	0.06	0.78	4.0	1.7	27	27	9	6.2	9600	0.03	0.59	1.4	2.1	5.8	20	312	0.19	1340
15	0.04	0.60	2.7	1.9	22	<15	5	4.3	7800	0.03	0.18	2.0	2.1	4.6	22.2	24	0.07	1150

—, Not determined.

selenium concentrations is also the observed enrichment of the element in the aqueous phase. This is in agreement with earlier results.[7]

When boiling fresh fish it has been shown that the arsenic is enriched in the aqueous phase.[6] These results are confirmed in the present investigation. Especially in the halibut samples a very high content of arsenic is found in the aqueous phase.

About 1 month after neutron activation, it was possible to measure the radioactive mercury isotope ^{203}Hg together with the radioactive selenium isotope ^{75}Se (see Figures 1 and 2). The γ-spectra of all samples show the same tendency of the mercury content to be reduced in the dehydrated aqueous phase compared with the content in meal produced from the same sample. As indicated by Table 2, where some samples of the aqueous phases have been analysed separately by flameless atomic absorption spectrophotometry, this effect is especially pronounced for the halibut samples. This effect has also previously been observed for mackerel and herring.[7] It is difficult to say whether the mercury, which presumably is mainly present as methyl-mercury,[5] is released from the combination with proteins in the aqueous phase and evaporates; or whether the methyl-mercury is mostly localised proteins or compounds which are insoluble in water when treating the raw material as described in this investigation.

The results of the trace element determinations in the lipids extracted with hexane and with hexane/isopropanol, respectively, are shown in Table 3. The purpose of dividing the extraction of the lipids into two stages was to obtain some fractionation between the bound and the unbound lipids in the tissue. The concentration of phosphorus, (i.e. the phospholipids), in the two fractions indicates that such a fractionation is obtained, although some of the hexane extracts contain a relatively high amount of phospholipids. The different steps of sample handling prior to extraction may have some influence on this fractionation.

It has been shown previously that the presence of metals such as zinc, copper and cobalt in the oil phase seems to be correlated with the simultaneous occurrence of phospholipids.[13] This was also observed in the present investigation, iron and zinc were found to be present in the lipid phase in both the hexane extracts and the hexane/isopropanol extracts where phosphorus also was observed. This was particularly so in the case of tunny. It should, however, be recognised that the iron and zinc levels in the oil will be dependent upon the content of iron and zinc which is available in the fish for this reaction.

The results show furthermore than the fat-soluble arseno and bromo organic compounds are to a certain extent concentrated in the hexane/isopropanol extract. This means that some of these compounds are probably more strongly bound to the tissue than others. This is most evident in samples from fish with a high content of oil but the tendency also prevails in the other samples. The amounts of these compounds associated with the tissue is, however, rather small compared with the total amount of these compounds present in the oil.

The results of the selenium determinations show an enrichment in the lipids extracted with hexane/isopropanol which is in accordance with earlier findings.[10] Except for two samples 2 and 7, the level of selenium in tunny is of the same order as has been reported,[9] while the level in the lipid phase from halibut is lower. The great variations of the selenium content in the lipid phase compared with the meal phase should be noted. In

TABLE 3. The content of trace elements, phosphorus and sulphur (parts/million) in the lipid phase of tunny and halibut

Sample no.	Hexane extract								Hexane/isopropanol extract							
	Yield of lipids (%)	As	Br	Se	Zn	Fe	P	S	Yield of lipids (%)	As	Br	Se	Zn	Fe	P	S
1	0.2	1.2	38	0.96	7.8	29.7	9300	—	0.3	7.4	20.1	0.97	2.9	3.0	14200	—
2	1.1	5.0	5.3	3.4	5.8	33.0	970	88	0.5	7.8	13.0	5.9	4.6	56	5400	—
3	0.4	4.0	3.5	0.72	4.5	12.6	1480	—	0.3	5.6	10.9	0.61	3.1	15	7400	—
4	42.2	2.6	1.8	0.05	0.12	—	13	10	0.6	9.8	7.0	0.30	6.6	10.2	3300	131
5	8.4	3.2	3.9	0.07	0.66	—	98	85	1.7	6.2	4.2	0.28	1.4	2.1	2020	1180
6	21.2	3.6	2.0	0.19	1.4	4.5	169	600	1.1	12	7.8	0.85	4.9	4.3	3200	3525
7	5.1	5.8	5.4	7.6	4.7	42.8	1230	900	1.5	19	12.5	13.9	6.0	12.0	9000	16400
8	4.0	2.4	4.2	0.03	0.22	0.3	117	—	1.7	4.8	10.7	0.066	7.3	0.74	2810	2530
9	3.7	1.6	3.5	0.06	0.26	0.3	167	950	1.0	4.4	9.6	0.20	0.4	—	3550	14500
10	9.0	2.2	3.2	0.036	0.1	0.3	41	240	3.5	1.8	4.8	0.040	0.42	—	660	95
11	5.3	2.2	9.1	0.02	0.1	0.3	10	—	2.1	4.6	9.9	0.030	0.21	—	77	990
12	6.7	4.2	5.6	0.032	0.25	0.3	71	440	2.4	6.2	7.0	0.035	0.44	1.5	490	560
13	8.1	2.4	6.5	0.025	0.1	0.3	10	40	2.3	4.0	7.7	0.031	0.50	—	600	155
14	8.4	5.4	4.6	0.03	0.1	0.3	344	60	0.9	9.8	12.4	0.090	4.0	12	3900	—
15	7.0	3.6	2.5	0.043	0.1	0.3	148	780	1.3	4.8	4.7	0.010	0.48	—	3500	6990

—, Not determined.

order to get some more information about the nature of the organic selenium compounds, the sulphur : selenium ratio was calculated both in the lipid and in the non-lipid phase, see Table 4. These results show a good correlation between sulphur and selenium in the different samples of meal, where the sulphur : selenium ratio appears to be rather constant, Se : S ~ 1.5 to ~4.0 × 10^{-4}. No such agreement is observed for the sulphur : selenium ratio in the lipid phase. The lipid-soluble selenium compounds seem here to be formed independently of the sulphur organic compounds. The selenium :

TABLE 4. The ratio of Se/S, Se/Hg and Cd/Zn in fractions produced from tunny and halibut

	Lipid phase Se/S		Meal phase			Dehydrated aqueous phase		
Sample no.	Hexane extract ($\times 10^{-3}$)	Hexane/isopropanol extract ($\times 10^{-3}$)	Se/S ($\times 10^{-3}$)	Se/Hg	Cd/Zn (10^{-3})	Se/S ($\times 10^{-3}$)	Se/Hg	Cd/Zn (10^{-3})
1			0.48	0.55	2.8	3.3	3.4	3.1
2	38		1.4	1.17	3.3	41.3	112	2.3
3			0.38	0.73	3.7	1.8	19	1.9
4	5	4.5	0.39	0.86	1.4	3.7	18	0.6
5	0.8	0.47	0.35	0.86	3.5	9.1	26	2.4
6	0.3	0.48	0.42	0.63	2.1			9.6
7	8.4	1.7	5.8	4.8	3.7	18.2	81	4.0
8		0.05	0.26	1.6	0.6	0.9	11	1.6
9	0.06	0.02	0.31	0.36	4.5	0.7	18	7.1
10	0.15	0.83	0.25	0.44	0.9	3.2		12.1
11		0.06	0.16	1.2	2.4	2.0		3.7
12	0.07	0.13	0.22	0.52	2.0	2.9		8.5
13	0.62	0.4	0.17	1.7	1.5	2.5	35	10.8
14	0.5		0.18	0.28	2.2	1.6	11	5.2
15	0.05	0.002	0.25	0.45	1.8	1.8	30	6.5

sulphur ratio in the aqueous phase indicates an enrichment of selenium compared with sulphur.

The high sulphur contents found in both lipid fractions were quite surprising, since to the author's knowledge presence of typical fat-soluble sulphur compounds in such quantities have previously not been observed in marine organisms. At the present time nothing is known about the nature of these compounds. They seem to separate between lipids extracted with hexane and lipids extracted with hexane/isopropanol in a pattern similar to the phospholipids, i.e. they are enriched in the fraction of lipids which are more closely bound to the tissue. The relatively large amounts of sulphur observed may indicate a specific biochemical function.

Lately, interest has been focused on a possible connection between selenium and methyl-mercury in tunny.[12] It is proposed that the selenium acts as a detoxification agent against methyl-mercury and also that the fish has an ability to regulate the uptake of selenium corresponding to the level of methyl-mercury in the fish. In Table 4 is presented the mercury : selenium ratio in tunny and halibut which shows considerable

variations. In view of the rather constant selenium content, see Table 2, the above suggestion that the content of methyl-mercury should influence the uptake of selenium seems, therefore, dubious.

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