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Studies of Extractable Organohalogens in Northern Pink Shrimp, *Pandalus borealis*

by

Christina Sheila Bottaro

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
July 1999

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

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by Christina Sheila Bottaro

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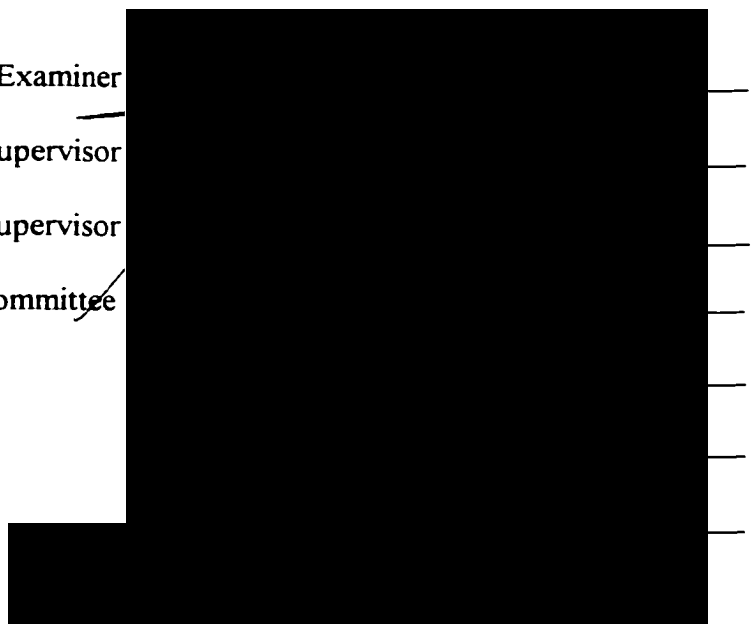

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ABSTRACT

Extractable organohalogens (EOX) are organic compounds that have halogen substituents and can be extracted from a sample using any one of a variety of extraction techniques. Some components of EOX, especially extractable organochlorine (EOCl) have known toxic effects, and have been a source of concern for many years. The literature suggests that up to 95% of the chlorine in EOCl cannot be accounted for as known organochlorine (OCl) compounds. The objective of this thesis has been to study extractable organohalogens in the Northern Pink Shrimp *Pandalus borealis*.

One of the primary aims has been to evaluate various methods for the extraction of EOX from shrimp. This work has indicated that of the solvent systems studied, a mixture of acetone and hexane (1:1) gave the highest relative extraction efficiency for lipid. Other factors investigated included: extractability of EOX using different apparatus, efficiency of washing to remove salt, drying, solvent evaporation, and degradation due to storage. A neutron activation analysis (NAA) method has been developed for the determination of total EOX with detection limits of 32, 2.0 and 0.68 ng mL^{-1} for Cl, Br and I, respectively. A quality assurance program was also applied to the work to ensure that results were reliable.

Shrimp muscle and roe from several areas in the North Atlantic were analyzed for EOX. The EOX levels in shrimp roe from the Coast of Labrador showed an increasing trend from North to South. There was also a strong correlation between the levels of extractable organobromine (EOBr) and extractable organoiodine (EOI) in muscle and roe. This high correlation was not evident for EOCl and EOBr or EOI.

Studies were conducted to characterize EOX. Size exclusion chromatography on extracts from shrimp muscle and roe showed that the distributions of EOX were highly correlated to that of lipid. A strong relationship was found between the distributions of EOBr in different roe extracts and also for EOI in extracts of muscle and roe. Both NMR and MS were used to characterize roe extracts containing high levels of EOCl. The results revealed that the extracts were primarily composed of acylglycerols and fatty acids. The primary chlorinated compound is believed to be a mono-unsaturated fatty acid with a double bond at the n-2 position and a terminal chlorine.

LIST OF ABBREVIATIONS AND SYMBOLS

σ	standard deviation
AOX	adsorbable organohalogen
APcI	atmospheric pressure chemical ionization
ASE	accelerated solvent extraction
B.P.	boiling point
BCF	bioconcentration factor
BDL	below detection limit
BE	Bleidner vapour phase
BHC	see HCH
BKME	bleached kraft pulp mill effluent
Bq	disintegrations s^{-1}
BVE	Bleidner vapour phase extraction
CAD	Canadian Dollars
CHL	chlordane compounds
CNAA	cyclic neutron activation analysis
CRM	certified reference material
d.w.	dry weight
DDD	1,1-dichloro-2-(*o*-chlorophenyl)-2(*p*-chlorophenyl) ethane
DDE	1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene
DDT	dichlorodiphenyl trichloroethane or 1,1,1-trichloro-2,2-bis-(p-chlorophenol) ethane
ΣDDT	DDT, DDD, DDE
DDW	distilled de-ionized water
DFO	Department of Fisheries and Oceans
ECD	electron capture detector
ELCD	electrolytic conductivity detector
EOBr	extractable organobromine
EOCl	extractable organochlorine
EOI	extractable organoiodine

EOX	extractable organohalogen
EPA	Environmental Protection Agency
EPS	Environmental Protection Service
EROD	ethoxyresorufin-*O*-deethylase
eV	electron volts
FDA	Food and Drug Administration
FID	flame ionization detector
FTIR	Fourier transform infrared
GC	gas chromatography
Ge(Li)	lithium drifted germanium
HCH	hexachlorohexane
HOBr	hypobromic acid
HOCl	hypochloric acid
HPLC	high performance liquid chromatography
HRGC	high resolution gas chromatography
INAA	instrumental neutron activation analysis
K-D	Kuderna-Danish evaporator
K_{ow}	partition coefficient
LC	liquid chromatography
L_D	critical detection level
LD_{50}	lethal dose for 50% of a population
MAE	microwave assisted extraction
MFO	mixed function oxidase
MS	mass spectrometry
MSE	methanolic saponification
MW	molecular weight
N/A	not applicable
NAA	neutron activation analysis
ND	no data available
NH_3PICIMS	positive ion ammonia chemical ionization mass spectrometry
NIST	National Institute of Standards and Technology

NMR	nuclear magnetic resonnance
NR	no roe
N_t	number of target nuclide atoms
OBr	organobromine
OCl	organochlorine
OCP	organochlorine pesticides
OI	organoiodine
P. borealis	Pandalus borealis
PAH	polycyclic aromatic hydrocarbons
PAM	Pesticide Analytical Manual
PBB	polybrominated biphenyl
PCB	polychlorinated biphenyl
PCB-SO_2CH_3	PCB-methylsulfone
PCC	polychlorinated camphene
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
pcp	pentachlorophenol
PFE	pressurized fluid extraction
POP	persistent organic pollutants
ppb	parts per billion
ppm	parts per million
QA	quality assurance
QC	quality control
r.b.	round bottom
RSD	relative standard deviation
SD	standard deviation
SDE	steam distillation extraction
SE	Soxhlet extraction
SEC	size exclusion chromatography
SFE	supercritical fluid extraction
SIM	single ion monitoring

slph	standard litres per hour
t_c	length of count
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
t_d	duration of decay
t_i	duration of irradiation
TIC	total ion chromatogram
TLC	thin layer chromatography
TOC	total organic carbon
ton	2 000 pounds
tonne	1 000 kg
TQMP	total quality management plan
US EPA	United States Environmental Protection Agency
USD	United States Dollars
USE	ultrasonic extraction
UV	ultra violet

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1. INTRODUCTION

1.1 Organohalogens and the Environment

The term "organohalogen" refers to any organic compound that possesses one or more fluorine-carbon, chlorine-carbon, bromine-carbon and iodine-carbon bonds. Extractable organohalogens (EOX) are the halogenated organic compounds that can be extracted from samples by any one of a number of methods of liquid/liquid (*e.g.* hexane-water) or liquid/solid (*e.g.* hexane-fish) extraction. As the number of organohalogens that contain fluorine found in natural marine systems are few, they will not be considered in this work. The majority of the current literature cites the determination of specific components of total EOX, mainly in terms of extractable organochlorine (EOCl). The focus of most research is on compounds which have been targeted by the United States Environmental Protection Agency (US EPA) as short-term or long-term toxins. Examples of these include: polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dichlorodiphenyltrichloro-ethane and its metabolites (ΣDDTs), hexachlorocyclohexanes (ΣHCHs), chlordane compounds (ΣCHLs), and various other halogenated pesticides. Extractable organobromines (EOBr) like polybrominated biphenyls (PBBs) are also reported in the literature, but to a lesser extent.

The emphasis on chlorinated compounds is not a mystery. Chlorine is abundant and is used extensively for industrial purposes [1]. Fifty percent of all commercial chemical processes include the use of chlorine and as much as 75 billion pounds of chlorine are used each year by chemical companies such as Dow [2]. There is also

significant non-commercial use of chlorine. Municipalities use chlorine products for disinfecting drinking water and treatment of sewage. The use of chlorine for water processing is extensive. European use has been estimated to be 1 g per person per day and 4 g per person per day for the residents of Halifax-Dartmouth area [3]. Many towns and cities also unintentionally produce organohalogens during waste incineration and other forms of combustion [4].

The abundant production and use of organohalogens is not the only reason there is so much interest; some organohalogens can also be extremely toxic. For example, the most toxic organohalogen known, the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has an LD_{50} of only 0.001 mg kg^{-1} intravenously injected in guinea pigs [5] and 0.022 mg kg^{-1} given orally in male rats [6]. In comparison, the oral LD_{50} for potassium cyanide in rats is 10 mg kg^{-1} and for ethyl alcohol in rats it is 7.06-10.6 g kg^{-1} [6]. Other organohalogen compounds, like DDT, are generally much less toxic (113 mg kg^{-1} orally in rats [6]).

Aside from the acute toxicity, organohalogens present in the environment might be mutagenic or carcinogenic [7, 8]. They may also act synergistically, or have unidentified cumulative effects. It has been suggested [9, 10] that organohalogens may be responsible for reduced egg and larval viability in marine fish, thus reducing commercial potential of stocks. There is also some evidence indicating possible long term toxic effects and accumulation of organohalogens in humans [11, 12].

For these reasons, there is significant pressure from environmental groups and the community at large to ban the use of chlorine and chlorinated products. Clearly, the necessary use of chlorine must be balanced with the need to protect the environment. It

must be determined if the current use can be reduced through new technologies and also if it is actually a threat to the environment. This can only be established through further research.

1.2 Naturally Occurring Organohalogens

Generally, the public perception of organohalogens, especially organochlorines (OCl), has been that they are all manmade, highly toxic, and dangerous to all life. Although many of the organohalogens found in the environment are in large part due to the activities of man, many are also produced in nature. Since the number and widespread presence of natural organohalogens is compelling, they must be considered in any discussion of EOX.

To date more than 2 000 naturally occurring organohalogens have been reported in both marine and terrestrial systems [13]. Although the discovery that organohalogens are created naturally is relatively new, the production is not. Some naturally formed halogenated organic compounds (*e.g.* halogenated fulvic acid) have been found in samples dating back several hundred years [14]. Generally, chlorine and bromine dominate as substituents in these organic compounds. Occasionally iodine is present, but examples of fluorine in naturally produced organohalogens, except those produced by volcanoes, are rare [15].

Many of the natural organohalogens are believed to be produced as a deterrent to predation. Other compounds have been reported to show natural anti-microbial and anti-tumor actions, which benefit the organisms that produce the compounds. Organohalogens may also serve other functions in an organism. For example, the

hormones thyroxine and triiodothyronine are organoiodine compounds that are derivatives of tyrosine. They are produced by the thyroid gland and are involved in the control of metabolism [16]. The functions of natural organohalogens indicate that organohalogens as a group are neither good nor bad. However, they should still be quantified because of their potential adverse effects and to track trends in their levels.

1.3 Analysis for EOX

There are thousands of identified EOX compounds running the gamut in size, polarity, and complexity. They range in size from simple halomethanes to larger, naturally produced compounds that may weigh several hundred daltons. Many compounds are of the same molecular weight and in some cases co-elute in chromatographic columns as is the case with many PCB congeners. Although an overwhelming number of EOX compounds have been detected and characterized, a large percentage of detected EOCl cannot be identified.

Methods like neutron activation analysis (NAA) measure total EOX, *i.e.* all chlorine, bromine and iodine that is extracted in an organic solvent. Most other methods quantify individual components which are summed to arrive at a value for total EOX. When both methods are applied to a sample, the results of summing all known components account for only a small percentage of total EOX as measured by NAA. This approach was applied to the determination of EOCl, and it was found that only ~15% of EOCl compounds in fish, and ~5% of EOCl in sediments can be identified by gas chromatography–mass spectrometry (GC-MS) [17]. On the other hand, Mu *et al.* [18] have reported that 70–90% of EOCl determined in some fish species can be

identified as chlorinated carboxylic acids. Techniques such as GC-MS currently employed for quantification can neither measure nor account for total EOX [17]. It is for this reason, techniques such as NAA must be employed to measure the total amount of EOX.

1.4 Neutron Activation Analysis

Neutron activation analysis was discovered in 1936 by Hevesy and Levi while performing experiments involving rare earth elements and neutron irradiation. It was found that the rare earth elements would become radioactive if irradiated by neutrons. The early work using NAA involved the qualitative determination of elements by their half-lives [19]. In 1944, the first research reactor with high neutron flux was built and served as an excellent source of neutrons for NAA. While other detectors served as an adequate means of detecting γ-rays, the next vital advancement for NAA as an analytical method was the invention of lithium-drifted germanium semiconductor (Ge(Li)) detectors in the early 1960's. Coupled with a multi-channel pulse height analyzer (with as many as 4096 channels), Ge(Li) detectors provided a good efficiency γ-ray detection with excellent energy resolution [20].

The underlying principle of NAA involves the bombardment of a sample with neutrons. If the energy of the neutrons is above the threshold energy of the reaction, then a nuclear reaction occurs. In this way, stable isotopes are made radioactive *i.e.* they become radionuclides. Part of the decay process for many radionuclides includes the emission of gamma (γ) radiation. The energy of the γ-rays is generally unique for the decaying nuclide and the number of events is proportional to the amount of the

radionuclide in the sample. Modern detectors collect the radiative events as counts, and can easily differentiate the energies of the γ-rays. This allows the simultaneous, quantitative determination of many elements.

The activity generated by a given nuclide in a sample can be determined using the activation equation [21]:

$$A_t = N_t \delta \phi (1-e^{-\lambda t_i}) \qquad [1.1]$$

Where: A_t is the induced activity in Bq (disintegrations per second),
N_t is the number of target nuclide atoms (calculated from the mass of element in the sample),
ϕ is the neutron flux (neutrons cm^{-2} s^{-1}),
δ is the cross-section for neutron capture (cm^2),
λ is the decay constant (s^{-1}), and
t_i is the irradiation time in seconds

If one also includes the gamma branching ratio (γ), the detector efficiency (ε), and factors governing the reduction in activity for the decay time (t_d) and the counting time (t_c), then a workable equation relating counts (C) to the number of target nuclides can be derived:

$$C = (Nm\theta/M) \phi \delta \varepsilon \gamma (1-e^{-\lambda t_i})(e^{-\lambda t_d})(1-e^{-\lambda t_c}) \qquad [1.2]$$

In this case N is Avogadro's Number, m is the mass of the element, θ is isotopic abundance and M is the atomic weight of the element.

The factors governing counts detected are numerous and some are difficult to measure; therefore the use of a less complex method is desirable. By using comparator standards, the activation equation can be simplified. In such a case, a known amount of the element of interest is irradiated, allowed to decay and then counted under identical conditions as the sample. The irradiation, decay and counting times are strictly

controlled, as is the counting geometry. The counting geometry is related to the physical size (*e.g.* 500 μL of sample in a 1.2 mL counting vial) and homogeneity of the sample, as well as its position in relation to the detector. When these parameters are kept the same for the sample and the comparator standard, then most terms in the activation equation cancel out (N, θ, M, ϕ, δ, Θ, ε, γ, t_i, t_d and t_c). The resulting equation [1.3] is a simple relationship between the counts and the mass of the element present in the sample and standard:

$$m_{unknown} = \frac{C_{unknown}\, m_{standard}}{C_{standard}} \qquad [1.3]$$

This relationship was used to determine the levels of halogens in the samples throughout this project.

1.5 Unknown Components of EOX

The detection, identification and measurement of the unknown components of EOX are of paramount interest to analytical and environmental chemists, biologists and toxicologists. There is some research to indicate that the unidentified components may be high molecular weight compounds like lipids, while the anthropogenic organohalogens are generally less than 300 daltons. Watanabe *et al.* [22] reported that 60% of the organochlorine found in the adipose tissue of Japanese citizens could be accounted for by known compounds. The remaining 40% of organochlorine was isolated in the fat fraction separated on Florisil® and concluded to be similar to fats. Some of the EOBr and extractable organoiodine (EOI) compounds were also found in the fat fraction [22]. The organobromines isolated in the fat fraction were thought to be brominated fatty

acids although no data were presented [22]. However, reports in the literature suggest that it might be the case. In separate papers, Lunde [23] as well as Tinsley and Lowry [24] reported brominated fatty acids in oils from marine animals. Since the Japanese diet is high in fish, this may be the source of the brominated compounds. Recently, dichloromyristic acid, a chlorinated fatty acid, was identified as a major component of total EOCl in lobster digestive gland, accounting for as much as 20% of the total [25]. The remaining unidentified organohalogens may result from conjugation of known organohalogens to lipid molecules like cholesterol or other large membrane or storage molecules.

A great deal of emphasis has been placed on the separations of organohalogens using gas chromatography (GC), liquid chromatography (LC), thin layer chromatography (TLC), *etc.* These methods are especially useful when one has some idea of the types of compounds present in a given extract. They can also be valuable in determining some physical characteristics of the unknown EOX compounds. For example, size exclusion chromatography coupled with NAA can be used to determine distribution and relative size of chlorinated compounds.

1.6 Extraction of EOX

One of the most important steps in the determination of EOX is likely the extraction method itself. For this reason a great deal of attention has been paid to the extraction methods applied to EOX in a variety of matrices. Much of the literature is related to extraction methods that can be applied to sediments and water. Solid phase extraction is most commonly reported in the literature for the extraction of EOX from

water [26-28]. One of the most commonly employed methods for the extraction of EOX from biological samples and sediments involves the use of Soxhlet extraction (SE) [29-30]. Since SE is time consuming, the Polytron apparatus is a preferable alternative for the extraction of EOX from tissues; it homogenizes and sonicates the sample simultaneously resulting in a better extraction [32, 33]. Microwave-assisted extraction is a promising technique as it can provide a high recovery using a relatively small amount of organic solvent [34, 35]. This is usually applied to the extraction of stable compounds from sediment samples and it may not be appropriate for the determination of the unknown portion of EOX because of the possible decomposition of some compounds. Part of the drive to develop new extraction methods is the need to reduce the quantity of hazardous waste generated through the reduction of the amount of solvents used in analytical and other laboratories. There is also some interest in reducing the time required to complete an extraction with no accompanied reduction in extraction efficiency.

1.7 Selection of the Marine Species for Study

The northern pink shrimp (*Pandalus borealis*) was selected for study in this thesis. There are several reasons for this selection. First, it is a commercially valuable species. The yearly landings of this species are estimated at somewhere near 270 000 tons, 50 000 tons in Canada alone; and in 1997, the retail price demanded for shrimp ranged from USD 3.25 to USD 5.00 per pound [36]. Second, since a commercial shrimp fishery already exists, it was fairly easy to obtain samples needed for this project. Third, populations of this shrimp species can be found throughout the entire North Atlantic

region allowing for studies of any spatial trends that may exist. Fourth, due to its potential toxic effects, the quantification of EOX in shrimp is of interest as it is consumed by human beings. Finally, shrimps live long enough (*viz.* 4-11 years) so that there is a significant potential for bioaccumulation of EOX.

1.8 Summary of Objectives

Most biological samples are inherently heterogeneous, therefore it is necessary to apply methods of sampling and sample preparation that yield results representative of the sample population. It is also important that the homogenization method employed be suitable for use with fatty, biological samples. Additionally, numerous samples for analyses are required and since all the analyses cannot be completed immediately, the resulting mixture needs to be easy to handle, weigh and store.

Since little information is available in the literature comparing the relative extraction efficiencies of various solvents, one of the objectives of this project was to determine the conditions that would result in the highest extraction efficiency of EOX from shrimp. Although the relative extraction efficiencies of a few solvent systems were compared by Martinsen *et al.* [37], more research on the extraction efficiencies of a range of solvent mixtures for EOX in shrimp was considered necessary. Additionally the comparison between the leaching method (*i.e.* solid-liquid extraction with protracted shaking) described by some authors [37, 38] and Polytron assisted solid-liquid extraction was of interest.

Complete removal of any residual halide ions from the organic phase is essential for the accurate measurement of total EOX by most methods. The washing process must

be effective in removing all inorganic halides, but at the same time, it should not cause any loss of organohalogens from the organic phase. The efficiency of the post extraction wash does not seem to have been adequately addressed in the literature. Washing methods have been described which employ distilled water [39], solutions of salts or weak acids [37]. Water washing alone may not be sufficient because it is possible that halogenated fatty acids may occur as sodium or potassium salts which are significantly more soluble in water than the native acids. In addition, if the extract contains polar lipids, some anions in the form of the halides may not be removed by the water wash. Thus, one of the objectives of this project was to develop a washing regime that would consider these factors. Experiments were performed to ensure the efficiency of the washing step. The various methods for removal of water from the organic phase and for reducing its volume were also evaluated here for ease and efficiency.

Neutron activation analysis (NAA) is a useful technique for the simultaneous determination of total amounts of chlorine, bromine and iodine in a sample, and is the method of choice for the analysis of EOX in this thesis project. If the previous steps (extraction, washing, *etc.*) were efficient, then the results produced using NAA should be an accurate reflection of the total amount of organohalogen in a given sample and this can be guaranteed by the use of proper quality assurance (QA) program. Therefore, it was important to include an extensive QA program in this work which consisted of the regular analysis of elemental comparator standards, certified reference materials, and reagents; the use of control charts and procedural blanks to ensure that the overall process was under statistical control.

It appears that there is very little information reported in the literature on organohalogens in shrimp. Therefore, one of the objectives of this project was to use the optimized method to complete a survey of the levels of organohalogens present in *P. borealis* collected from a number of regions in the North Atlantic. From this data, the distribution of EOCl, EOBr and EOI in shrimp tissue and roe could be evaluated. Additionally, it could be determined if any spatial trends for EOX in these compartments existed.

The final goal of this research was to attempt to characterize some of the components in EOX. This was accomplished through the examination of the distribution of EOX based on molecular weights using size exclusion chromatography (SEC). The molecular-weight fractions containing the highest concentrations of EOX were further investigated by ^{1}H, ^{13}C, and ^{31}P nuclear magnetic resonance (NMR) spectroscopy, other modes of separation, and positive and negative ion modes of atmospheric pressure chemical ionization (APCI) MS in an attempt to identify some of the components in these higher molecular weight fractions.

2. LITERATURE SURVEY

The body of literature covering identification, determination, and toxicology of organohalogens is extensive and the sheer number of publications makes it prohibitive to provide an exhaustive discussion of the literature. The intent of this chapter is to report the most relevant references from the available literature in a manner that will give a complete picture of the current state of research in the field related to the objectives of this thesis.

There are many papers on individual organohalogen compounds; however, literature describing research on total extractable organohalogens is limited. The topics discussed below include the types of organohalogens and their sources, toxicology, methods of extraction, methods of analysis, methods of solvent evaporation, and quality assurance. Literature regarding the shrimp species studied in this work is also presented here.

The organically bound halogens that have been the focus of most research are chlorine and bromine, of these, chlorine has been studied most extensively. There is also some minimal interest in organoiodine, but since EOI is usually present in lower concentrations and generally is less toxic, research on this topic is less extensive. There is even less interest in organofluorine. An overview of the major types of compounds that have been identified in the environment is given below.

2.1 Classification of Organohalogens

Many organohalogen compounds have been identified in the environment and these can be classified in many ways. The most obvious way is simply to group them in terms of chlorine, bromine and iodine. This is an appropriate approach, especially when applying NAA as it is only able to identify the total amount of the elements present with no information on their speciation. Another broad method of classification is to group them on the basis of their origin, *i.e.* whether naturally produced or anthropogenic, which is followed in this chapter.

The question of naturally produced organohalogens enters the fray when determining whether organohalogens constitute contamination by man. The naturally occurring compounds can contribute significant amounts of organohalogens to the environment and in some circumstances greater than the amounts from anthropogenic sources [15] and they can also be just as noxious. On the other hand, some natural organohalogens can be beneficial and could not be considered contaminants.

2.1.1 Anthropogenic Organohalogens

In the early 1960's, health concerns arose over the use of chlorinated pesticides when it was reported that DDT was causing high mortality in birds of prey. Public concern was highlighted in popular books like "Silent Spring" by Rachel Carson [40]. Since then, the US EPA and its Canadian counterpart, the Environmental Protection Service (EPS), have targeted a considerable number of compounds as dangerous pollutants. One example of a group of compounds that are of particular interest is dioxins which are highly toxic to certain animals; there is some indication of long term

carcinogenic effects in humans [41]. There have been more than 17 000 reports of possible dioxin linked cancers in humans since the discovery of dioxins in the late 1960's [2].

The number of targeted compounds that have been studied and considered potentially harmful is overwhelming. In the case of PCBs, there are hundreds of possible congeners and of these, only some are toxic. Because PCBs are similar in overall shape to dioxins and their functions as toxins are similar, they are often compared to dioxin. The toxicity and carcinogenicity depend on the position and degree of chlorination: 3,3',4,4',5-pentachlorobiphenyl is one of the most toxic PCBs. Its substitution pattern makes this specific PCB extremely toxic, it is one-tenth as toxic as TCDD, the most toxic dioxin known; other PCBs are not as toxic [42].

2.1.1.1 Types of Anthropogenic Compounds

Many man-made organohalogens have been identified in the environment and most of these are organochlorines. These can be broken down into several significant classes: chlorinated alkanes, chlorinated fatty acids, chlorinated ethers, chlorinated pesticides, chlorinated aromatics, chlorinated heterocyclic aromatics, chlorinated polycyclic aromatics (*e.g.* polychlorinated terphenyls and napthalenes), and polychlorinated biphenyls (PCBs). Brominated and iodinated analogues of the organochlorines fall into similar categories. The main classifications and examples are listed in Table 2.1. It should be noted that most of the evidence for the presence of organoiodine is found through methods of analysis that have detected iodine in nonpolar solvent extracts or by measuring organoiodine adsorbed onto carbon. Few of the specific

organoiodine compounds found in these fractions have been isolated and identified [14, 22, 43].

2.1.1.2 Sources of Anthropogenic Organohalogens

The chemical industry produces vast quantities of chlorinated compounds and billions of pounds of chlorine are consumed each year by the chemical industry [2]. Thirty-five percent goes into making pesticides, herbicides, pharmaceuticals and other chemicals, 28% for plastics, 18% for solvents, 14% in the pulp and paper industry, and 5% for water and waste purification [37]. In Canada alone, the chlorine industry is worth CAD8.5 billion per year, and approximately 85% of all pharmaceuticals and about 95% of pesticides contain chlorine [44].

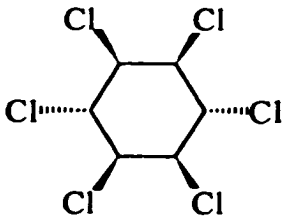
Although water treatment only accounts for 5% of the total use of chlorine there is still concern over the production of harmful organohalogens during the drinking water disinfection and renovation processes. Oyler *et al.* [45] reported some of the possible undesirable compounds formed during the reaction of active chlorine species with polycylic aromatic hydrocarbons (PAHs) such as 9-chlorophenanthrene.

Other important anthropogenic sources of organohalogens are pesticides and herbicides. They have the potential to significantly effect the environment, as they are toxic and can adversely affect not only the targeted organism but also other organisms that may co-exist in its ecosystem. There is significant concern over the effects of pesticides and herbicides on fish (especially fish of commercial value), mammals, and humans. Many of these toxic compounds may persist for a long time and so, may pose

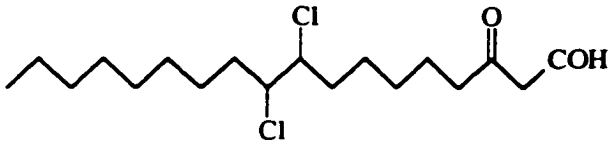
problems long after their input into the environment has ceased. Some of the research concerning these effects will be described in the section on toxicology.

Table 2.1 Anthropogenic Organohalogens

Category	Common Halogenation	Typical Compounds
Halogenated alkanes	Cl, Br, I	chlorinated paraffins [46], halogenated methane, halogenated ethane, Lindane (γ-hexachlorocyclohexane) (Fig. 2.1A)
Halogenated fatty acids	Cl, Br, I	9,10-dichlorostearic acid [47] (Fig. 2.1B)
Halogenated ethers	Cl, Br	brominated phenyl ether [48], chlorinated dibenzofurans, chlorinated dibenzo-*p*-dioxins (Fig. 2.1C)
Halogenated pesticides/ herbicides	Cl, Br	DDT (Fig. 2.1 D), DDE, DDD, Dieldrin (Fig. 2.1E), Chlordane, [49]
Halogenated aromatics	Cl, Br, I	chlorinated phenol, chlorinated guaiacol [50] (Fig. 2.1F)
Halogenated polycyclic aromatics	Cl, Br	chlorinated terphenyls, chlorinated naphthalene, chlorinated phenanthrene
Halogenated biphenyls	Cl, Br	PCBs, PBBs (Fig. 2.1G)
Halogenated heterocyclic compounds	Cl, Br	chlorinated and brominated thiophenes [51] (Fig. 2.1 H)

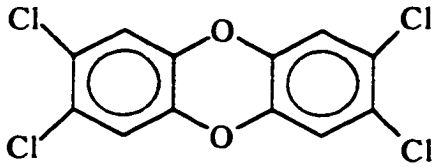


Lindane
(γ-hexachlorocyclohexane)
Fig. 2.1A

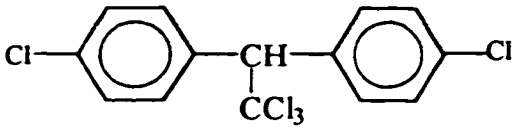


9,10-dichlorostearic acid

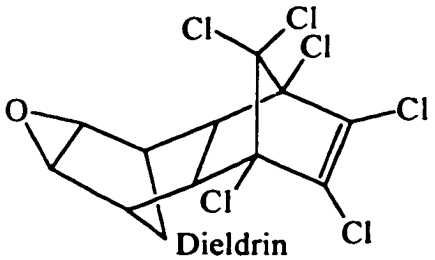
Fig. 2.1B



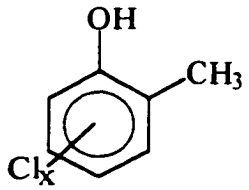
2,3,7,8-tetrachlorodibenzo-*p*-dioxin
Fig. 2.1C



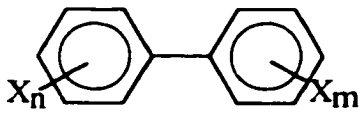
DDT
Fig. 2.1D



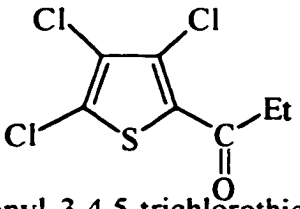
Dieldrin
Fig. 2.1E



chlorinated guaiacol
Fig. 2.1F



polyhalogenated biphenyls
(X= Cl or Br)
PCBs or PBBs
Fig 2.1G



2-propionyl-3,4,5-trichlorothiophene

Fig 2.1H

Fig. 2.1. Examples of Anthropogenic Organohalogens

There are also other more indirect sources of anthropogenic organohalogens and the current literature on anthropogenically produced organohalogens and their sources is summarized in Table 2.2. Biogenic compounds that are formed by *in situ* transformations of anthropogenic compounds, *e.g.* DDT metabolites DDD and DDE; as well as those formed by transformations of anthropogenic compounds in the environment are also considered to be of anthropogenic origin.

2.1.2 Natural Organohalogens

The observation that natural organohalogens constitute a major portion of global organohalogen levels is relatively new. As late as 1990, a researcher commented that "some types of synthetic compounds, including halogenated hydrocarbons such as PCBs, are not found in nature" [52]. An earlier publication in 1968 reported more than 30 naturally produced chlorinated organic compounds and, furthermore, a few natural organohalogens containing bromine, iodine and fluorine were also found [53]. In a 1973 review, Siuda *et al.* [54] reported approximately 200 natural organohalogens from sources including fungi, higher plants and ferns, and marine flora and fauna. To date, nearly 2 400 naturally produced organohalogens have been identified [55], of these at least 1 500 are found in marine organisms [15]. The typical marine sources are red algae, sponges, seaweed, molluscs and microbes. The non-marine compounds are produced in soils (typically by bacteria), terrestrial plants, in fresh water systems, and by volcanic activity. It can be concluded from the literature, that naturally produced halogenated hydrocarbons should be a major consideration in any discussion of organohalogens. The natural organohalogens can be divided into two major categories depending on their

sources: terrestrial (includes fresh water systems) and marine. Of these, the largest source, by far, is the latter.

Table 2.2. Anthropogenic Organohalogens and Their Sources

Identity	Source	Reference(s)
PCDD/PCDF (polychlorinated dibenzo-*p*-dioxin/polychlorinated dibenzofuran)	• by-products in chlorinated hydrocarbon production (e.g. pesticide production)	2, 56, 57
	• incineration of municipal and hazardous waste	2, 56, 57
	• photochemical dimerization of chlorophenol	56
	• compost and sewage sludge	57, 58
	• pulp and paper chlorine bleach process	2, 59
	• peroxidase enzyme-catalyzed oxidation of chlorophenol	56, 60, 61
chlorinated fluorenes and chlorinated alkylfluorenes	• pulp and paper chlorine bleach process	62
brominated aromatic hydrocarbons (brominated biphenyls, biphenyls oxides, benzenes, bromotoluene)	• flame redardant production	49, 63
chlorinated thiophenes	• incineration	4, 64-66
	• car exhaust	64, 65
	• pulp and paper chlorine bleach production	52, 64, 65, 67
chlordane compounds	• wood preservatives	68
chlorinated fatty acids	• pulp mill effluent	69-71
halogenated fatty acids	• water disinfection	72

Table 2.2. Anthropogenic Organohalogens and Their Sources (*contd.*)

Identity	Source	Reference(s)
PCBs (polychlorinated biphenyls)	• Domestic and industrial waste water	73
	• Target compound of manufacturing	74
chlorophenols	• domestic and industrial water waste	50
	• anti-stain agents for wood	75
	• pulp and paper bleaching process	49, 76-78
	• municipal incinerators	50
	• lumber mills	78
	• waste products in electrochemical industry	78
	• solvent and chemical intermediate, heat transfer fluid, flame retardant, and pesticides	69
DDT (1,1,1-trichloro-2,2-bis-(p-chlorophenol) ethane)	• Pesticide formulations	79

2.1.2.1 Terrestrial Organohalogens and Their Sources

In the late 1980's Asplund, Grimvall and Petterson [14] surveyed adsorbable organohalogens (AOX) in surface water, groundwater, and soil. They found that the total amount of AOX in a raised Swedish peat bog was 300 times greater than the annual deposition of AOX from rain. In a separate study [43], Swedish bogs were estimated to contain at least 300 000 tonnes of AOX, whereas the total annual deposition in terms of contaminated rainfall is only 3 700 tonnes. The levels of organohalogens in fulvic acid

from groundwater samples dating back 5 200 years ranged from 230 to 370 μg AOX g^{-1} fulvic acid [14]. The ratio between AOX and total organic carbon (TOC) in present-day surface waters has been reported to be between 730 and 8 600 μg g^{-1}. More recently, naturally produced chlorobenzoic and trichloroacetic acids have been isolated from bog pond water and sediment [80].

Organohalogens in soils have been found globally, and the ratio of AOX to TOC (180-2 800 μg Cl g^{-1} C) appears to be relatively constant throughout the world as shown in Table 2.3 [43].

There is little information about the chemical structure of AOX. It was suggested in 1989 that a large part of the material is made up of relatively high molecular weight humic acids (MW>500) [81]. Research by Hesketh *et al.* [82] described experiments to determine binding energies of chlorinated anthropogenic compounds and humic acid. The results suggested that some of the total AOX may have been due to halogenated anthropogenic compounds bound to humic acid.

The marine and terrestrial biomass annually produces about 5 million tons of chloromethane; man-made chloromethane emissions on the other hand are only 26 000 tons per year [13]. The terrestrial sources of halomethanes are volcanoes (fluoro-, chloro-, bromo- and iodo-methanes, ethanes and ethenes), forest fires (chloromethane), fungi (chloromethane), as wells as land-based higher plants and trees (trichloromethane) [15]. Soil bacteria also produce halogenated hydrocarbons and broad-spectrum antibiotics such as chlortetracycline [1]. Naturally produced PCBs, which are isomerically different than anthropogenic PCBs, have been isolated from volcanic ash [15]. It has also been reported that Canadian forest fires are the source of 130 pounds of

polychlorinated dibenzo-*p*-dioxin each year [15]. Other compounds have been isolated from terrestrial animals; for example, epibatidine, a chlorinated alkaloid, was isolated from the Ecuadorian poison frog, *Epipedobates tricolor* [83, 84].

Table 2.3. Concentration of AOX in Soils From Various Locations

Sampling Site	Vegetation	AOX ($\mu g\ Cl\ g^{-1}$ dry weight)	AOX/TOX ($\mu g\ Cl\ g^{-1}\ C$)
Mochudi, Botswana	Steppe	5	2 400
Manos, Brazil	Campinarana	145	500
N.E. Alberta, Canada	peat bog 0-10 cm	254	700
Ayer Itam, Malaysia	deciduous forest	49	820
Dalir, Iceland	Arctic heath	290	1 300
Hörby, Sweden	beech forest depth 5-10 cm	348	2 400
Antalya, Turkey	Aleppo pine forest, depth 0-5 cm	68	180
Antalya, Turkey	Aleppo pine forest, depth 5-10 cm	170	900
Cockle Park Farm, Great Britain	meadow	224	1 900

2.1.2.2 Marine Organohalogens and Their Sources

At an international conference held in 1993 on the topic of natural organohalogens in the environment, papers on terrestrial sources dominated [83]. Only two papers, by Abramson and Ekdahl [86] and Grimvall [87], dealt with the formation and distribution of organohalogens in the marine environment; other papers referring to marine organohalogens were concerned with quantification. In contrast, the literature

indicates that the majority of the world's natural production of organohalogens takes place in the marine environment [13, 15, 53, 54, 88] and so, directly affects the marine fauna. As this thesis is concerned with organohalogens in shrimp, it seems that the input of organohalogens into the environment by marine sources would be highly relevant.

Literature on the subject of naturally produced organohalogens in the marine environment does not refer to total organohalogens or even AOX (as is done with fresh water systems). Instead, specific organohalogens present in marine organisms and marine ecosystems are identified and/or quantified.

Gribble [15] published an extensive survey of the some 611 naturally produced organohalogens discovered between 1980 and 1991, the majority of which were from the marine environment. It also included halogenated versions of well-known natural compounds. Some of the organohalogens mentioned include the halogenated forms of the following compounds: simple alkanes, terpenes (*e.g.* Fig. 2.2A), amino acids, peptides, fatty acids, lipids, steroids, nitrogen heterocycles (pyrroles, indoles (*e.g.* Fig 2.2B), carbazoles, carbolines, quinolines), nucleic acids, phenols, phenolic ethers (*e.g.* Fig. 2.2C), and others [15, 89]. Gribble published additional papers in 1994 on the subject of natural (marine and terrestrial) organohalogens. One of these focused on the production of chlorinated compounds [88] while another provides a good review of the magnitude of natural organohalogen production [13].

The principal source of marine production of organohalogens appears to be algae. From one species of red algae (*Rhodophyta)* alone, more than 250 halogenated compounds (ketones, alcohols, carboxylic acids, *etc.*) have been identified [15]. Seaweed is also a rich source; for example, almost 100 halogenated compounds have been isolated

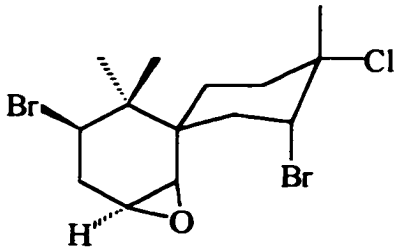
from an edible Hawaiian seaweed (*Asparagopsis taxiformis)* [15, 16]. The sponge (*Dendrilla sp.*) produces a rare halogenated furan, 2-bromofuran. Imre *et al.* [90] reported the isolation for the first time of polybrominated C_{15} non-terpenoid compounds (two acetylenic cyclic ethers and an allenic cyclic ether) in red seaweed species (*Laurencia paniculata* and *Laurencia obtusa)*. There are also many examples of marine organohalogens being produced in animals, *e.g.* fatty acid chlorohydrins from edible jellyfish have been characterized (Fig. 2.2D) [91], as well as sesquiterpenes in sea hares [92].

Other interesting patterns have also arisen through the study of natural organohalogens. Recently, Führer and Ballschmiter [93] have described bromoanisoles (Fig 2.2E) which can be attributed to natural production whereas the chlorinated analogues are known to be of anthropogenic origin.

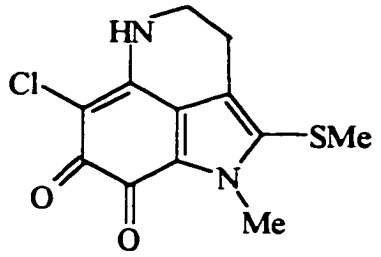
New organohalogens from unknown, but in all probability, natural sources continue to be discovered. Tittlemier *et al.* [55] reported a novel nitrogen heterocyclic compound (Fig. 2.2F) 1,1'-dimethyltetrabromodichloro-2,2'-bipyrrole in seabird eggs. This compound was found in eggs from birds that fed in the Atlantic and Pacific Oceans but not those from the Great Lakes. This seems to suggest that the source is the marine environment although no evidence for the production of this compound by marine organisms could be found in the literature.

It has been suggested that all natural products serve some purpose for the organisms that produce them [94]. The probable reason for natural production of organohalogens is defense against predation and parasite infestation, which includes

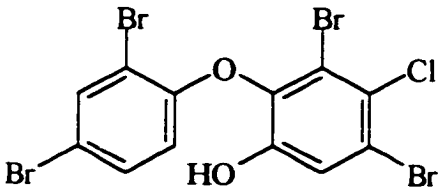
microbial infections. There is also some evidence that some of these compounds have herbicidal, anti-tumor, and anti-fungal properties [15].



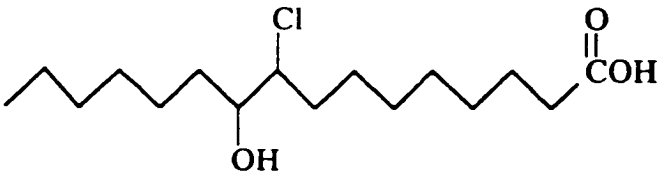
chamigrene epoxide
Fig. 2.2A



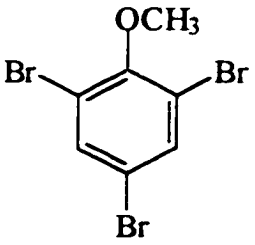
batzelline A
Fig. 2.2B



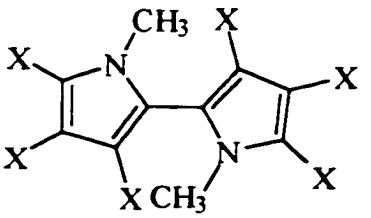
bromochlorodiphenyl ether
Fig. 2.2C



9-chloro-10-hydroxypalmitic acid
Fig. 2.2D



2,4,6-tribromoanisole
Fig. 2.2E



1,1'-dimethyltetrabromodichloro-2,2'bipyrrole
X=Br or Cl, so that
total Br=4 and total Cl=2
Fig. 2.2F

Fig. 2.2. Some Naturally Occurring Organohalogens

Aside from the potential toxic effects of natural organohalogens on other organisms, there may also be an additional threat. Hoekstra and de Leer [1] indicated that marine algae synthesize chloromethane, bromomethane, bromoform and iodomethane in large quantities; these can enter the stratosphere through the marine boundary layer. Once there, they interact with UV radiation and ozone to form oxygen (O_2) and an oxohalide radical. These unstable halogen compounds are then recycled back into the halogen cycle. In this way, natural organohalogens can contribute to ozone depletion.

No reports in the literature referring to the natural production of organohalogens in the shrimp *P. borealis* could be found.

2.1.2.3 Mechanisms for Formation of Natural Organohalogens

As the literature suggests that an enzymatic pathway is one of the major ways halogens can be incorporated into natural organic compounds, this pathway has been studied extensively and is considered responsible for organohalogens produced *in vivo* [1, 43, 88].

Most naturally formed halogenated organics are produced through biosynthesis within a living organism; the group of enzymes that make this possible are haloperoxidases. According to Hoekstra and de Leer [1], there are three main types of haloperoxidases: chloroperoxidases (which also can oxidize bromide and iodide), bromoperoxidase (which also oxidizes iodide), and iodoperoxidase. In the presence of peroxide and a halide (usually not fluoride) these enzymes are capable of halogenating organic material. The major haloperoxidases described by Hoekstra and de Leer, and the

halides they are able to oxidize (*in vivo* or *in vitro*) are summarized in Table 2.4 [1]. Boeynaems *et al.* [95] describe the formation of an iodolactone from iodination of docosahexaenoic acid by lactoperoxidase.

Table 2.4. Haloperoxidases

Class of Haloperoxidase	Oxidizable Halide	Examples of Haloperoxidases
chloroperoxidase	Cl^-, Br^-, I^-	Eosinophile peroxidase, myeloperoxidase, verdo-peroxidase,
bromoperoxidase	Br^-, I^-	lacto peroxidase, ouo peroxidase
iodoperoxidase	I^-	Thyroid peroxidase, horseradish peroxidase

Gribble [88], on the other hand, did not classify all enzymes capable of chlorinating organic compounds, chloroperoxidases; instead, he wrote of chloroperoxidase as being one enzyme and then described other enzymes capable of performing the same action. Enzymes like vanadium peroxidase and horseradish peroxidase can chlorinate phenols and phenol ethers in the presence of chloride. Horseradish peroxidase is also responsible for enzymatic conversion of chlorophenols into polychlorinated dibenzo-*p*-dioxins. Myeloperoxidase (found in human eosinophils and neutrophils), in the presence of peroxide and halogen, can rapidly produce active halogens (such as HOBr and HOCl). These compounds are able to cause the death of foreign microorganisms within the cell [88].

2.2 Toxicology of Organohalogens

Toxicology is an important and complex component of the study of organohalogens and a large number of publications are devoted to this subject. As part of a complete discussion of toxicology, one must also include the acute and toxic effects, metabolic treatment of contaminants and bioaccumulation. While acute effects have been studied extensively, most organohalogens present in the environment are at levels significantly below that required to elicit acute effects and therefore the survey of this subject will be relatively brief.

2.2.1 Acute Effects

Acute toxic effects are those limited responses that are manifested within a short time; this can be within minutes or may take several days. The effects can be lethal or sub-lethal (*e.g.* a rash, lightheadedness, *etc.*). The acute effects of exposure to organohalogens can vary greatly. In some cases there is nearly no effect, *e.g.* chlorinated paraffins are metabolized by birds and mammals with no apparent toxic effects [47], while others are lethal in small doses, as is the case with TCDD in rodents [5]. The factors dictating this wide range of responses depend somewhat on the type of compound, the dose, the length of exposure, and on the metabolism of the species of animal exposed [96].

For humans, the proven acute responses to TCDD are mainly limited to the skin, and include interfollicle hyperplasia, hair follicle atrophy, and sebaceous metaplasia [97]. For obvious ethical reasons it difficult to determine the effects of toxins on people, so most data are limited to reports of accidental exposures. One of these types of incidents

occurred in Japan, where rice oil produced for human consumption was contaminated with PCBs and PBBs. The acute effects included chloracne, other dermal changes, damage to the eyes, neurological damage, and respiratory problems. In cases where there have been occupational exposures, the symptoms are related to chronic effects, although at lower levels than those found with dietary exposure [96].

With regard to acute exposure in animals to PCBs and PBBs, several features of these effects have been documented. First, the toxicity of the various commercial mixtures is quite similar. Second, it takes about 1 to 3 weeks after exposure for a toxic response to develop. Third, the actual degree of toxicity depends on the gender, strain, and age of animal exposed. Fourth, multiple doses elicit a greater toxic effect than a single dose. Last, the symptomology of PCB and PBB exposure is similar to that associated with other toxic compounds like PCDD/Fs, and polychlorinated naphthalenes [96]. In fact, some research suggests that it may actually be the chlorinated naphthalenes that occur with the PCBs that cause a number of the toxic responses [12]. Another important discovery in the study of PCBs and PBBs, is that the brominated biphenyls are more potent toxins than their chlorinated analogues, and the iodinated compounds are more potent than the brominated analogues [96]. The acute effects of exposure to various organohalogens on humans are summarized by Manahan [98].

Since global fish stocks (including *P. borealis*) are valuable resources, many studies of the toxic effects of organohalogens have focused on fish, and the potential effect that organohalogens may have on the viability of certain species. Salmon are an important food source and are easily studied during their passage through fresh water streams and rivers. Therefore, many experiments have been conducted to determine the

effects of bleached kraft pulp mill effluent (BKME) [99] and herbicides [100-103] on salmon. The results help to set acceptable levels of discharge to fragile systems. The exposure of juvenile shrimp (*Penaeus vannamei*) to a sub-lethal dose of pesticides causes increased respiration and decreased levels of protein (28.5-41.8%) indicating that pesticide pollution causes a significant threat to commercial fisheries [104].

In addition to the risks to juvenile and adult fish in contaminated environments, fish eggs are also threatened. Research has shown that eggs that have been exposed to toxins like TCDD and PCB126 have elevated sac-fry mortality [9]. Evidence also suggests that the effects are additive; and repeated exposure to small amounts of many toxic compounds can yield the same effect as a large dose of one toxic compound.

2.2.2 Chronic Effects

Chronic effects are those effects that continue for a long period of time. These are of great interest to researchers, but are also problematic to study due, in part, to the fact that long-term effects are often only manifested after extended periods of exposure or long after a single exposure incident; and also because as much as 85% of the organohalogens present in fish may be unidentified. Chronic effects are more difficult to study than the acute effects. Since most fauna are likely exposed to low organohalogen levels for extended periods or to single exposures at sub-lethal levels, the chronic effects can be more significant than acute effects.

Impairment of reproductive function is a serious effect of chronic exposure to halogenated xenobiotics. This type of impairment threatens the viability of animal-based food stocks and survival of endangered species. Since organohalogens are lipophilic,

there is a tendency for them to partition to organs with high levels of lipids; the reproductive organs contain high levels of lipids and, therefore, are at risk. For flounder and herring, high levels of PCBs in the ovaries are correlated to reduced viability of offspring [10]. An extensive review of the effects of PCB and PBB exposure on a variety of organisms is given by Safe [96]. Some of the reproductive effects described include: decreased progesterone levels, low birth rate, increased frequency of non-productive pregnancies, birth defects, low birth weight and sterility in rhesus monkeys; and thinning of the eggshells and a reduction in the number of hatchlings in birds [96].

High levels of DDE in the ovaries were also correlated to reduced viability of herring and whiting offspring; and high levels of Dieldrin in the ovaries were correlated to reduced viability of cod offspring [10]. There are also indications of potential reproductive effects on humans and other mammals exposed to halogenated hydrocarbons [96, 105, 106]. Chlordane compounds have been shown to have significant effects on the testicular tissue of mice after relatively short periods of exposure, causing gross atrophication and seriously impaired spermatogenesis [107].

Long-term exposure to BKME has been shown to cause chronic liver disease and has mutagenic effects on rainbow trout [108]. Single doses of TCDD have produced cleft palates in mice fetuses [97, 109]. In terms of carcinogenic effects, farmers exposed to 2,4-dichlorophenoxyacetic acid have 6 to 8 times the incidence of non-Hodgkin's lymphoma [86]. Organohalogens such as DDT and its metabolites have been linked to breast cancer and other effects that are a result of an estrogenic action [8, 11]. Chlorinated naphthalenes also have been shown to exhibit similar estrogenicity [110].

Metabolism is closely related to the chronic effects of organohalogen exposure, because inability to metabolize the compounds or slow metabolism can actually result in long-term exposure. Additionally, the metabolite, not the parent compound, may actually induce the effect.

2.3 Metabolism of Organohalogens

The metabolism of xenobiotics is thought to occur in two steps. In Phase I, an enzyme system oxidizes (but may also hydrolyze or reduce) lipophilic compounds to make them more polar [111]. If the metabolite is not excreted then it undergoes Phase II where conjugation to a hydrophilic group may make it polar enough to be excreted. This pattern is not always followed, often the organohalogen is stored directly, or the products are still lipophilic and accumulate in lipid stores.

The Ah receptor ligand (or 'induction receptor protein') plays an important role in metabolism of organochlorines. Suitable molecules can interact with the Ah receptor and form a complex that is capable of interacting with the chromatin on DNA, inducing the activation of the gene expression sites for several different enzyme proteins. One of the main proteins produced is cytochrome P450. Compounds with geometry similar to TCDD, which is planar and have four chlorine substituents making up the vertices of a rectangle about 10Å long, seem to fit best and also are more toxic. Organohalogens with this geometry (or those that can assume it *e.g.* PCB126) and with at least three of the four corners of the rectangle substituted with chlorine or bromine are more toxic than those with less halogen substituents or those that cannot assume a planar configuration [97].

There are different families of cytochrome P450, which metabolize compounds differently, and levels of each of these families vary from species to species [112]; this accounts for interspecies metabolic variability. These biomarkers can be used as an indication of exposure to organohalogens. For example, human exposure to the herbicide, chlortoluron induces cytochrome P450 3A4 and can be determined by measuring bound cytochrome P450 [113]. Other indicators of P450 activity such as mRNA [114] and hepatic microsomal EROD (ethoxyresorufin-*O*-deethylase) [110, 115] have been applied to the study of organisms' exposure to organochlorine compounds in pulp and paper mill effluent and other industrial effluent.

The cytochrome P450-based mixed function oxidase (MFO) system is one of the more important systems present for metabolism and elimination of lipophilic xenobiotics in fish and other vertebrates. The MFO system is also known to mediate the formation of PCB-methylsulfones (PCB-SO_2CH_3) and DDE-methylsulfones [116]. Some PCB-methylsulfones have been isolated from seal and mink tissues [117].

Metabolism of an organohalogen does not always result in a compound that can be excreted or that is benign. One example is chlordane, where the major components of chlordane are metabolized to oxychlordane, which is more lipophilic and more toxic than either parent compound [118]. Negative effects on hepatic lipoprotein metabolism, which can be caused through the metabolism of several organohalogens have been shown in test animals like rats [119]. Carbon tetrachloride has been shown to decrease lipoprotein production; chlorinated alkanes decrease triglyceride production; PCBs and TCDD increase plasma cholesterol in guinea pigs and rabbits; and TCDD also increases plasma triglycerides in guinea pigs and rabbits.

Another significant metabolic pathway for xenobiotic compounds is the formation of lipid conjugates [120, 121]. This pathway is potentially important in the case of organohalogens because much of the unidentified organohalogens are found in lipid fractions [22]. *In vivo* formation of hybrid triglycerides consisting of two fatty acids and a xenobiotic such as 4-benzyloxybenzoic acid have been described in rats [122]. Similarly, when 1-(4-carboxyphenoxy)-10-(4-chlorophenoxy)decane (CCD) was fed to rats, it formed a conjugate with cholesterol through an ester linkage (Fig. 2.3) [123]. *In vitro* studies using rat liver microsomal system fortified with coenzyme A, showed conjugation of trichloroethylene with palmitic acid as a major metabolic pathway [124].

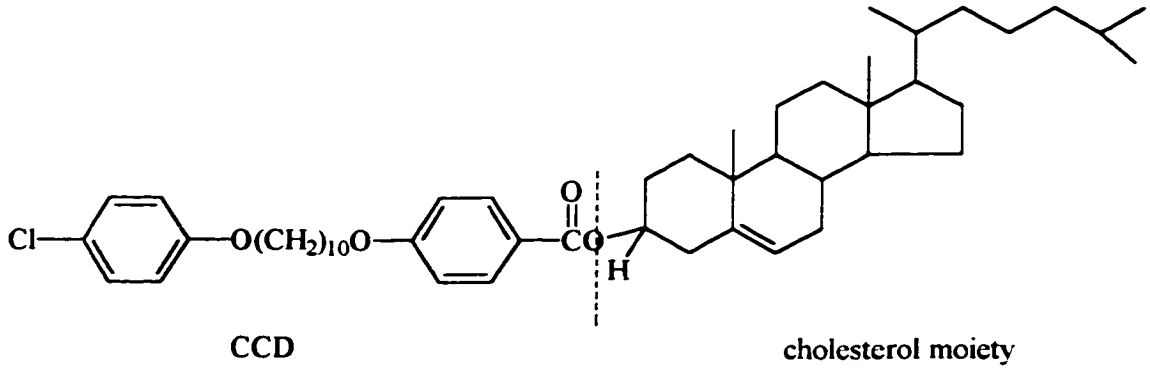


Fig. 2.3. Organochlorine Cholesteryl Ester

Miyamoto *et al.* [125] describe another cholesterol conjugate with the chlorinated pesticide, Fenvalerate. Dodds has published a review of the inclusion of xenobiotic compounds in lipid biosynthesis [126]. Included in this review are several organochlorines and organobromines which undergo elongation to form xenobiotic fatty acids, and form ester conjugates with endogenous alcohols resulting in xenobiotic-triacylglycerols, -cholesterol esters, -phospholipids and -glycolipids. A conjugate of

pentachlorophenol and palmitic acid has been isolated from human adipose tissue [127], however, it was suggested that this may have not actually formed by human metabolism, but may have originated in food that contained the fatty acid conjugate.

2.4 Bioaccumulation

Organohalogens and many of their metabolites are lipophilic in nature, and so there is a tendency for them to accumulate in lipid rich biological systems. They may be accumulated over time or be introduced into the body by one exposure and then retained for a long time. Since many are confirmed or suspected carcinogens and mutagens [86], understanding the degree, trends, and mechanisms of bioaccumulation and storage are important.

Devillers *et al.* [128] define several of the terms that are commonly used in the discussion of this topic. *Bioconcentration* is defined as "uptake, distribution and elimination of a chemical in a fish due to water-borne exposure", while "*bioaccumulation* includes all routes of exposure, and *biomagnification* deals with accumulation via the food chain". The tendency of a compound to accumulate or bioconcentrate is definitely linked to the hydrophobicity of the compound. Thus, the water-octanol partition coefficient (K_{ow}) is often used as predictor of the tendency of a compound to partition from water to lipid, and to be retained by an organism [129]. Other methods of modelling take into account growth of an organism, and uptake and elimination rates as well as K_{ow} [130]. It is important to note that in cases where the compound has a very high K_{ow}, (very hydrophobic) it may be so insoluble in water that is not available for

uptake. However, these compounds may be adsorbed on particulate and taken into the organism on that particulate matter.

Strictly speaking, the bioconcentration factor (BCF) for marine animals has been defined as the concentration of a compound in an animal relative to its concentration in seawater. Kawano *et al.* [131] found that the BCFs increased with trophic level from zooplankton (BCF=$5.4X10^6$ for chlordane compounds) to Dall's porpoise (BCF=$2.8X10^8$). Other authors use bioconcentration factors to relate the amount of a compound measured in an organism to that found in its food [132], however this is generally used as the definition for biomagnification factor (BMF) [133].

Organohalogens bioaccumulate differently (amounts and compound distribution) depending on the organ or the animal. One of the predictors of the differences in the bioaccumulation in different organs is the lipid/water partition coefficient based on phospholipids [134].

Animals exposed to waters containing organohalogens tend also to have measurable amounts of the same compounds in their systems. Pink and Chinook salmon from the Fraser River in British Columbia accumulate chlorophenols from pulp and paper effluents and fungicide contaminated run-offs [135, 136].

Selective accumulation by different organisms is related to interspecies metabolic variations [133]. For example, when PCBs were measured in Sea urchins in the arctic, the PCB profile was different from that found in the sediment. The selective accumulation was dictated by the ability of the urchin to metabolize and excrete compounds that have adjacent meta and para carbons on one (or both) of the biphenyl rings available [137]. In another study [138], the PCB profile of ribbed muscles, grass

shrimp, mummichogs and eels from New Bedford Harbor in MA, USA were compared to that of the water and sediments in their environment. The PCB profile found in the organisms of the lower trophic levels, namely mussels and mummichogs, was highly correlated to the PCB profile in the water and sediments. Accumulation of PCBs by mussels was strongly correlated to the concentration profile in water in which it lived and to K_{ow} [139].

Polychlorinated camphenes (PCCs) like toxaphene have been measured in Arctic narwhal and in freshwater turbot and those PCC with 9 chlorine substituents were found to accumulate preferentially. This is likely due to the inability of the organism to metabolize these compounds [140].

Fifteen organochlorines were measured in fish and invertebrates caught near a sewage outfall in Sydney, Australia [141]. In this study it was found that the organs with the highest lipid content had the highest concentrations of organochlorines, *e.g.* liver. In research on organochlorines in cod, the highest concentrations were found in the liver and ovaries, whereas organochlorines were virtually undetectable in the muscle [142]. Ungerer and Thomas [143] found that Atlantic Croaker exposed to PCBs and DDT preferentially accumulated the compounds in the ovaries but not the testes. There also appears to be some trends for preferential organochlorine accumulation related to gender. Japanese horseshoe crab males contained more than females and this difference was attributed to transfer of contaminants from tissues to roe by female crab [144]. A similar trend was also observed in seals, where adult female harp seals showed lower levels of PCBs, DDT, CHL and ΣHCH than male or juvenile seals [145].

Another interesting example of tissue related preferential bioaccumulation can be found in molluscs, which were found to partition organic compounds quite rigorously between the shell and soft tissues. Compounds detected in the shell were not found in the soft tissues and *vice versa* [146].

The feeding habits of organisms can also affect the extent of organohalogen accumulation. One study of PCBs and organochlorine pesticides in Walrus indicated that the animals that fed on seals had a higher concentration of contaminant than those whose food was from a lower trophic level [147]. This was likely due to the fact that seals biomagnify many organohalogens. Some differences have been reported in the degree of bioaccumulation for animals using contrasting methods of feeding, *i.e.* filter feeding *vs.* deposit feeding. No conclusive evidence was found to indicate whether these differences were actually related to the feeding method rather than metabolism. In general, the deposit-feeder bioaccumulated more organochlorines on a lipid basis than did the filter feeders [148]. This food based trend was also seen when chlordane compounds and their metabolites were measured in the plasma of people from Northern Quebec [149].

In 1975, Lunde and Steinnes [151] used NAA to determine soluble organochlorine, organobromine, and organoiodine in mackerel and herring oil. They found that there was some enrichment of the heavier halogens compared to relative abundance in seawater.

It appears that there is very limited information on organohalogens in shrimp and in particular on bioaccumulation in *P. borealis*. Hellou *et al.* [152] report results for PCBs and several organochlorine pesticides in *P. borealis* and found that higher concentrations of organochlorines are found in the hepatopancreas which also coincided

with the higher lipid content. They also found that the levels of organohalogens were roughly one-sixth of the levels measured in finfish from a similar area. One of the objectives of this project is to investigate the trends of accumulation and distribution of EOX with respect to muscle and roe in *P. borealis*.

2.5 Temporal and Spatial Trends

The use of pesticides such as DDT has been prohibited for many years, yet they continue to persist [153]. For this reason, studies of temporal trends were undertaken. Spencer *et al.* [154] reported a decrease in DDT and its metabolites in soil between 1971 and 1994. Most of this loss was attributed to volatilization of DDT and DDE, as well as removal of the water-soluble DDT metabolite, DDD, by rain and flooding. Paasivirta *et al.* [155] found that the DDE, DDT and PCB were decreasing in Finnish wildlife between 1967 and 1983, and lindane also between 1978 and 1983.

Many reports on the state of the St. Lawrence River basin and the animals that make their home there indicate that it is highly contaminated [156]. Organochlorine pesticides levels in lamprey larvae collected from the St. Lawrence in the late 1940's were compared to those collected in 1990. It was found that the levels of the PCBs, and the original pesticides (β-BHC, α-Chlordane, γ-Chlordane, *p,p'*-DDT, and Heptachlor) and the DDT metabolites DDD, and DDE were higher in the older sample. However, the amount of Mirex and the levels of DDD and DDE, relative to ΣDDT, were higher in 1990 [156]. Similar trends were reported in spottail shiners in the near shore waters of the Great Lakes [157].

Weber and Goerke [150] compared the levels of several organochlorines in fish from the Antarctic between 1987 and 1991. They found that *p,p'*-DDE and three PCB congeners increased during this period. It has been proposed that this increase was due to global redistribution.

Kannan *et al.* [29] reported the distributions of organochlorines in tropical Asian and Oceanic countries, and compared them to available data from other countries. The use of DDT has been banned in the US since the 1970's (total use up to that point was about 1 million tonnes). India is still using DDT and it has used approximately 500 000 tonnes, but DDT in fish in India is 10 to 100 times lower than that in the US. India has used 1 million tonnes of hexachlorohexane (HCH) to date. Despite these massive inputs to the local environment, the literature indicates that DDT is the only major organochlorine found in tropical fish. This discrepancy between emission and level of bioaccumulation is thought to be due to the difference in temperature of the regions and thus the tendency for organohalogens to be more volatile in tropical regions. DDT tends to preferentially partition from air to water and biota near the emission source, but HCH and chlordane stay in the atmosphere because they are more volatile than DDT. These semi-volatile organochlorines may contaminate the Arctic, Antarctic, and temperate regions through long-range transport.

Some spatial trends in the levels of organochlorine contamination have been noted for cod [142]: Arctic, Northwest Atlantic, West Atlantic, Norway < North Baltic, Nova Scotia, North Sea < South Baltic.

2.6 Mass Transport of Organohalogens

Organohalogens have been found in organisms and environments all over the world. Organochlorines are found in fish from the Antarctic [150], and even the most pristine British reed beds are found to contain organohalogens like PCBs [158]. Contaminants in areas where there is no local use or source of production can be attributed to mass transport. Spatial trends that have been noted may also be related to mass transport processes. In the late 1960's, scientists discovered that North Atlantic trade winds carried organochlorine pesticides on dust and these were likely deposited in the ocean [159].

More recently, Mackay and Wania [160, 161] have proposed comprehensive theories that attempt to explain how persistent organic pollutants (POPs) are transported. They suggested that POPs, such as organohalogens, are volatile enough to participate in a cycle of evaporation, fractionation and condensation that is responsible for the distribution of POPs globally. The warm conditions in tropical and subtropical regions favour the evaporation of chemicals like POPs, while in cooler climes, such as the temperate and arctic regions, these compounds are more likely to condense and become associated with particles which end up in soil, water and sediment. Once in the marine or terrestrial environment, the pesticides may be available for uptake by biota.

Throughout the evaporation and condensation process, fractionation is also occurring. Over time, a gradient develops in which the least volatile components are found closest to the point source. The levels then tail off as one moves away from the source. The more volatile compounds are found in low concentrations or not detectable near the origin, and are found in high levels in the colder environments. The most

volatile compounds continue to evaporate and condense even in the coldest climates; thus they are found globally in relatively stable concentrations [161]. The global distribution of organohalogens continues until the compounds are either destroyed, transformed, or no longer available to the cycle through permanent adsorption to soil and sediment or by bioaccumulation by plants and animals [161].

Once in the cold Arctic marine environment, volatilization for many organohalogens is unlikely and thus they are trapped. The experimental evidence bears this out, since flux values for northern seas are highly negative, indicating active transfer from air to water whereas tropical seas generally show positive fluxes [162].

Long- and short-range transport in air is not the only mechanism of organochlorine transfer; other pathways are also available. Rivers contaminated with run-off can contribute to levels of organohalogens in air and in the sea [163]. Marine fogs also have been shown to accumulate relatively high levels of pesticides and other contaminants and these fogs can transport and transfer organohalogens to water, soil and snow [164]. Ocean currents are hypothesized to be one of the other major pathways [165]. Currents move warm water from the tropics to the poles, thereby distributing heat and if the water were from regions where POPs are in high concentrations, then the water would be a significant method of mass transport. Some of the compounds that are thought to be transported in this way are: hexachlorocyclohexanes, DDT and some PCB congeners [162]. Coastal tidal and thermohaline fronts show enrichment of organohalogen compounds [166] which may be due to the accumulation of oil matter in these fronts that attract the hydrophobic compounds.

2.7 Methods of Extraction of Organohalogens

The first step in the study of extractable organohalogens is their separation from the matrix and emphasis must be placed on the development of a suitable method of extraction. Several methods can be employed for this purpose. The choices mainly depend on the relative extraction efficiency of a method for a desired analyte. An ideal method should take into consideration various parameters such as ease, speed, cost, amount of reagent, reagent blanks, and extraction efficiency.

The traditional method of extraction uses the Soxhlet extraction (SE) apparatus, where a ground sample is refluxed with either a solvent or a mixture of solvents. Due to the excellent recoveries and reliability associated with this method, it is often used as a standard for the evaluation of a new or modified method. A recent paper by Luque de Castro and García-Ayuso [167] reviews innovations in the use of Soxhlet extraction. These advances include the use of closed high-pressure systems, Soxhlet and microwaves in the forms of the Soxwav-100 extractor (single step) and Soxtet® extractor (which uses a three-step process). The results for these modified extraction systems were all superior to the traditional extractor. The authors [167] caution that, because Soxhlet extraction is performed at the boiling point of the solvent for a relatively long period of time, it may result in decomposition of some thermally labile compounds. They also comment that ultrasound-assisted extraction can meet or surpass the efficiency of Soxhlet extraction. A 1997 paper compares the extraction efficiency of supercritical fluid extraction (SFE), accelerated solvent extraction (ASE), ultrasonic extraction (USE) and methanolic saponification (MSE) for a variety of organopollutants [168]. Extraction of chlorinated

compounds from filter candles using ASE and USE was reported to be 102% compared to Soxhlet extraction.

Pressurized fluid extraction (PFE), a relatively new method of extraction introduced in 1996 [169], uses elevated temperatures and pressure with liquid solvents to enhance extraction from solid matrices. Schantz *et al.* [170] compared this method of extraction to Soxhlet extraction as applied to a variety of environmental reference materials. They found that the extraction efficiency for EOCl was similar to the Soxhlet, but offered the advantage of increased speed, reduction of solvent use, automation and concentration of extracts. The various methods of extraction reported in the literature are summarized in Table 2.5.

One of the objectives of this thesis was to compare the efficiency of extraction using a Polytron apparatus to that of a shaking board. Several different solvent systems were evaluated for extraction efficiency for EOX from homogenized shrimp using a Polytron. These experiments are described in Chapter 3.

Solid phase extraction (SPE) can be used instead of multiple solvent extraction methods to reduce contamination encountered in the clean-up step of seafood extracts and also as a method of extraction and concentration of organohalogens from water. A Florisil column is often used in SPE. Cleanup may be achieved by eluting the extract through the Florisil column and then extracting the desired material with solvent(s) such as 10% diethyl ether in petroleum ether [171]. Early studies of the use of columns to remove interferences included silica and alumina packings; silica columns were found to be the most effective [172].

Table 2.5 Methods of Extraction of Organohalogens

Method	Reported Analytes	Solvent	Efficiency	Notes	Reference
Supercritical Fluid Extraction (SFE)*	PCDD/Fs	CO_2	higher than SE	Uses an acid pretreatment for extraction from electrofilter ash.	184
	PCBs	CO_2 with 1.7-4% MeOH modifier	98% of efficiency of SE	Reports an independent inter-laboratory comparison of SE and SFE. Found that SFE uses less organic solvent and less time, no subsequent cleanup is required for gas chromatography. Can be applied to a variety of matrices.	185
	PCDDs, PAHs	CO_2	3 times faster than standard SFE method	This method uses a smaller extraction vessel and higher temperatures (up to 200 °C), overcomes strong matrix analyte interactions. Applied to fly ash, marine sediment and railroad bed soils.	186
	a variety of organo-chlorines (OCl)	Water	100%	Uses 400 °C, extracts test compounds from spiked sand.	187

* SFE is limited by impurities found in the CO_2, therefore the volume of gas used must be minimized [186]

Table 2.5 Methods of Extraction of Organohalogens (*contd.*)

Method	Analyte	Solvent	Efficiency	Notes	Reference
subcritical fluid extraction	OCl	Water	>90%	Performed at 250 °C on spiked sand samples. Found that for real samples the subcritical extraction worked better than SE.	187
Simultaneous steam distillation extraction (SDE)	Organochlorine pesticides (OCPs) from soil	Steam	~100%	SDE is better than SE and SFE for recoveries from soil, and is less laborious than SE	188
	OCPs from seeds and milk		85%	SE has better efficiency (92%) for extraction from fatty matrices, but SDE is less labour intensive	188
Bleidner vapour phase extraction (BVE)	Organochlorines in lake sediments	Isooctane/ water	N/A	This method eliminates the drying, extracting, and clean-up steps needed for SE	189
Direct extraction from solids packed in a chromatographic column	pesticides from fish, fish food, and mud	Several (acetonitrile, hexane, cyclohexane, diethyl ether, petroleum ether, and isooctane)	10% more efficient than extraction using a blender	Solids homogenized by grinding with dry ice, then dried with anhydrous sodium sulfate, resulting mixture is then eluted directly with solvent The major advantage is that it combines extraction, filtration and drying in one step	171

Table 2.5 Methods of Extraction of Organohalogens (*contd.*)

Method	Analyte	Solvent	Efficiency	Notes	Reference
Polytron with tandem C_{18} column pre-concentration and Florisil column clean-up	OCP and PCBs	acetonitrile	as good or better than those prescribed in the FDA Pesticide Analytical Manual (PAM)	Good for fatty matrices, reduces hazardous waste consumption by 85% as compared to PAM methods. Also, this method takes less time (3 minutes per extraction) to process samples.	32
Polytron	EOX from fish	acetone/ hexane	86% recovery from spiked sample	Takes only 3 minutes per extraction.	33
Microwave Assisted Extraction (MAE)	organochlorines	acetone/ hexane	as good or better than SE	MAE requires much less solvent than SE. It requires only 10 minutes total extraction time, and up to 12 samples can be processed at once.	34
	Halogenated phenols from soils	acetone/ hexane	performed as good or better than SE or sonication extraction	The advantages of using MAE for this purpose are the same as above.	36
leaching (*i.e.* solvent extraction with shaking-board)	EOX from solid biological matrix	cyclohexane/ acetone, cyclohexane/ isopropanol	82 –100% recoveries	The acetone mixture had higher recoveries and a lower blank, but precision was better for the isopropanol mixture.	37

In 1994, Schenck *et al.* [32] employed a tandem SPE method to clean-up non-fatty seafood products; this method has also been used in the cleanup of eggs [174]. Schenck and co-workers modified this method so that it could be applied to fatty fish and found that the results were comparable to the official AOAC method with recoveries ranging from 59-104% [173]. The extract was eluted through a C_{18} column which was then washed with water. The column was subsequently placed in tandem with a Florisil column and the compounds of interest were eluted with a variety of solvents. The results were as good or better than those obtained using methods described in the Pesticide Analytical Manual (PAM) of the US Food and Drug Administration (FDA). Solid phase extraction disks have also been investigated for the analysis of pesticides in high-moisture foods in an attempt to reduce the use of hazardous wastes such as methylene chloride [175]. Again, the reported recoveries from spiked samples were found to be adequate, in this case greater than 80%.

Clean-up can also be accomplished through a variety of other means. One method of clean-up includes pre-treatment of the sample with concentrated sulfuric acid [176]. This results in the destruction of certain organohalogens and, therefore, its applications are not as broad as the use of Florisil SPE. Gel permeation chromatography has also been used for clean-up [177, 178] and for separations of organohalogen samples [179, 180]. Semi-permeable membrane dialysis has been applied for large-scale isolation of organochlorines from lipids [181].

It should be noted that these methods of clean-up are only necessary when certain chromatographic separations are used (*e.g.* GC) where impurities may reduce the effectiveness of separation or subsequent separations. This type of clean-up is not

necessary when NAA is used for the analysis of samples unless information regarding molecular mass distribution of EOX is desired.

Post-extraction washing is an important step in the determination of EOX by NAA. Martinsen *et al.* [37] investigated washing procedures and found that sequential washing, using 3 portions of water followed by a wash with an aqueous solution of ammonium phosphate (0.1%) and sodium nitrate (0.9%). They found that most of the chloride was removed in the first water wash and also found very little chloride in the nitrate wash. The nitrate ion presumably replaced any inorganic chloride left in the non-polar phase. Other researchers reported the use of water washes to achieve the complete removal of inorganic halides from extracts [182, 183].

In the present thesis, a sequential wash procedure was used, which involved 6 washing steps, 3 with a 0.1% ammonium phosphate-0.9% potassium nitrate solution. Details are given in Chapter 3.

2.8 Solvent Evaporation

In 1981 Erickson *et al.* [190] evaluated several solvent evaporation techniques for the recovery of many organohalogens. For macro solvent evaporation, *i.e.* a reduction of volume from 200 to 10 mL, techniques used included Kuderna-Danish (K-D) concentrator, rotary evaporation, evaporation on a hot plate, and heated nitrogen blow-down. Nitrogen blow-down yielded the best recoveries of organohalogens from 15% methylene chloride in hexane or pure methylene chloride, but the 120-min required to achieve this was considered prohibitive. The K-D concentrator was superior to nitrogen

blow-down due to the shorter evaporation time of 10 to 15 min. The rotovap was deemed better for methylene chloride in hexane as total evaporation time was 15-20 min.

The K-D concentrator was also compared to the Zymark Corporation TurboVap, which uses evaporation under a nitrogen atmosphere. The K-D concentrator was found to perform better than the TurboVap for pesticide recovery, and marginally better for PCB recovery. Although the recovery is better, there are a few disadvantages of using the K-D concentrator. For example: it cannot be automated, it requires constant attention to prevent loss of sample due to bumping and to prevent the sample from drying, and it requires more than one step [191].

For micro solvent evaporation, *i.e.* a reduction of volume from 8 to 0.2 mL, techniques used include the micro K-D concentrator, nitrogen blow-down with Snyder column, and nitrogen blow-down without column. For methylene chloride as the solvent, the nitrogen blow-down with column method was clearly superior. Although the results using methylene chloride and hexane were not as clear, the micro K-D method was unacceptable due to poor recoveries [190]. In the present thesis, a rotary evaporator was employed for the reduction of solvent volume.

2.9 Methods of Analysis

Much of the research reported in the literature involves the determination of specific organohalogen compounds. Many of these analytes and methods of their analysis are listed in Table 2.6. The most common method of analysis for specific organohalogens is gas chromatography coupled with mass spectrometry (GC-MS). One of the problems associated with this method is that a prior knowledge of the compound of

interest is preferable so that the optimal conditions for separation by GC and subsequent ionization for mass spectrometry can be selected. Another complication is that extensive clean-up must be done on the sample before using GC, otherwise the sample matrix may cause a significant reduction in response. Also, the broad range of physicochemical properties of the analytes makes it difficult to optimize recoveries for all components [192]. One of the benefits of GC-MS is that it is very useful for the identification of specific organohalogen compounds.

The large number of organohalogens, generally present in a biological sample, makes the GC-MS technique rather impractical for routine determination of total EOX. The compound of interest must be reasonably volatile for use in gas chromatography. This may be difficult to achieve without combustion or decomposition of some of the high-mass halogenated molecules (*e.g.* lipids). There is also some indication that there may be thermal breakdown of organoiodine in the injection port when performing GC analysis [1]. An invaluable method for the simultaneous determination of total organohalogens with minimum handling of the extract is instrumental NAA (INAA). An INAA method has been developed and used in this thesis.

2.10 Quality Assurance

The term quality assurance (QA) should figure prominently in all analytical measurements since all activities involving sample material can affect the final result. To effectively manage analyses, a total quality management plan (TQMP) should be employed. This plan covers protocols for all critical steps from sample handling to data reporting (*e.g.* sample collection, preservation, storage, chemical separation, analysis,

data processing, *etc.*). Once this TQMP has been established then the task of quality control (QC) at each of the steps must be undertaken.

Most laboratories have little control over the sampling procedure. The main responsibility of the facility lies in the post-sampling quality assessment. A QA program includes such things as standard good laboratory practices, method validation, detection limit determinations, use of standards, control charts, analysis of blanks, blind and duplicate samples, methods for rejecting or reporting low level data, and traceability. These steps are important to ensure reliability of a result [237].

The Analytical Methods Committee of the Royal Society of Chemistry suggests practical approaches to quality control [238]. It also outlined the control of random error using replicate analyses, as well as the use of certified reference materials to control systematic errors. A reasonably in-depth review of univariate methods for quality control charting was covered by Howarth [239]. In this review, he covered several methods of reporting deviation among replicate samples (*i.e.*, chart for the mean, and chart for the standard deviation). Criteria for determining whether to take action on a given point or set of points of data were also described.

In 1995, Johnson *et al.* [240] described the criteria for validation of data produced using NAA as put forward by the US EPA Contract Laboratory Program. All laboratories that perform analyses for the EPA are subject to these guidelines, and the data are only considered valid if the criteria are met. The guidelines are very specific and cover holding times, calibration, blanks, control samples, duplicates, magnitude of flux, analyte quantification and detection limits, and system performance. An independent QA program has been applied to the work done in this thesis and is described in Chapter 3.

Table 2.6. Analytical Methods for the Determination of Organohalogens

Analyte	Method of Analysis	Reported Detection Limit	Notes	References
PCBs	HPLC/GC (ECD)	0.5 ppb	This method allows for automated sample cleanup and analysis.	193
		1 ppb	Uses an HPLC phase for one-step cleanup.	194
	HPLC/GC-MS	< 1 pg g^{-1}	On-line coupling of HPLC and GC-MS for determination of PCBs in human plasma.	195
		ND	Coupled two HPLC columns allowing for separation of PCB, dioxins, furans and Polycyclic aromatic hydrocarbons based on aromaticity and planarity	196
	GC/ECD	1 ng g^{-1} lipid	Dual column separation using two columns of different polarity.	197
	GC/ECD and GC/FID	0.032 μg kg^{-1}	Applies Florisil mini columns for pre-injection cleanup of extracts from mussel.	198
	multi-dimensional GC/ECD	0.5 μg kg^{-1}	This method reports the use of two separate columns (HP-Ultra 2 column and HP-FFAP column) to determine different PCB congeners	199
	HRGC/ECD and HRGC-MS	N/A	These researchers developed a dual column method for determination of congener based on a retention model.	200

Table 2.6. Analytical Methods (*contd.*)

Analyte	Method of Analysis	Reported Detection Limit	Notes	Reference
PCBs (cont.)	GC-MS	N/A	Reports qualitative separation of PCB on new smectic phase GC column.	201
		0.28-0.41pg g^{-1}	Authors describe the analysis of 92 PCB congeners, resulting in a higher ΣPCB values for reference materials than reported by other laboratories.	202
	GC/ECD and GC-MS	ND	Compares separations using GC-MS and 3 GC/ECD columns to one another for a large number of PCBs in marine standard reference materials.	203
	HRGC-MS/MS	N/A	Authors use tandem MS to determine low levels (10 ng/g) of PCBs in the presence of other chlorinated aromatics.	204
	GC/FTIR	50 ng (>99% identification through library match)	Employs GC/FTIR for identification and quantification of PCB congeners. If using only using single wavelength monitoring the detection limit is 20 ng.	205
	Photon activation analysis	50 ng g^{-1}	Determines PCBs and dioxins using selective separation, activation by high energy photons, and then Cl is determined using the 146 keV peak of the ^{34m}Cl isotope	206
	Capillary electrophoresis	ND	Separates PCB congeners using cyclodextrin-modified micellar electrokinetic chromatography.	207
PCB and DDE Methyl sulfones	GC/ECD, GC/SIM, GC/TIC	24.2 pg, 2.1 pg, 44.4 pg, respectively	Compared the methods of analysis and found ECD and TIC should be used for quantitative analysis as they have better precision and reproducibility.	208

Table 2.6. Analytical Methods (*contd*)

Analyte	Method of Analysis	Reported Detection Limit	Notes	Reference
PCDD/Fs	GC-MS/MS	0.500 pg per μL injected	GC with ion-trap MS used to determine TCDD in environmental matrices. The results were comparable to HRGC with triple quadrupole MS.	209
PCDD/Fs (cont.)	HPLC/GC/ECD	3 pg g^{-1}	Allows one-step cleanup and separation of PCDD/Fs in human milk.	194
	HPLC/GC-MS	ND	Separates of PCDD/Fs according to aromaticity using two HPLC columns.	196
Pesticides (*i.e.* DDT, aldrin, dieldrin, *etc.*)	GC/ECD	5 ng g^{-1} lipid	Uses two different parallel GC columns for the separation of organochlorine pesticides in Mediterranean fish.	197
		ND	Eggs are extracted and then cleaned up on tandem C_{18} and Florisil columns and then analyzed by GC/ECD.	174
		4 ng g^{-1}	Uses simultaneous extraction and clean-up of human milk leading to two fractions containing pentachlorophenol and neutral pesticides.	210
		ND	Collaborative study of determination of organochlorine pesticides (OCP) in chlorine disinfected drinking water.	211
	GC/ECD	0.05 pg g^{-1}	OCP are adsorbed from fresh water on to Amberlite XAD-2 macroreticular resin, and then eluted and determined by GC/ECD.	212

Table 2.6. Analytical Methods (*contd.*)

Analyte	Method of Analysis	Reported Detection Limit	Notes	Reference
Pesticides (cont.)	GC/ECD and GC-MS	ND	Results from the analysis of OCP in marine reference materials using GC/ECD (three different columns) and GC-MS are compared.	203
	GC-MS	ND	Samples containing different OCP in different matrices are analyzed using GC-MS in selected ion monitoring mode and compared to other detection methods.	213
	LC/GC/ECD	<2 ppb	A new on-line method for analysis of OCP residues in fat	214
		0.1 ng mL^{-1}	This method was applied to the determination of DDE in human adipose tissue; recoveries were 91% and eliminates the need for analysis cleanup.	215
	HPLC/GC/ECD	1-5 ppb	A method for automated sample cleanup of human milk using column switching and HPLC.	193
	chiral HPLC and chiral and achiral HRGC-MS	ND	Uses results from two different methods of separation to determine the composition of technical DDT.	216
	Immunoassay	0.01 ppb	Reviews the use of antibodies in pesticide detection, chromatography, and immunolabeling.	217
		N/A	Describes immunosorbents for pesticide separations and determinations.	218
		N/A	Reviews the use of immunoassays for pesticide analysis.	219

Table 2.6. Analytical Methods (*contd.*)

Analyte	Method of Analysis	Reported Detection Limit	Notes	Reference
Pesticides (cont.)	GC-MS	0.02-0.2 ppm	A method for determination of a large number of pesticides in fruit and vegetables.	220
		1 ng L^{-1} or less	Combines solid phase microextraction with GC-MS to determine 60 pesticides in water.	221
Herbicides	GC-MS	3-4 ng g^{-1} lipid	Method for determining chlorophenoxy herbicides in fish tissue.	222
	LC/high flow electrospray/MS	0.01-0.03 ppb	Automated method for determining acidic herbicides in environmental water.	223
Fatty Acids	GC/ELCD	50 pg of chlorine	Method for identifying and determining fatty acids in fish lipids using Hall electrolytic conductivity detection (ELCD).	224
	GC/ NH_3 PICIMS	ND	Uses positive ion ammonia chemical ionization mass spectrometry (NH_3 PICIMS) in selected ion monitoring mode to determine chlorinated fatty acid methyl esters.	225
AOX/EOX	microcoulometry	0.18 μg Cl	Combusts sample at 1000 °C and titrates halides produced microloumetrically.	226
AOX	NAA	5 ppb (OCl) 0.5 ppb (OBr)	Determines AOX from water adsorbed on activated carbon.	14, 227, 228
EOX	NAA	20 ppb (Cl) 5 ppb (Br), 3 ppb (I)	Organic chlorine, bromine, and iodine in environmental samples are measured.	229

Table 2.6. Analytical Methods (*contd.*)

Analyte	Method of Analysis	Reported Detection Limit	Notes	Reference
EOX (cont.)	NAA (cont.)	ND	Organochlorine and organobromine are measured in different lipid fractions of Herring Gull Eggs	230
		ND	EOCl, EOBr and EOI are measured in extracted fish lipids.	23, 37, 231
		30 ng	Organochlorine in fish lipid is determined.	182
		5-10 ng	Organobromine in measured in marine and terrestrial oils.	23
	cyclic NAA	0.185 ppm (Br) 0.082 ppm (I)	Bromine and iodine in fish, plant and mammal oils are determined.	232
Miscellaneous	TLC on silica for chlorinated paraffin, transferred to aluminum oxide, detected with silver nitrate spray	50 ng	Used for the determination of chlorinated paraffins in water, sediment and biological samples.	233
	GC-MS (negative chemical ionization)	0.1 ng g^{-1} wet weight	Polychlorinated terphenyls in measured in aquatic biota.	234
	GC/FID	ND	Determination of 60 priority chlorinated pollutants using two liquid-modified adsorption GC columns.	235
	vacuum distillation/GC-MS	<1 ppb	Applied to the measurement of volatile organochlorines in environmental samples.	236

2.11 *Pandalus borealis*

The evaluation of organohalogens in *P. borealis* is necessary for several reasons. There is a valuable commercial fishery of *P. borealis*, [36] and the shrimp are distributed over a wide geographical area. Also, the species occupies an important position in the food chain as a consumer of flora and fauna and as a food resource for fish, other crustaceans, marine mammals and humans [241, 242].

The distribution of *P. borealis* is extensive. In the Pacific, the range includes the north and north-east coast of Japan, west to the Bering Sea, and in the east, from the Aleutian Islands south to the Washington-Oregon coast [243]. In the Atlantic, the range includes the north coast of Norway extending into the Arctic Ocean, part of the northern coast of Russia, the south-eastern and -western coasts of Greenland, the coast of Iceland, the Labrador Sea, the coast of Newfoundland, the Gulf of St. Lawrence, and the Gulf of Maine [241]. The limits of the range of the *P. borealis* are dependent on water conditions, which include depth, salinity, substratum and temperature. The preferred depths are between 50 and 500 metres. At higher latitudes, the population occurs in deeper waters. *P. borealis* requires waters of relatively high salinity, ranging from 23.4‰ to 35.7‰. The preferred substratum is generally muddy or sandy and the shrimp population is directly related to the organic content of the substratum. *P. borealis* has been found in water temperatures ranging from -1.6 ° up to 12 °C, but the usual range is between 0 and 5 °C. [241]

This species is a *protandric hermaphrodite*, meaning it begins sexual function as male, goes through a transition period and then functions as a female [243]. Male *P. borealis* generally reaches maturity at 1.5-3.5 years. The females become mature

between 1.5 and 5.5 years. The average female carries approximately 1 700 eggs [243]. The average age of *P. borealis* is 4 to 5 years or more and in some colder regions of the Arctic, the maximum age may be as high as 11 years or more as found in Iceland [241].

Transformation from male to female has been suggested to be dependent on size and adult mortality rate. Since the shrimp fishery mainly harvests females (as they are the largest), this causes a decrease in female population. To compensate, the length of time a shrimp spends as a male may be shortened. In fact as many as half of all the shrimp in heavily fished areas (*e.g.* Burrard Inlet, BC) may mature only as females [244].

The distribution within the population with respect to distance from the shore and depth is dependent on sex, age, temperature variation in the region, and the times of year and day. Observations of shrimp migration in Maine and Alaska found that ovigerous females migrate inshore in fall and early winter to spawn and then the eggs are hatched sometime in late winter or early spring. The female shrimp carry the fertilized eggs for 4.5-10 months and this varies with temperature. After the eggs are hatched, the females migrate offshore. The juveniles spend two seasons inshore then migrate as males offshore. The mature males stay offshore until they pass through the transitional phase and become females. The shrimp migrate up the water column during the evening hours. The smaller shrimp move up to the shallowest levels and the ovigerous females do not as their ability to swim is decreased [241].

Age determination in *P. borealis* is difficult as they have no hard parts that remain after molting [242]. The age must be determined by sexing (based on gender related differences on the first pleopod) and size (based on the length of the oblique carapace). To determine the age of shrimp, measurements must be obtained for a sample of at least

500 shrimp. The results must be processed by computer to determine length-frequency distribution. This procedure is cumbersome and time consuming and for this reason any age-dependent factor is difficult to assess accurately.

One study reported an analysis of lipid composition for whole shrimp caught in August of 1965 [245]. These results are likely to be representative of the species. *P. borealis* were found to contain approximately 2.3% lipid. Of the recovered lipid, 47% was phospholipid, 43% triglyceride and 10% sterol. After saponification it was found that the lipids consisted of 75% fatty acid and about 6.5% non-saponifiable material. The distribution of fatty acids in the whole shrimp depends on whether the shrimp is berried (*i.e.* egg bearing) or not. Non-berried shrimp contained about 21.5% saturated fatty acids, 45.8% monounsaturated fatty acids, and 32.6% polyunsaturated (mostly with 5 or 6 as the degree of unsaturation). The berried females contained less of the saturated (20.3%) and monounsaturated (43.7%) fatty acids and more polyunsaturated fatty acids (36.0%) but with the same overall distribution in each category. The increase in the amount of polyunsaturated fatty acids is probably associated with the roe lipids. Variations in total lipid content of *P. borealis* in Norwegian waters were found to be seasonal with the maximum in late summer (August/September) and the minimum in late winter (February/March) [245].

In this thesis, all shrimp used (except those from Canso Hole) were female as indicated by the fact that they carried eggs. The shrimp from Canso Hole did not carry eggs, however their size was consistent with females from other locations.

3. METHOD OPTIMIZATION AND QUALITY ASSURANCE

The work presented in this chapter describes the experiments performed to achieve optimal extraction as well as determination of EOX from shrimp. The equipment and chemical reagents are given, as is the description of the procedures for sample preparation and analysis. The preparation of standards, the irradiation and counting schemes are given here. An extensive quality assurance (QA) program was carried out to ensure the reliability of the method developed. Experiments related to QA presented in this chapter included the evaluation of reagent blanks, and the use of comparator standards, control charts, and standard reference materials.

The determination of the most efficient and practical method for the extraction of lipid and organohalogens from shrimp was of paramount interest in this thesis project. Several steps were evaluated including the choice of solvent system, the extraction method, the wash to remove inorganic halides, as well as the drying and solvent reduction steps. The levels of halides determined in the procedural blanks were also used in selection of the solvent system.

3.1 Chemicals

The comparator standards were prepared using Fisher Scientific Company Certified ACS grade solid potassium chloride (KCl) and potassium iodide (KI). The potassium bromide (KBr) was AnalaR grade supplied by BDH Chemicals Canada Limited. Potassium is not as sensitive as sodium in neutron activation and so the potassium salts of the halide ions rather than the sodium salts were used in order to

minimize background activities. About 0.5g of BDH AnalaR grade potassium hydroxide (KOH) per litre was added to the above comparator standard solutions. The added KOH made the solution basic and thus stabilized the iodide in solution, a procedure which has been successfully used in this laboratory in the past [247]. Without this precaution, the iodide levels will decline due to the formation of the volatile iodine (I_2) species.

Concentrated nitric acid was ACS reagent grade from Fisher. Sulfuric acid was Ultrex® brand from J.T. Baker Chemical Co. Dibasic ammonium phosphate was reagent grade sold by Baker and Adamson. Analytical reagent grade potassium nitrate was purchased from BDH. The anhydrous sodium sulfate was ACS grade, and produced for use in pesticide analysis by Fisher Scientific.

All water used in this work was quartz-distilled. De-ionized distilled water (DDW) was made by passing distilled water through a Barnstead ultrapure ion-exchange column, and was stored in a capped 25-L Nalgene carboy. All of the organic solvents used were purchased from Anachemia and were glass-distilled, pesticide grade solvents. The solvents purchased were acetonitrile, acetone, chloroform, cyclohexane, hexane, isooctane, petroleum ether (30-60°C boiling range), dichloromethane, ethyl acetate, and toluene. All solvents were analyzed by NAA for chlorine, bromine and iodine before use. The dielectric constants and boiling points of the solvents [248] are shown in Table 3.1.

Table 3.1. Dielectric Constant and Boiling Points of Solvents of Interest

Solvent	Dielectric Constant (measurement temperature)	Boiling Point (°C)
ethyl acetate	6.02 (25 °C)	77.06 °C
acetone	20.7 (25 °C)	56.5 °C
acetonitrile	38.8 (20 °C)	81-82 °C
petroleum ether	low*	30-60 °C
hexane	1.89 (20 °C)	69 °C
isooctane	1.94 (20 °C)	98-99 °C
cyclohexane	2.02 (20 °C)	78 °C
toluene	2.38-2.44 (0 °C)	110 °C
chloroform	4.81 (20 °C)	60.9 °C
dichloromethane	9.08 (20 °C)	40 °C

* exact value not reported

3.2 Procedure for Cleaning Glassware and Vials

Glassware (also vials and Teflon labware) was first cleaned thoroughly with distilled water and liquid soap, rinsed with distilled water three times. It was soaked in 2M nitric acid (made with DDW) for 24 h, after which the glassware was rinsed thoroughly (at least four times) with DDW. The glassware was then rinsed three times with pesticide grade acetone followed by three times with hexane, and was then covered and left to air dry. The dry glassware was stored covered, and the clean vials were stored in pre-cleaned polyethylene sample bags.

3.3 Sample Collection and Handling

The samples of *P. borealis* were collected by Fisheries and Oceans Canada, Newfoundland region, during commercial fishing by the vessel "Otter". The samples used for the optimization of the methods were caught off the coast of Labrador (latitude 55°00', longitude 54°24') in 1995 January. Seven kg of shrimp were quick frozen, wrapped in polyethylene, stored in a box, and kept frozen until arrival in St. John's, Newfoundland. The shrimp were then packed in dry ice and liquid nitrogen and transported by air to Halifax. For long-term storage, the shrimp were kept at –25°C.

For much of the optimization work, whole shrimp was employed. Where EOX were determined in muscle or roe, the following procedure was used to separate shrimp tissue from the whole shrimp. First, the head was broken off from a partially thawed shrimp; most of the organs were also removed by this action. Stainless steel dissecting scissors were used to make a dorsal cut on the thin shell. The muscle was removed and subjected to the dry ice homogenization procedure described below. Eggs, when present in female shrimp, were analyzed separately from the shrimp muscle.

3.4 Homogenization

To provide a batch of tissue that would be suitable for comparison of extraction efficiencies, a large amount of tissue had to be homogenized so that all samples would have similar composition. The dry ice method given below was selected because it resulted in solid homogenate and did not alter the composition of the matrix, which is not always true with some other methods such as those using sodium sulfate. It was also

suitable for application to fatty matrices because the dry ice aided in keeping the sample frozen and lipids solid.

Approximately 500 g of shrimp were prepared for use in the optimization procedure. Batches of whole shrimp or of shrimp muscle were subjected to homogenization with dry ice in a large blender. The roe were not subjected to this procedure, as they were not used in the optimization experiments.

The plastic blender and its titanium blades were washed with distilled water and liquid soap (Fisher Versa-Clean) three times, rinsed thoroughly with DDW, and air-dried. The blender (Robot-Coupe, Inc.) was assembled with the two blades at 180° to one another. Frozen shrimp (whole or muscle only) were placed in the blender with an equal volume of chunked dry ice. The blender, operating at 220V, was set at 1 500 revolutions per min (RPM) during the first stage of the homogenization process; and was continued until the solids were small pieces (< 5-mm diameter). It was then switched to 3 000 RPM and processing was continued until the material resembled a granular powder (1-2 mm in diameter). The entire process took about 10 min. The blender container, with cover and blades in place, was then transferred to a freezer at -25°C. At this temperature, the dry ice sublimed leaving a shrimp homogenate. Individual samples were taken from this homogenate, weighed, and stored (at -25°C) in clean glass vials with Teflon-lined tops. These samples were used for individual extraction experiments.

The degree of contamination that might have been associated with the homogenization process was determined in the following manner using Mazola food grade corn oil. This oil was selected because it contained low levels of halogens. Twenty-five mL of the Mazola oil, 250 mL of water and approximately 250 mL of dry

ice were homogenized in the same manner as the shrimp samples. After sublimation of the dry ice, a portion of the homogenate was extracted, as described in Section 3.11. The organohalogen content was determined by NAA as described below and this result was compared to untreated corn oil and is presented in Section 3.10.3.

3.5 Lipid Determination

The lipid content of a shrimp sample was determined by pipetting 1 000 µL of the sample extract into a tared, 1.2-mL polyethylene vial. The vial was placed in a fume hood, and the solvent was allowed to evaporate to dryness and constant weight. The sample was left for no more than 24 h because of the possibility of oxidation of unsaturated lipids causing the mass determination to be artificially high. The vial containing the extract residue (lipid) was weighed to four decimal places, and the mass of the lipid contained in 1 000 µL was calculated. This can be used to calculate the percent lipid in the individual samples.

3.6 Elemental Comparator Standards

The potassium halide salts were dried in an oven for at least 24 h at 110°C, then transferred to and stored in a desiccator. These salts were used to produce all aqueous comparator standards used in this work.

The concentrations of the elemental standards should approximate the levels found in actual samples. Typically, samples contained more chlorine than bromine, and more bromine than iodine. Conveniently, this trend was inverse to the gradient of sensitivities for the halogens *i.e.* iodine is more sensitive than bromine, and bromine is

more sensitive than chlorine by NAA. Therefore, the lower levels of bromine and iodine were still easily detected.

Stock solutions were made for each of the halogens studied, *i.e.* chlorine, bromine, and iodine. For chlorine, 2.103g of KCl was weighed and transferred to a 1 000-mL pre-cleaned volumetric flask, and made up to the mark with DDW yielding a 1 000 ppm solution. A KBr primary stock solution was made by dissolving 3.270g of KBr in DDW and was made up to the mark in a 500-mL volumetric flask. Then a secondary stock solution of 43.92 ppm of KBr was made from the above solution. A similar procedure was carried out with 1.489g of KI measured into a 500-mL volumetric flask, 0.25g of KOH was also added before DDW. A secondary stock solution of 22.77 ppm KI was made from the primary stock solution, and about 0.5g of KOH was added. The final standard solution used for irradiations contained the following concentrations of halides: 10.0 ppm chlorine, 0.439 ppm bromine, 0.227 ppm iodine, and 0.5g L^{-1} of KOH.

Vials containing these aqueous standards were prepared, irradiated, transferred and counted in the same way as the samples. The sensitivities (*i.e.* counts μg^{-1} of analyte) of the halides were calculated and presented in Section 3.9.

3.7 Preparation of Samples for Irradiation

Aliquots of approximately 750 μL of the sample solution or standard solution were pipetted into pre-cleaned, 1.2-mL polyethylene vials. These vials were immediately sealed and placed inside a larger polyethylene vial along with a spacer vial and sealed again. The spacer vial is used to stop the sample vial from moving about during transfer

to and from the reactor. If the sample moves too much, the seal may break, leading to a lost sample and possible contamination of the irradiation site.

3.8 Irradiation, Transfer, and Counting Schemes

The method used for the determination of halogens in liquid samples was previously developed in this laboratory [33] and applied to the determination of chlorine in fisheries samples. This method was slightly modified and employed for the simultaneous determination of chlorine, bromine and iodine.

All irradiations were performed at the Dalhousie University SLOWPOKE-2 reactor. Samples, standards, and blanks were irradiated in the inner sites with a constant neutron flux of 5×10^{11} cm^{-2} s^{-1}.

Each sample, standard, or blank was irradiated for 30 min, and allowed to decay for 5 min. During the decay period, the vial was carefully opened and a 500-μL portion of the irradiated liquid was transferred into a fresh inactive (*i.e.* non-irradiated) vial. Since the irradiation vials contained variable amounts of chlorine, the use of a fresh vial helped minimize the blank contribution. The sample was then counted for 30 min. The 1642.4- and 2167.5-keV photopeaks of ^{38}Cl were used for the quantification of chlorine, the 617-keV photopeak of ^{80}Br for bromine, and the 442.9-keV photopeak of ^{128}I for iodine. The nuclear data [249] such as isotopic abundance, cross-sections for neutron capture, half life, *etc.* for the elements of interest are presented in Table 3.2. The two photopeaks used for the chlorine determination originate from the same nuclide and have similar sensitivities. They can be and were used for quality control (QC) purposes.

Table 3.2. Characteristics of Halide Nuclides used in this Work

Parameter	^{38}Cl	^{80}Br	^{128}I
stable isotope	^{37}Cl	^{79}Br	^{127}I
isotopic abundance	24.23%	50.69%	100%
cross-section: for thermal neutron capture	428×10^{-27} cm^2	8.5×10^{-24} cm^2	6.2×10^{-24} cm^2
for epithermal neutron capture	213×10^{-27}cm^2	148×10^{-24}cm^2	147×10^{-24}cm^2
nuclear reaction	$^{37}Cl(n,\gamma)^{38}Cl$	$^{79}Br(n,\gamma)^{80}Br$	$^{127}I(n,\gamma)^{128}I$
half life ($t_{1/2}$)	37.24 min	17.68 min	24.99 min
most intense photopeak(s)	1642.40 keV, 2167.50 keV	617.00 keV	442.9 keV

Active samples were counted on one of the two lithium-drifted germanium semiconductor detectors (Ge(Li) #1 and Ge(Li) #2). The active volume of Ge(Li) #1 is 60 cm^3. It was manufactured by Canberra and attached to an Aptec multichannel analyzer (MCA) card. This detector had a resolution of 1.88 keV at the 1332-keV photopeak of ^{60}Co, a peak-to-Compton ratio of 35:1 and an efficiency of 9.5%. The Ge(Li) #2 has an active volume of 40 cm^3. This detector, manufactured by Princeton Gamma-tech, was connected to an Aptec MCA card. The Ge(Li) #2 detector had a resolution of 2.02 keV at the 1332-keV photopeak, a peak-to-Compton ratio of 30:1, and an efficiency of 7.1%.

3.9 Sensitivity, Detection Limits and Counting Error

The sensitivity of a given nuclide for NAA depends mainly on neutron flux, cross-section for neutrons, isotopic abundance, detector efficiency, γ-branching ratio, the

decay constant and the irradiation, decay and counting schemes [250]. Of these, most of the variables are intrinsic to the element of interest while others like neutron flux and detector efficiency usually remain constant over time. The main parameters that can be varied to optimize sensitivity are the irradiation, decay and counting schemes. Longer irradiation times with shorter decay times may lead to increased sensitivity, but may also increase background when other elements are also present in a sample and thus gives poorer detection limits.

Detection limits are directly related to sensitivity and background. If the sensitivity is high and the background is low, the detection limit will be low. Since the background is variable (mainly from contributions from other elements and the sample matrix), therefore the detection limit is also variable. The detection limits were lower for extracts from shrimp muscle than from the whole shrimp and shrimp roe. Shrimp muscle tended to be low in EOX levels and often had a correspondingly low background. Shrimp eggs contained the highest levels of EOX but the detection limits were also high because of the higher background. The sensitivities and typical detection limits are presented in Table 3.3.

The limit of detection for any photopeak detected depends on the mean of the counts for the blank (μ_B) for that photopeak. The detection limit (L_D) is the true net signal level that has a magnitude sufficient to lead to detection. This is defined by Currie [251] as:

$$L_D = 2.71 + 4.65\,(\mu_B)^{1/2} \qquad \text{Eq. 3.1}$$

The blank should be exactly the same as the sample of interest except the element of interest is not present. Since it is difficult to determine exactly the composition of the

matrix in real samples, it is useful to use the background counts (B) instead of the blank counts for this determination. Using this system, Currie's equation can be rewritten as:

$$L_D = 2.71 + 4.65\,(B)^{1/2} \qquad \text{Eq. 3.2}$$

This criterion was used to determine the validity of results in this work. As the background varies from sample to sample, this limit was determined on a case by case basis.

Table 3.3. Sensitivities and Detection Limits

	Cl 1642.4 keV (2167.5 keV)	Br 617 keV	I 442.9 keV
Sensitivity (counts μg^{-1}) Ge(Li) #1	1 013 ± 1.6% (1 031 ± 1.9%)	21 876 ± 3.5%	70 240 ± 2.1%
Sensitivity (counts μg^{-1}) Ge(Li) #2	993 ± 3.2% (974 ± 3.3%)	20 065 ± 4.0%	74 026 ± 3.4%
detection limits	32 ng mL^{-1}	2.0 ng mL^{-1}	0.68 ng mL^{-1}

The estimated counting error (or uncertainty) associated with a given photopeak is proportional to the total number of counts (net signal plus background signal) under that photopeak, and is defined as:

$$\text{Error} = (\text{total counts under photopeak})^{1/2} \qquad \text{Eq. 3.3}$$

As peak size increases the absolute error increases, but the relative error decreases.

3.10 Quality Assurance

A QA program is a necessary part of any analysis. If there is no proof that the error and bias are in control, then reported results have little value. This is particularly important for this thesis work, as there are a number of variables that contribute to each result. There is also considerable possibility for variation due to the inherent inhomogeneity of biological samples. For these reasons, an extensive QA program was undertaken to address these concerns.

3.10.1 Internal Quality Assessment and Control Charts

Elemental comparator standards were prepared and analyzed by INAA. To maintain quality assurance, attempts were made to irradiate at least one comparator standard on every other day of irradiations. Sufficient data were collected to make a reliable determination of sensitivity and to provide a means to ensure that there were no significant problems with the NAA method used on any given day.

These were used to determine the sensitivities for chlorine, bromine, and iodine. By analyzing standards over the duration of the experiment, the results also served as a means of monitoring performance. These data were plotted and presented as control charts.

The control charts for the two photopeaks for chlorine can be seen in Figs. 3.1 and 3.2. Each major horizontal division is one unit of standard deviation (σ) and the upper and lower warning limits are given by $\pm 2\sigma$. Results more than $\pm 3\sigma$ from the mean are beyond the control limit and considered to be influenced by determinate errors. The elemental sensitivities used to calculate the concentrations in samples were up-dated for

the first 25 values of comparator standards. The sensitivities for chlorine using the Ge(Li) #2 detector were 993 counts μg^{-1} ± 3.2% for the photopeak at 1642.4-keV and 974 ± 3.3% for the photopeak at 2167.5-keV. The standard deviations were acceptable, as the error associated with the counting alone was 1.6%.

Bromine and iodine were treated in the same manner. The sensitivity for bromine using the Ge(Li) #2 detector was 20 065 counts μg^{-1} ± 4.0%. This is an acceptable standard deviation as the error associated with the counting was 2.2%. The control chart for bromine is shown in Fig. 3.3.

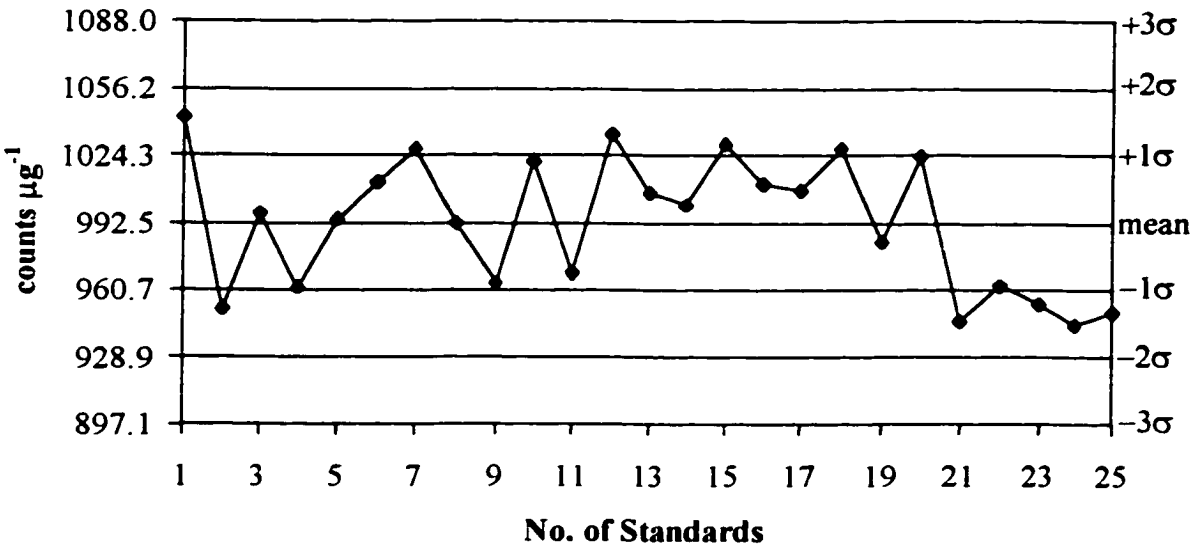


Fig. 3.1. Internal Quality Assessment Chart for Cl Standards at the 1642.4-keV Peak

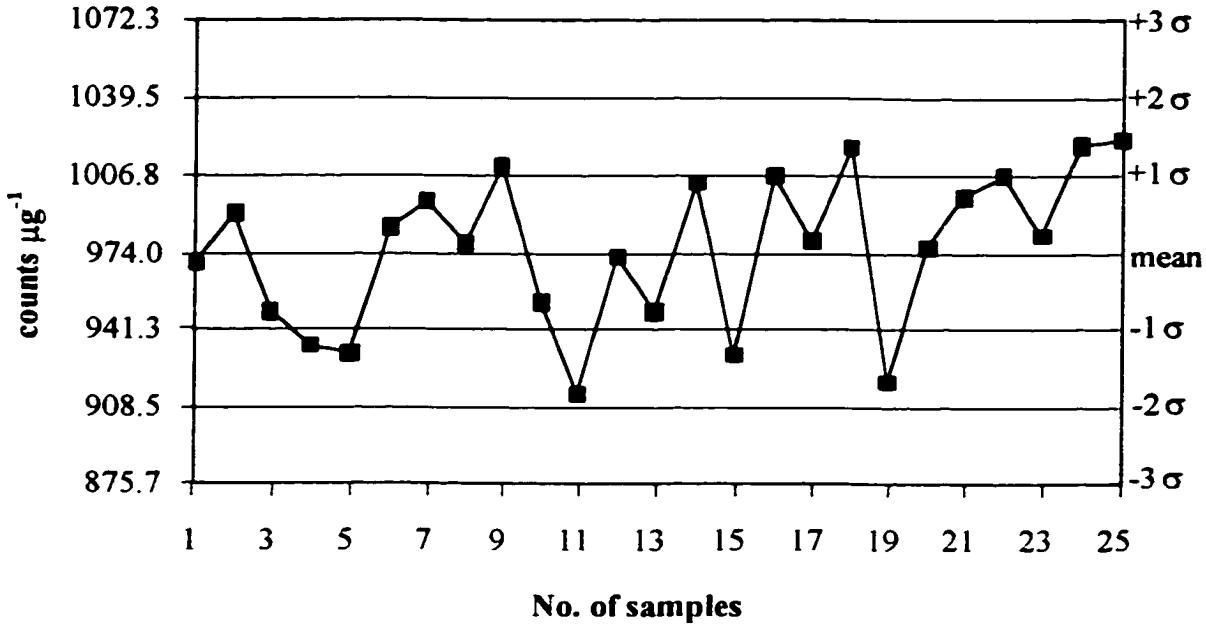


Fig. 3.2. Internal Quality Assessment Chart for Cl Standards at the 2167.5-keV Peak

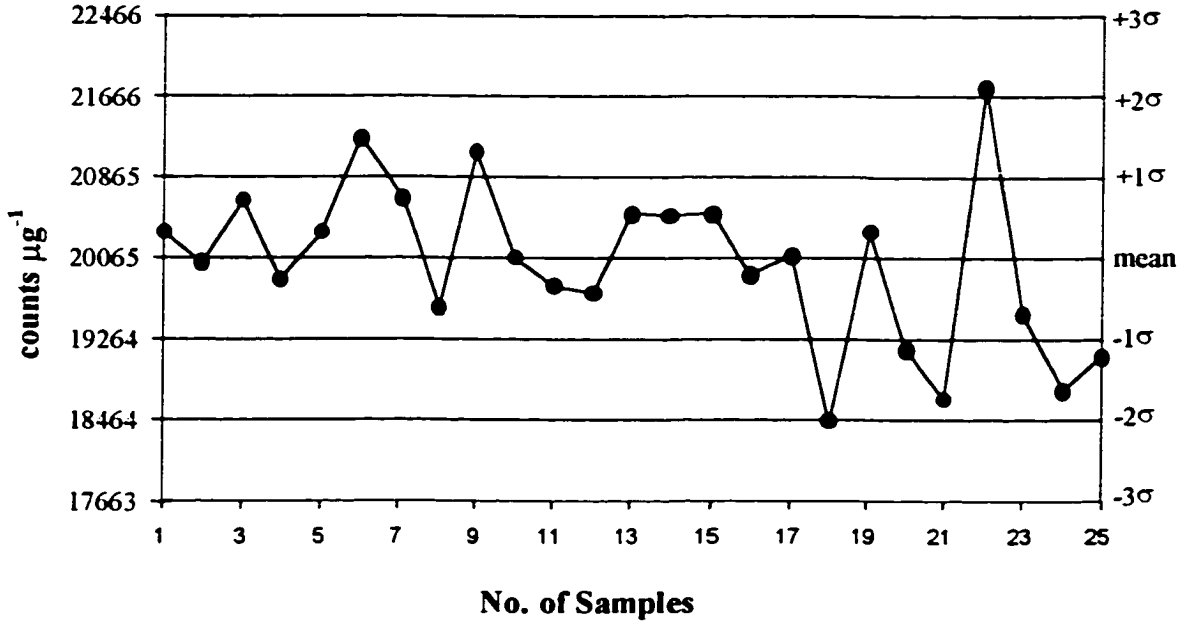


Fig. 3.3. Internal Quality Assessment Chart for Br Standards at the 617-keV Peak

The sensitivity for iodine using Ge(Li) #2 was 74 211 counts μg^{-1} ± 2.1%. The standard deviation is reasonable, as the error associated with the counting alone was 1.5%. The control chart for iodine is shown in Fig. 3.4.

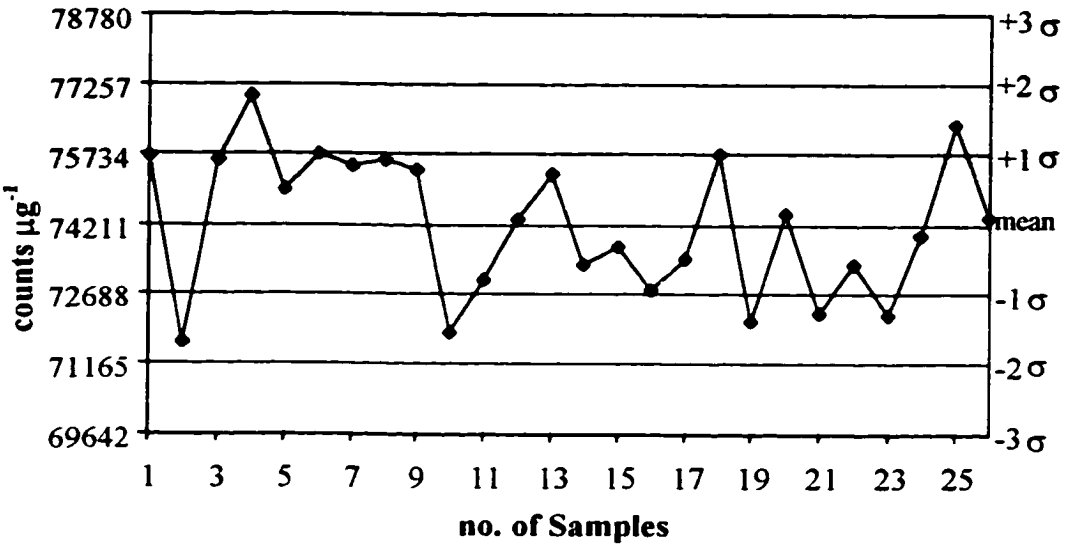


Fig. 3.4. Internal Quality Assessment Chart for I Standards at the 442.9-keV Peak

In later experiments the Ge(Li) #1 detector was also used. The sensitivities and relative standard deviations were similar to those reported for Ge(Li) #2. For chlorine, the sensitivities were 1 013 counts μg^{-1} ± 1.6% for the peak at 1642.4 keV and 1 031 counts μg^{-1} ± 1.9% for the peak at 2167.5 keV. The sensitivity for bromine was 21 877 counts μg^{-1} ± 3.5% for the peak at 617 keV and for iodine it was 70 240 counts μg^{-1} ± 2.1% for the peak at 442.9 keV.

3.1.2 Halogen Levels in Glass Distilled Solvents

The next step was to determine by NAA the concentrations of EOX present in each solvent to ensure large quantities of contaminants were not being introduced through the solvents.

The values given in Table 3.4 represent the average of 5 individual aliquots of each solvent. The detection limits were calculated for each individual result, and were based on the respective background counts. Most of the solvents contained very low levels of halogens as evident from Table 3.4. For example, chlorine content varied between 0.08 and 1.36 ppm with the exceptions of ethyl acetate and toluene. Toluene was found to contain 4.45 ppm of chlorine. This contamination may be due to co-distillation of an OCl (*e.g.* chlorobenzene, B.P. 112.5°C) which has a boiling point very similar to that of toluene (B.P. 110.6°C). Toluene may be used for the extraction but it must be completely removed (and the contamination with it) before the sample can be analyzed.

The levels of organobromine (OBr) and organoiodine (OI) measured in the solvents should not interfere with the analysis of organohalogens in real samples. Procedural blanks were performed to ensure that carry-over from solvent was not too great, and these are discussed in Section 3.11.2.

Acetone and petroleum ether (pet ether) were too volatile for direct irradiation in the reactor; so they were first dried in air in a fumehood and then the residue was dissolved in hexane to determine the amount of non-volatile organohalogen. This residue is most important in the present work as the final extract can be made up in a solvent with a low halogen content. Chloroform and dichloromethane, being OCl themselves, were

also analyzed for halogens in the residue. The determination of organohalogen residue was accomplished in the following manner. Exactly 750 μL of the solvent was pipetted into a cleaned glass vial and evaporated in a fumehood. The resulting residue was then dissolved in an equal amount of hexane, transferred to a vial, and irradiated.

Table 3.4. Concentrations of Organohalogen in Solvents

Solvent	Chlorine ($\mu g\ g^{-1}$)	Bromine ($\mu g\ g^{-1}$)	Iodine ($\mu g\ g^{-1}$)
ethyl acetate	2.41 ± 0.07	0.007 ± 0.006	<0.0007
acetone*	0.09 ± 0.03	0.010 ± 0.01	0.0020 ± 0.002
acetonitrile	0.42 ± 0.04	0.006 ± 0.007	0.0025 ± 0.0002
petroleum ether*	0.47 ± 0.03	0.009 ± 0.003	0.0061 ± 0.004
hexane	0.08 ± 0.02	<0.002	0.0015 ± 0.0006
isooctane	0.91 ± 0.03	0.009 ± 0.002	<0.0007
cyclohexane	0.21 ± 0.01	<0.002	<0.0007
toluene	4.45 ± 0.04	<0.002	0.0017 ± 0.0002
chloroform*	1.36 ± 0.08	0.037 ± 0.009	0.0108 ± 0.0008
dichloromethane*	0.47 ± 0.1	<0.002	<0.0007

* residue dissolved in hexane

3.1.3 Bulk Homogenization Process Blank

To ensure that no significant contamination was introduced during the bulk homogenization process, the homogenization procedure and extraction process was applied to corn oil. The concentrations of halogens in the processed oil were compared to those in the unprocessed oil. As can be seen in Table 3.5, the processed oil had more

chlorine, bromine, and iodine than the unprocessed oil. The contamination, however, was attributable not just to the homogenization process but also to the extraction, evaporation and drying processes. The amount of organohalogen in the corn oil (on a lipid basis) was small compared to the concentrations of extracted organohalogens found in the shrimp lipids (Section 4.3). For this reason, the homogenization method was considered acceptable.

Table 3.5. Contamination due to Homogenization

Sample	μg Cl g^{-1} lipid (RSD)	μg Br g^{-1} lipid (RSD)	μg I g^{-1} lipid (RSD)
unprocessed corn oil	0.99 (10.2%)	<0.002	0.0032 (33.5%)
processed corn oil	1.25 (3.6%)	0.031 (6.3%)	0.088 (3.1%)

3.1.4 Recovery of Corn Oil

An important step in ensuring that the method was sound was to ensure that recoveries and loses were not unreasonable. One way this could be evaluated, at least in terms of lipid recovery, was to spike dry tissue with corn oil. Tissue that had been extracted by the method used in this thesis work was reserved. The dry tissue was ground to a powder, and 1 g of corn oil was added to 4g portions of the powder and the lipid was allowed to absorb for 1 hour. Then 5 mL of DDW were added to yield a sample of ~10g and the sample was then extracted as described in Section 3.11.1. The amount of lipid extracted was determined as described in Section 3.5. The recovery for

corn oil was 99.27% and the relative standard deviation was 1.2% for the 4 samples prepared.

While the recovery for the method was very good, the results are only an approximation of the true recovery from a real sample due to certain intrinsic limitations of the experiment. The corn oil lipids could not be incorporated into the sample cell membranes, therefore the extraction of corn oil does not accurately replicate the extraction of membrane lipids which account for a large portion of lipids in shrimp samples. An additional shortcoming of this method was that corn oil does not accurately approximate the shrimp lipid composition. Corn oil is composed of triacylglycerols [252], whereas shrimp primarily contain a mixture of triacylglycerols and phospholipids [241]. The corn oil lipid may also interact with the dried tissue in ways that may not occur with sample that has not been dried. For example, Berdié and Grimalt [253] report that recovery of organohalogens from lyophilized samples was lower than those ground with sodium sulfate. It is possible that if organohalogens have lower recoveries under those conditions, then the same may hold true for lipids. Nevertheless, the experiment does suggest that losses due to the procedure are small.

3.1.5 Effect of Storage on EOX Levels

To ensure that levels of EOX measured in shrimp that had been stored for extended periods were reliable, EOX were measured in a fresh crab sample and then compared to the results determined after periods of storage. Since live shrimp are not available at all and live crab are easily obtainable from local fish markets and since their lipid content and composition should be somewhat similar, crab was used. A crab was

purchased, killed and the soft tissue collected. The tissue was frozen with liquid nitrogen and then homogenized with dry ice as described in Section 3.4. The homogenate was weighed into clean glass vials and stored in the same manner as the shrimp homogenate. Samples were extracted and the levels of EOX were determined the next day and again after 109 days of storage. They were also measured in samples that had been stored frozen for 109 days and were then kept for an additional 10 days at 8°C. The sample kept for the additional time at a higher temperature had decomposed to the point that it was off gassing and portions had turned black, likely due to microbial growth. The results of those experiments are presented in Figs. 3.5, 3.6 and 3.7 for chlorine, bromine and iodine, respectively.

The mass of tissue recovered from the crab allowed for the extraction and analysis of only three samples for each time period. There is significant variability from sample to sample, making it difficult to make conclusive statements on the stability of EOX during storage. Much of the variability can be attributed to the lack of homogeneity in the sample. Still, when taking this variation into account some conclusions can be drawn. For EOCl, there is no statistical difference between the samples that were measured in tissues that had not been stored (3.1 ± 1.0 μg g^{-1} tissue) and those that had been stored frozen for over 3 months (4.7 ± 0.8 μg g^{-1} tissue). It seems unlikely that EOCl would be gained during storage and since the samples were pre-weighed before storage, it also seems unlikely that the higher concentration reported after storage would be due to a relative increase in mass to mass ratio because of loss of moisture in the sample. The samples that were stored for an additional 10 days at 8°C do seem to have a loss in EOCl (1.9 ± 0.1 μg g^{-1} tissue). The deviation is also much lower in these samples suggesting

that the remaining EOCl might be distributed more homogeneously in the organism. These findings are consistent with reports in the literature that found no change in EOCl in herring and pike stored at –20°C for two years and no change in lipid for the herring, but a slight change in lipid content in pike [254]. De Boer and Smedes [255] reported that for biological samples stored for 2 years or less, storage at –25°C was sufficient to prevent degradation of triglycerides in the matrix. For longer storage times –75°C was recommended.

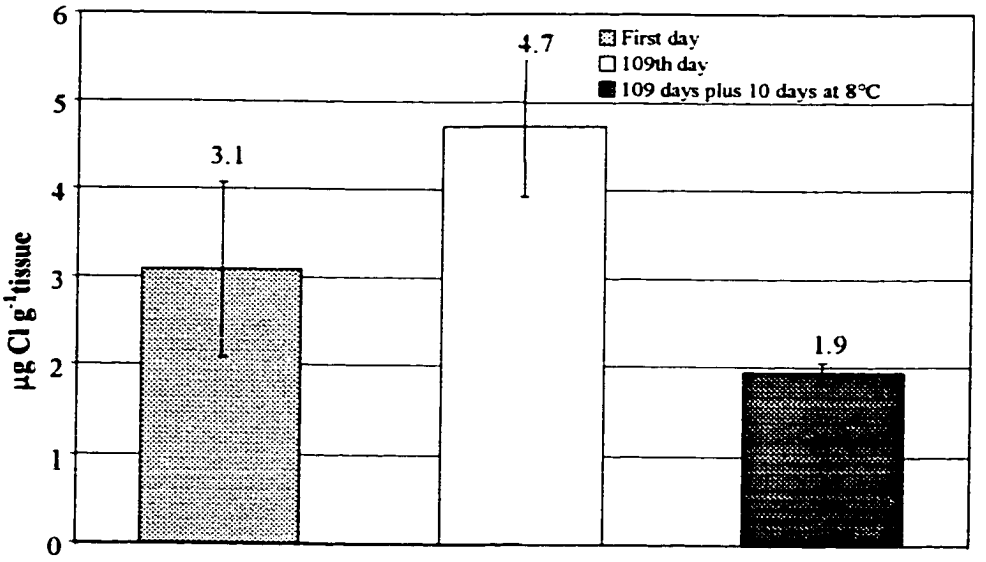


Fig. 3.5. Stability of EOCl in Crab during Storage

For EOBr there appears to be no statistical difference between the levels in the fresh samples (0.69 ± 0.06 µg g^{-1} tissue) and those which had been stored (0.63 ± 0.03 µg g^{-1} tissue for 109 days and 0.81 ± 0.08 µg g^{-1} tissue for 119 days). Storage at temperatures above freezing seemed to have affected the levels of EOI in the same way

as EOCl, showing lower values, whereas those stored at freezing differed little from the fresh samples.

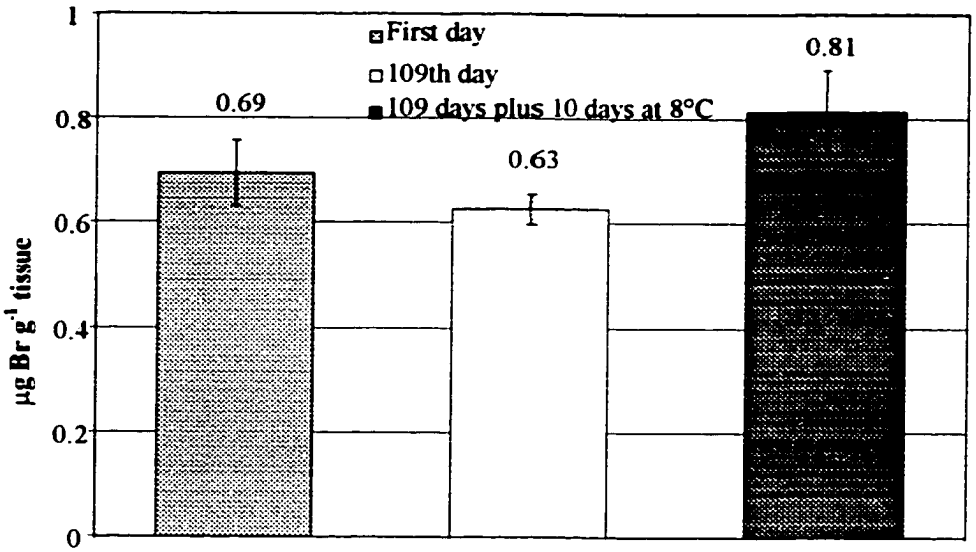


Fig. 3.6. Stability of EOBr in Crab during Storage

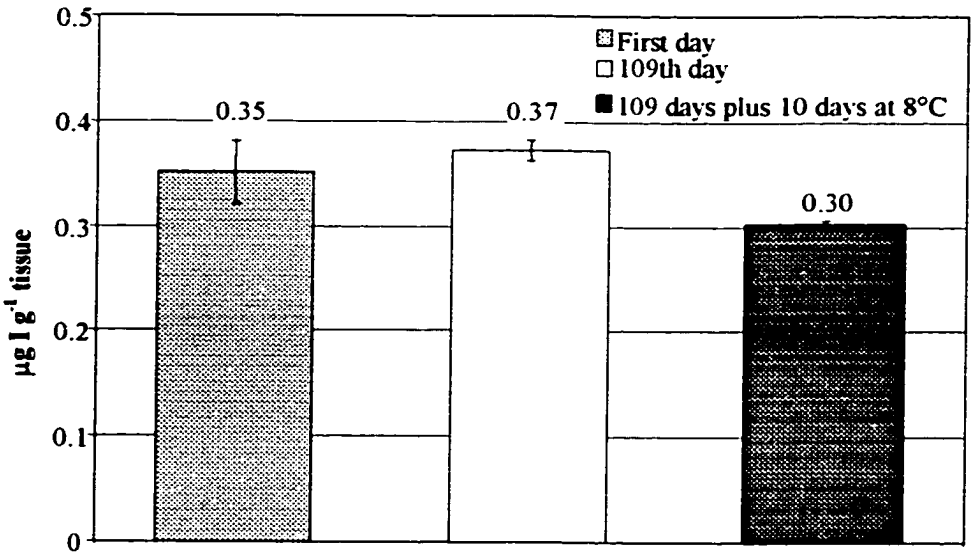


Fig. 3.7. Stability of EOI in Crab during Storage

3.1.6 Variability due to Pipetting

One of the steps for the determination of halogens in liquid samples involves the transfer of the radioactive sample from an active vial to one that has not been irradiated. This transfer was accomplished using a Barnstead/Thermolyne Corp. P250 Poppett® displacement micropipetor. The design of this type of pipet is particularly useful for use with samples and solvents with low or high viscosities. Part of the experimental error in sample determination is related to the error in the pipet. This error was determined by pipetting and weighing 500 μL of distilled water 25 times using the same tip and then repeating this process using several different tips. Distilled water was utilized because it has a well-established density and it was the solvent used to make the comparator standards.

The mean values of 25 replicate weighings ranged from 0.4950-0.4971 g, with a standard deviation of 0.0015 g. The overall mean for all weighings was 0.4965 g. The standard deviation associated with the individual tips was 0.005 g. The error associated with the balance was 0.7% and the relative standard deviation in the pipetting, as determined by weighing, was 1.02%. This value is very similar to the error associated with the weighing itself, which indicates that the precision of the pipet was very good. The negative bias from 0.5000 g for the mass of 500 μL of water can be ignored as it is eliminated by the use of the same pipet for the standards and the samples.

3.1.7 Certified Reference Materials

Certified reference materials (CRM) are particularly useful for validation of a method, since they ensure that the method meets the requirements of accuracy. The two

CRMs used in this project were the U.S. National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 1818aI and 1818aII (lubricating oils) certified for chlorine. The results are summarized in Table 3.6 for 5 replicate analyses. The errors in determination are due to sources such as pipetting, counting error, error in comparator standard preparation and analysis, and variation in the integration of peaks and so forth. The determined values were in good agreement with the certified values indicating that the NAA method was reliable for the determination of chlorine.

Table 3.6. NIST Standard Reference Materials: Chlorine in Lubricating Oils

NIST SRM Number	Determined Value ($mg\ kg^{-1}$)	Certified Value ($mg\ kg^{-1}$)
1818aI	29.6 ± 0.6*	31.6 ± 0.9
1818aII	58.0 ± 0.7*	60.0 ± 2.3

* 5 replicates

Although these SRMs are not certified for bromine, they did contain that element and so the bromine was quantified. SRM number 1818aI was found to contain 1.44 μg Br g^{-1} ± 0.6%. SRM 1818aII contained 4.19 μg Br g^{-1} ± 1%.

3.1.8 Half-Life Determination

The determination of half-life of a nuclide can be useful in demonstrating the absence of spectral interferences. The half-lives for ^{38}Cl, ^{80}Br and ^{128}I were determined in this work. Comparator standards were employed for this determination as they had

sufficiently high concentrations to produce measurable counts over more than 2 half-lives. Actual samples contained relatively low concentrations of halogens. They also contained very few additional nuclides when compared to the standards, hence, no unknown sources of spectral interferences.

Standards were irradiated for 30 min and transferred to a clean vial. After a 5-min decay, the samples were counted for 10 min, then recounted several times with 1-min intervals between. The count rate was calculated at the median of each count and this was used to plot a graph of the natural log counts against time. Linear regression was used to determine the half-life for each nuclide. The calculated half-lives are reported in Table 3.7.

Table 3.7. Half-life Determinations

Nuclide	Calculated Half-life (min)	Reported Half-life (min)
^{38}Cl	37.3 ± 1.4	37.24
^{80}Br	18.9 ± 0.6	17.68
^{128}I	24.6 ± 0.6	24.99

The calculated half-lives for chlorine and iodine are within 1σ of the reported half-lives while the calculated value for bromine seems to have a positive bias but still falls within 2σ of the reported value. The bias might be related to the contribution to the 617-keV peak for ^{80}Br from the double escape peak for ^{38}Cl at 620.4 keV. In actual

samples the relative concentration of chlorine compared to bromine is lower than that of the standards and in most cases the relative contribution from the chlorine 620.4-keV peak was negligible. A γ-ray spectrum of an actual sample is presented in Fig. 3.8; it can be seen that it consists primarily of the nuclides of interest as well as some that are found in the sample (such as ^{42}K) and in background (^{41}Ar).

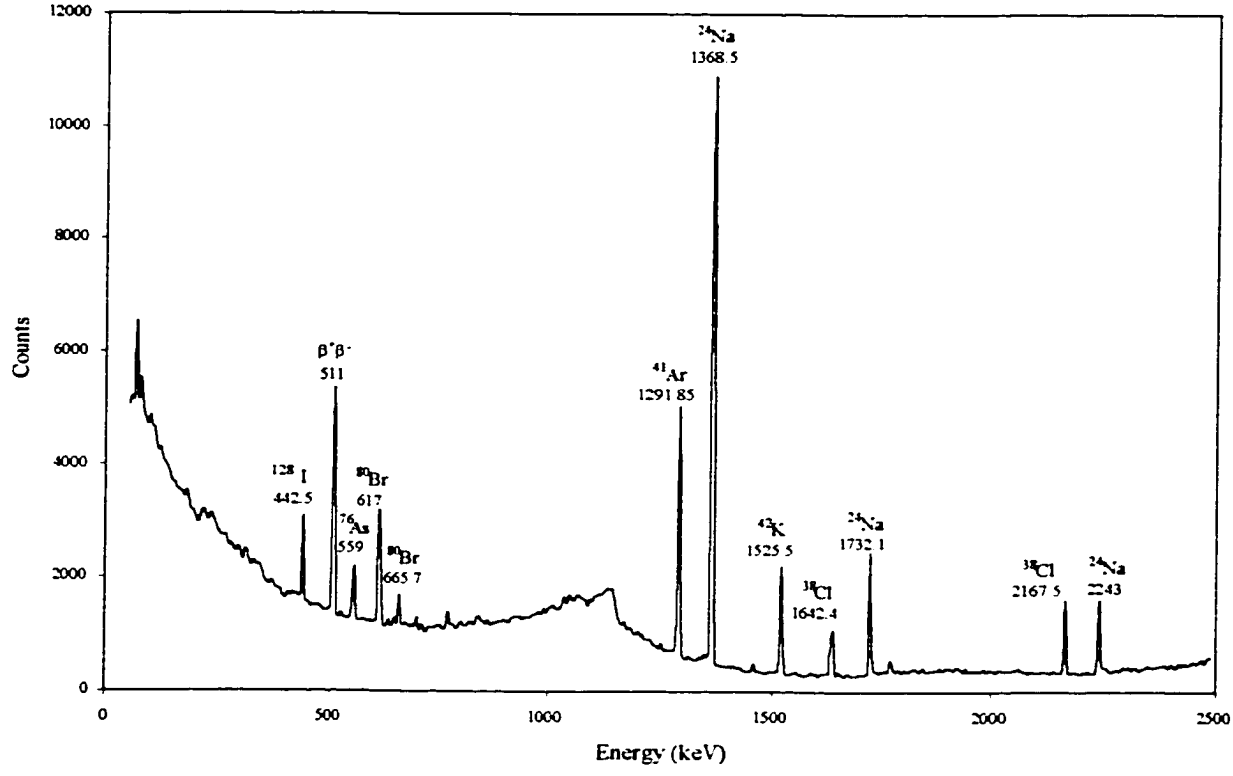


Fig. 3.8. γ-ray Spectrum of an Actual Sample

3.1.9 Effects of Shorter Irradiation Times and Dilution Effects

Shorter irradiation times and dilutions basically yield the same effect, which is to produce a sample with a smaller amount of active nuclide than a sample that has been irradiated for a longer time or for one that is of higher concentration. Usually it is best to

avoid changes to the irradiation scheme as they result in values that have a higher counting error. There are, however, situations when changing the scheme might be desirable or necessary. For example, if a sample has a high concentration of the target nuclide and thus a very high activity then the detector dead time may be too large, in which case it would be useful to reduce the amount of activity. This can be done either by dilution or reduction in irradiation time. Both require some mathematical corrections so that the result can be related to the values determined using the normal scheme. In the case of dilutions, the calculation is the simple act of multiplying the result by the dilution factor. To account for the shorter irradiation time, the corrected counts are calculated using a modification of the activation equation:

$$counts_{corrected} = counts\ (1-e^{-\lambda t_i})/\ (1-e^{-\lambda t_i'}) \qquad \text{Eq. 3.3}$$

where counts are the net counts,
t_i is the usual irradiation time,
t_i' is the actual irradiation time, and
λ is the decay constant.

Some experiments were done to determine whether analyses using these corrected counts could be considered reliable. For the dilution experiments, standards were transferred as usual but during the transfer step 250 μL of the active sample was combined with 250 μL of water. This was repeated 5 times so that a 2-fold dilution was obtained. To determine the effect of a reduced irradiation time, 5 standards were irradiated for 10 min instead of the usual 30 min. The samples were then transferred and counted in the usual way. The results are presented in Table 3.8. As expected the corrected sensitivities were comparable to those found using the normal scheme, but the relative error was slightly higher for the diluted samples and for the samples irradiated for

shorter times. It can be concluded that, when necessary, it is acceptable to use these changes. If the sample contained high levels of EOX, the increase in error would not occur as the net counts would still be high relative to the background.

Table 3.8. Sensitivities for Halide Using Corrected Counts

	I (counts μg^{-1}) 442.9 keV	Br (counts μg^{-1}) 617 keV	Cl (counts μg^{-1}) 1642.4 keV	Cl (counts μg^{-1}) 2167.5 keV
dilution (2-fold)	72 551 ± 4.3%	21 885 ± 5.1%	996 ± 3.8%	985 ± 3.8%
10-min irradiation time corrected to 30-min	73 399 ± 3.9%	21 940 ± 6.0%	986 ± 3.8%	969 ± 3.7%

3.11 Method Optimization

In the study of EOX in any given species, perhaps the most important step is to determine how to recover the analytes of interest most efficiently from the matrix. Organohalogens are very lipophilic; Donato *et al.* [256] reported that xenobiotics like DDT and DDE strongly partition into membranes and extracted lipids. Therefore, complete extraction of the lipid from the tissue is important. There is significant interspecies variation of lipid composition; therefore, the most efficient solvent mixture for one species may not be the most efficient for another. It is for this reason that a variety of solvent mixtures must be evaluated for extraction of lipids from shrimp.

Many standard procedures for the extraction of lipids include the use of chlorinated solvents for extraction (and this was attempted). However, when using NAA for EOCl determination, these types of solvents should be avoided because if they are not

completely removed, they will contribute significantly to the amount of chlorine determined. Of course, all other steps leading to a sample suitable for analysis must be evaluated for their efficiency to ensure the best and most accurate recoveries free of contamination.

A few researchers [37, 170] have reported the comparison of relative extraction efficiencies of individual solvents and mixed solvent systems. Schantz *et al.* [170] compared pressurized fluid extraction (PFE) using three different solvent systems and Soxhlet extraction using dichloromethane. The solvents used with PFE were dichloromethane, acetonitrile and hexane-acetone (1:1). The recoveries for PCBs and chlorinated pesticides for all the solvent mixtures used with PFE were very similar to the results for the Soxhlet extraction. This is particularly important as the traditional means of extracting lipids and organohalogens has involved the use of chlorinated solvents, and the fact that less hazardous solvents can be employed with equal results is encouraging. However, the number of solvent systems compared were few and no reports comparing solvent efficiencies using Polytron-assisted extraction were found.

3.11.1 Extraction Procedure

Solvent extractions were performed using a Brinkman Polytron. The Polytron apparatus homogenizes by both mechanical and sonic means. Approximately 10-20 g of shrimp were extracted with 80 mL of a 1:1 solution of a non-polar and a polar organic solvent. The mixture of sample and solvents were extracted in a square shaped glass extraction container for 5 min. If there was an emulsion in the top layer (the non-polar solvent phase), then the mixture was transferred to clean solvent-resistant centrifuge

tubes. These were centrifuged for 10 min using a Damon/IEC Division clinical centrifuge and the top layer was transferred to a 500-mL Teflon separatory funnel. If no emulsion formed, then the top layer was directly transferred to the separatory funnel. All solids and aqueous phase remaining after the first extraction were re-extracted and centrifuged in the same manner as described above. All the non-polar extracts were combined in the separatory funnel. These extracts were then washed to remove any inorganic halides by the procedure described in Section 3.11.7, dried using sodium sulfate as in Section 3.11.8, and the excess solvent was removed by rotary evaporation as in Section 3.12. The halogens were determined as in described in Sections 3.7 and 3.8.

3.11.2 Solvent Mixtures for Extraction of Lipid and EOX from Whole Shrimp

One of the more important steps was to determine which solvent mixtures gave the best relative extraction efficiencies for lipid and EOX. The best solvent mixture would provide high extraction efficiency, with low procedural blanks and good reproducibility. Multiple controlled extractions employing a variety of non-polar organic solvents in a 1:1 mixture with acetone were performed. The non-polar solvents used were hexane, cyclohexane, pet ether, toluene and isooctane. Acetonitrile and ethyl acetate in combination with hexane (1:1 ratio) were also evaluated for extraction efficiency. An attempt was made to use dichloromethane and chloroform, but there was significant variability in the results for chlorine possibly due to a small amount of solvent carry-over. Additionally, the question of residual chlorinated solvent as artifacts in the values determined could not be ignored, so further attempts to use these solvents were abandoned.

3.11.3 Relative Extraction Efficiencies of Solvents for Lipid from Whole Shrimp

The seven solvent mixtures (1:1, non-polar:polar) described above were each used to extract samples of whole shrimp. Five samples of homogenized whole shrimp were extracted using each solvent mixture. The lipid was determined for each extraction in the manner described in Section 3.5 and the results are presented in terms of percentage lipid present in wet, whole shrimp homogenate. This is the most convenient representation as it accounts for variations in tissue mass used in each extraction. These results are presented in Fig. 3.9.

It appears that the hexane-acetone mixture, which yields 5.97% lipid in the shrimp on the wet weight basis, extracts the highest amount of lipid. This mixture also shows a fairly low standard deviation of 0.30%. The hexane-acetonitrile mixture extracts the lowest amount of lipid with only 4.32% lipid in the tissue isolated. If one considers the errors associated with the extractions, no differences in extraction efficiency for lipid can conclusively be detected between the hexane-acetone mixture and the pet ether-acetone or cyclohexane-acetone mixtures. In terms of the amount of lipid extracted, any of these solvent mixtures seems to be adequate. The low extraction efficiency for ethyl acetate-hexane may be due to the fact that mixture was not polar enough and so could not extract as much of the various types of lipids.

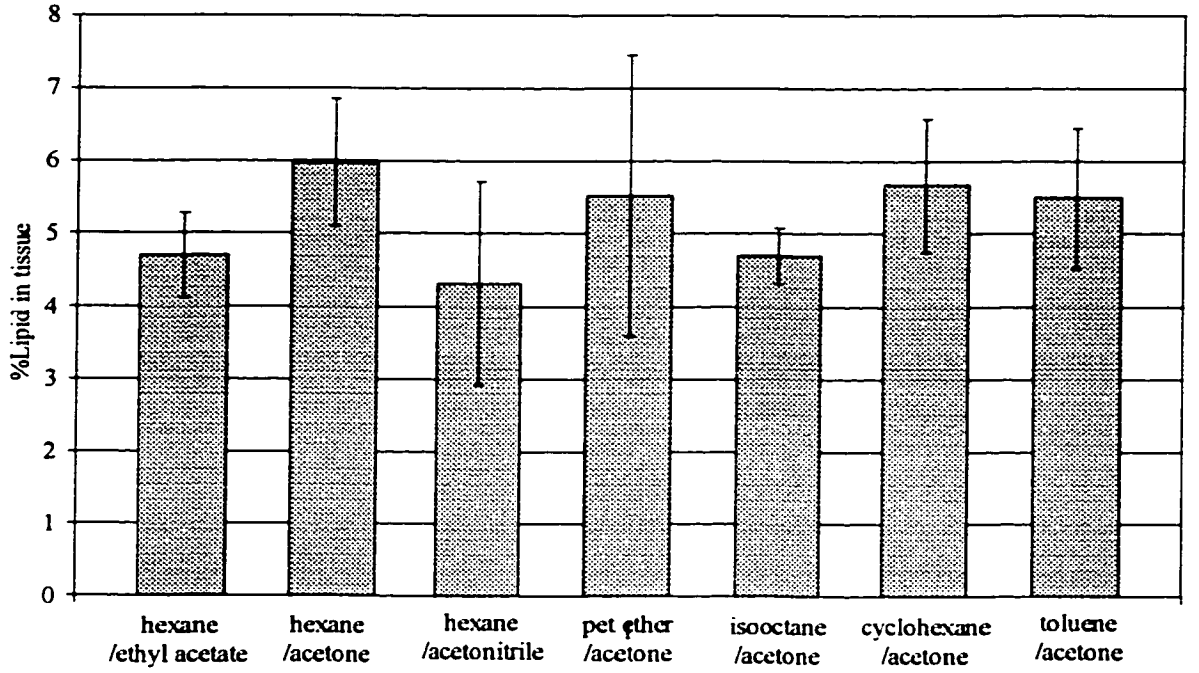


Fig. 3.9. Percent Lipid Extracted by Solvent Mixtures

3.11.4 Comparison of Solvent Systems for Extraction of EOX from Whole Shrimp

Lipid is an important part of determining the extraction efficiency for EOX as the components of EOX are lipophilic, and in other research organohalogens have been detected in lipid fractions of extracts. Of course lipid is not the only measure of good extraction efficiency, of greater interest is the extraction of EOX. The levels of EOX were measured in each of the extracts that were produced in the previous section. Procedural blanks (blank obtained from following all steps of the procedure in absence of the sample) were also determined to ensure that the levels of EOX measured were not due to contamination. The results of the analysis of the procedural blanks combined with the relative deviation among the results for each solvent were used to evaluate the various

extraction methods. The best solvent system combines good extraction efficiency with high precision and low procedural blanks.

The results for EOCl are presented in Figs. 3.10 and 3.11, and the procedural blanks in Fig. 3.12. Results for EOX in shrimp (wet weight basis) are also reported in Table 3.9. The results for the corresponding procedural blanks can be found in Table 3.10.

The results for the extraction of EOCl using the acetonitrile mixture are not presented in the figures (but are reported in Table 3.9) as both the value and its absolute deviation are very large. Acetonitrile was not miscible with the hexane so no solution that would act as a mixture of solvents was formed. The great difference in the amount of EOCl for this solvent system indicates that there might be some inorganic chlorine carried over in the solvent.

Table 3.9. Relative Extraction Efficiencies for EOX in Whole Shrimp (Wet Weight Basis)*

Solvent Mixture	Cl $\mu g\ g^{-1}$	Br $\mu g\ g^{-1}$	I $\mu g\ g^{-1}$
hexane-ethyl acetate	4.43 ± 61%	0.731 ± 31%	0.218 ± 20%
hexane-acetone	2.37 ± 9.7%	0.827 ± 9.6%	0.264 ± 9.6%
hexane-acetonitrile	62.7 ± 24%	1.03 ± 21%	0.211 ± 6.6%
pet ether-acetone	5.24 ± 9.8%	0.768 ± 10%	0.268 ± 4.2%
isooctane-acetone	4.09 ± 15%	0.742 ± 8.0%	0.225 ± 17%
cyclohexane-acetone	1.95 ± 11	0.932 ± 14%	0.234 ± 15%
toluene-acetone	2.71 ± 5.2%	0.959 ± 3.3%	0.281 ± 1.6%

*all results based on 5 sets of extractions, with five replicate irradiations per extraction

These results appear to indicate that the hexane-ethyl acetate, pet ether-acetone and the isooctane-acetone mixtures extract EOCl most efficiently (4.43, 5.25 and 4.09 μg Cl g^{-1} shrimp respectively). However, the procedural blank for hexane-ethyl acetate system (1.8 μg Cl g^{-1}) is high, as is the relative standard deviation (61%). Statistical differences between standard deviations were confirmed with a one-tailed-tailed F-test (P = 0.05). The pet ether-acetone system also has a high procedural blank (0.73 μg Cl g^{-1}) and, therefore, would not be the most suitable of the solvent mixtures. The isooctane-acetone system has a reasonable relative standard deviation (15%) and it displays a low procedural blank but it shows relatively low extraction efficiency for lipids. Since the primary interest is in the higher molecular-weight EOCl, the isooctane-acetone system may not be most desirable for this work. However, it might be better than the other mixtures for the analysis of other EOCl components such as PCB's.

Table 3.10. Procedural Blanks for Chlorine, Bromine and Iodine*

Solvent Mixture	Cl μg mL^{-1}	Br μg mL^{-1}	I μg mL^{-1}
hexane-ethyl acetate	1.8 ± 32%	0.008 ± 43%	0.002 ± 72%
hexane-acetone	0.26 ± 15%	0.005 ± 77%	0.003 ± 44%
hexane-acetonitrile	0.47 ± 12%	0.003 ± 36%	0.003 ± 48%
pet ether-acetone	0.73 ± 35%	0.009 ± 88%	0.003 ± 33%
isooctane-acetone	0.22 ± 8.0%	0.015 ± 56%	0.001 ± 17%
cyclohexane-acetone	0.901 ± 16	0.003 ± 47%	0.005 ± 19%
toluene-acetone	0.63 ± 18%	0.002 ± 77%	0.003 ± 30%

*all results based on 5 sets of extractions, with five replicate irradiations per extraction

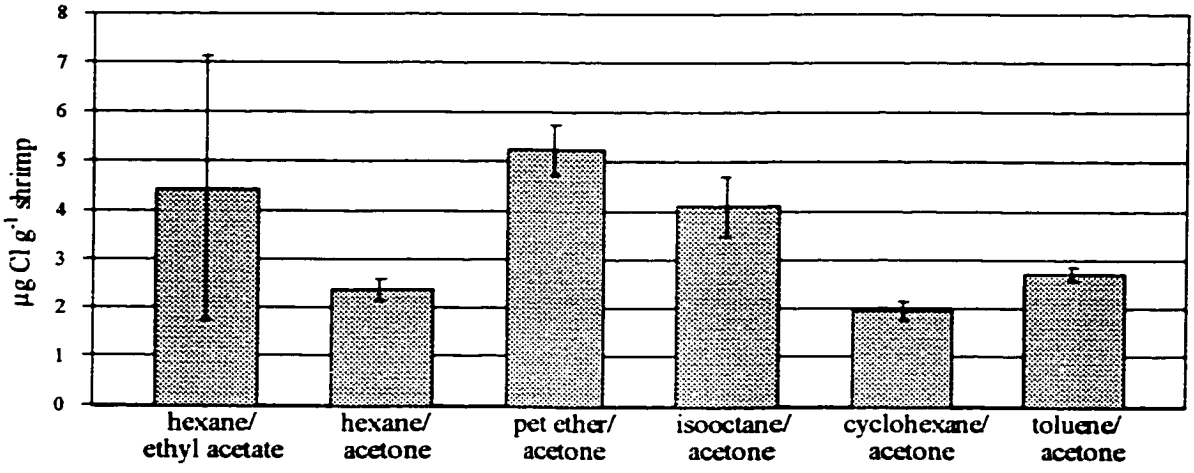


Fig. 3.10. Extraction Efficiencies of Solvents for EOCl from Whole Shrimp (wet weight basis)

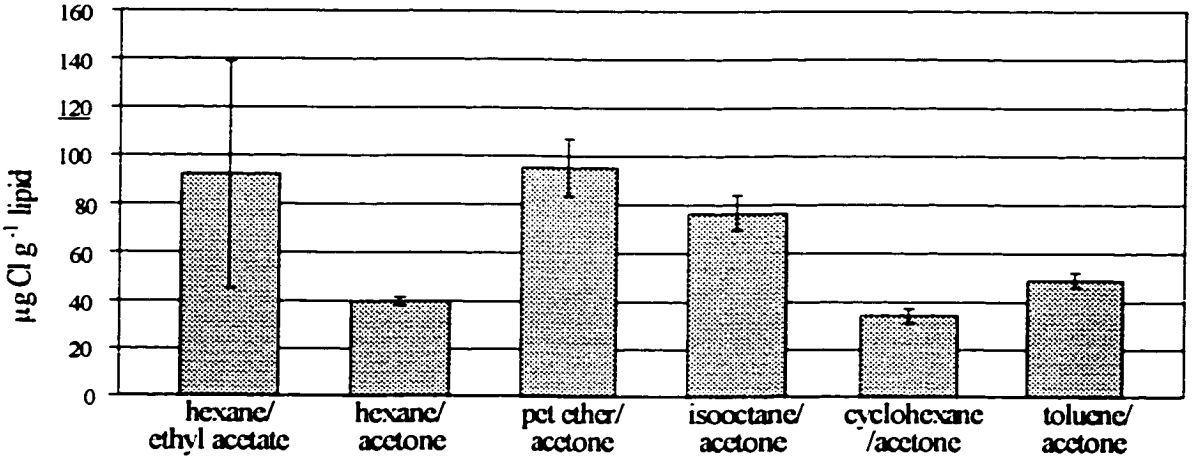


Fig. 3.11. Extraction Efficiencies of Solvents for EOCl from Whole Shrimp (lipid basis)

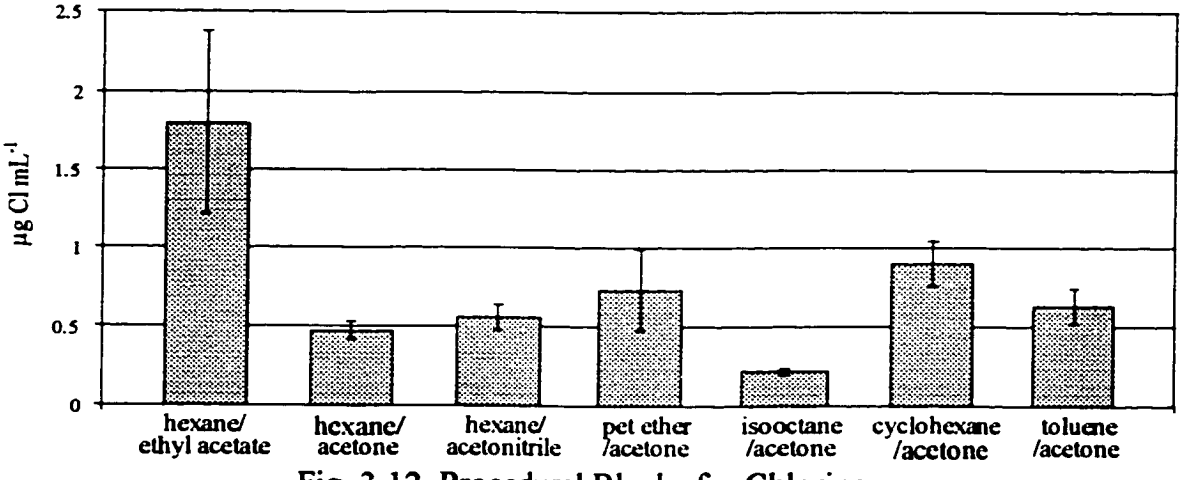


Fig. 3.12. Procedural Blanks for Chlorine

The toluene-acetone and cyclohexane-acetone systems show relatively high procedural blanks that make them undesirable as solvent mixtures. Although the hexane-acetone system appears to show a relatively low efficiency for the extraction of EOCl (2.37 μg Cl g^{-1} shrimp), it is accompanied by a low relative standard deviation (9.7%) indicating good precision. It also has a low blank (0.26 ppm Cl), with good extraction of lipid which makes it an appropriate choice for this work despite the fact the isooctane-acetone mixture yielded higher overall extraction of EOCl. Some of the variations may be due to inhomogeneity of EOCl distribution in the biological samples used. A greater number of samples might yield more conclusive results. No trend seems to exist with relation to extraction efficiency and polarity of the solvent mixture. The differences in extraction efficiency may be related to the type of compounds being extracted. It would be possible to determine whether this is a real effect through further analysis of the extract with a technique such as GC-MS.

The results for bromine are presented in Figs. 3.13, 3.14, and 3.15 and in Tables 3.9 and 3.10. The results for iodine are presented in Figs. 3.16, 3.17 and 3.18 and in Tables 3.9 and 3.10. For bromine, the results of the shrimp extracts are significantly higher than the corresponding procedural blanks. The best extraction efficiency was found using hexane-acetonitrile, 1.03 μg Br g^{-1} shrimp. However the relative standard deviation of 31% was much higher than the others which were typically around 10%. If one takes into account the relative standard deviation among the samples there is no discernable difference between the various solvent mixtures and their efficiencies for extraction of OBr. The differences appear to be independent of the amount of lipid extracted.

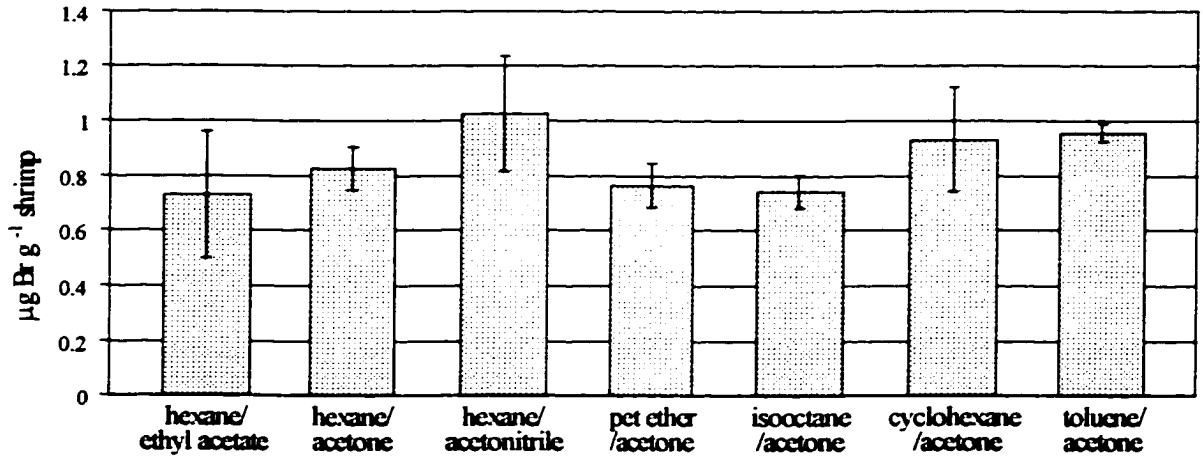


Fig. 3.13. Extraction Efficiencies of Solvents for EOBr from Whole Shrimp (Wet Weight Basis)

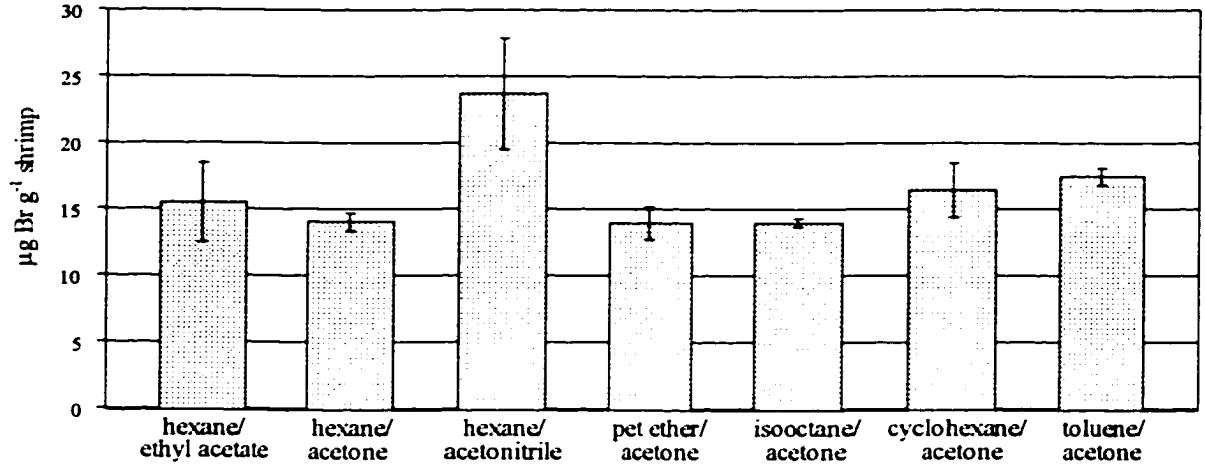


Fig. 3.14. Extraction Efficiencies of Solvents for EOBr from Whole Shrimp (Lipid Basis)

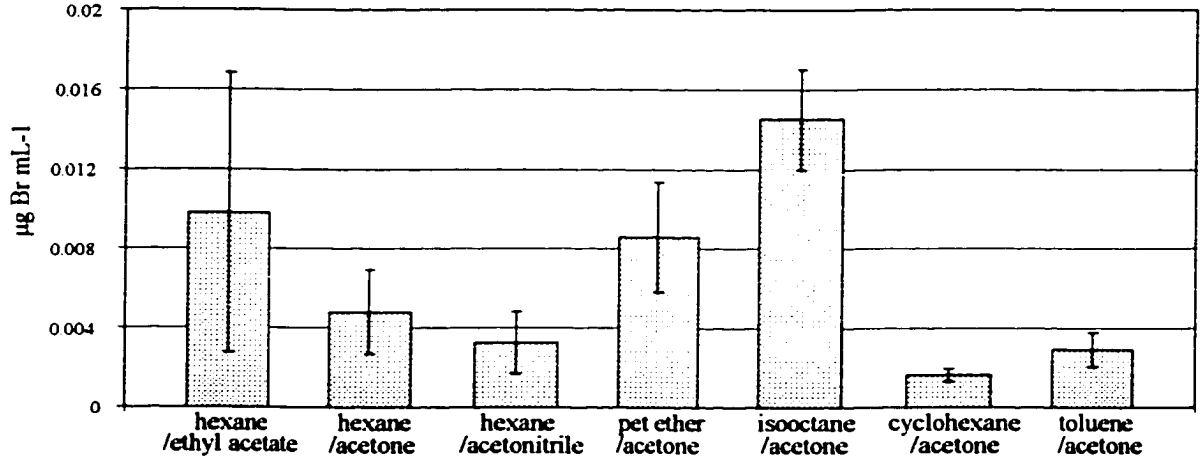


Fig. 3.15. Procedural Blanks for Bromine

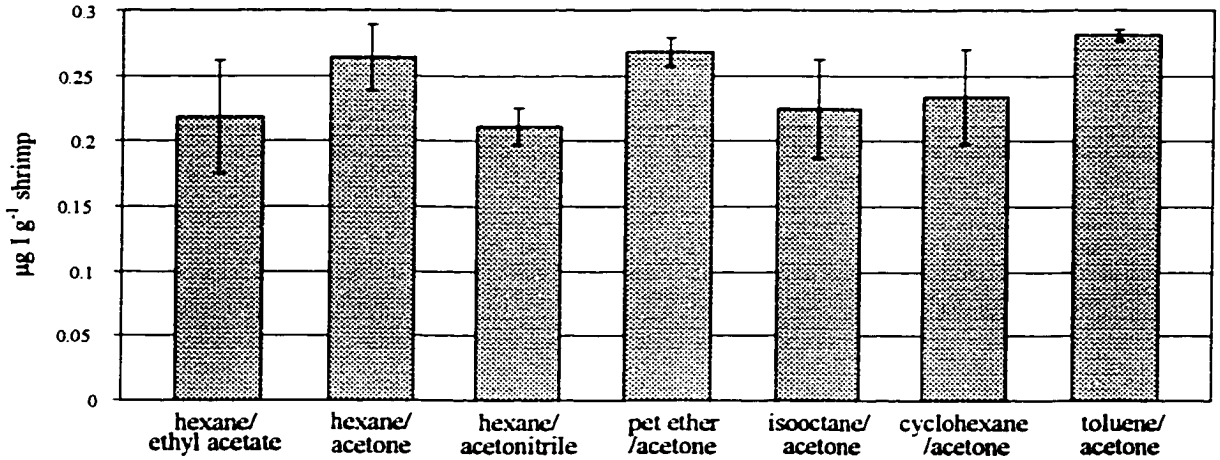
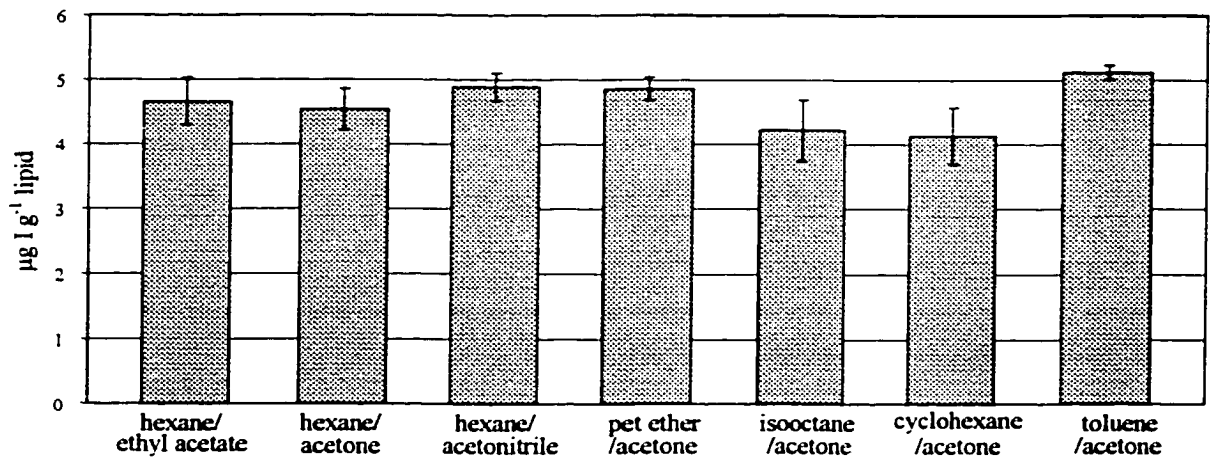


Fig. 3.16. Extraction Efficiencies of Solvents for EOI from Whole Shrimp



(Wet Weight Basis)

Fig. 3.17. Extraction Efficiencies of Solvents for EOI in Whole Shrimp (Lipid Basis)

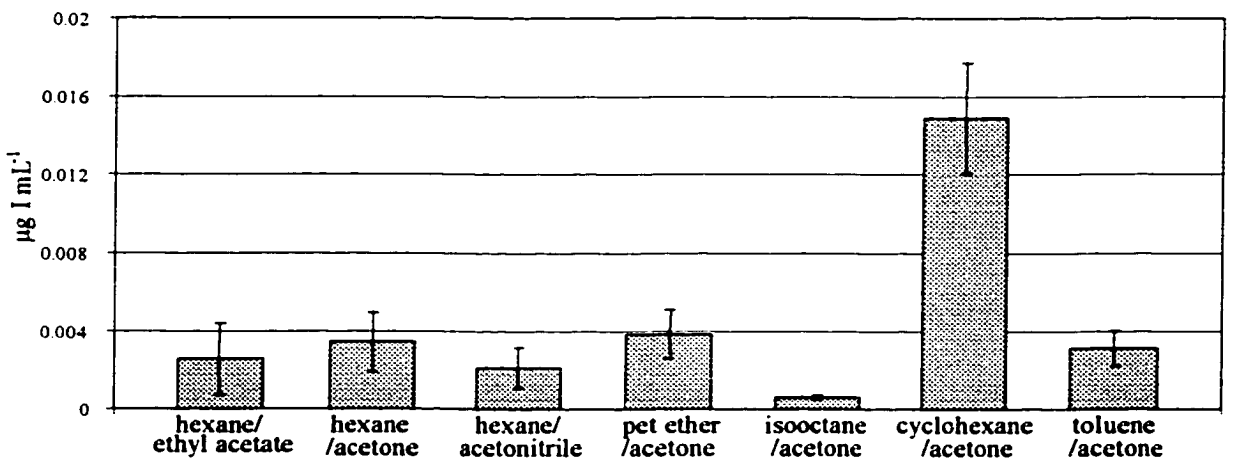


Fig. 3.18. Procedural Blanks for Iodine

Similarly, there is no discernable difference between the various solvent mixtures for the extraction of OI if one takes into account the RSD and the corrections on a lipid basis. The values for RSD range from 1.6% to 20%, with toluene-acetone providing the lowest value and hexane-ethyl acetate showing the highest. The procedural blanks are very low for iodine (0.001-0.005 ppm) and play no part in the evaluation of the solvent mixtures.

3.11.5 Extraction Efficiencies of Solvents for EOX from Shrimp Muscle

Cyclohexane, hexane, pet ether and isooctane each combined with acetone in a 1:1 ratio were used to extract ~10g of shrimp muscle. Each solvent mixture was used for at least three extractions, five aliquots from each extract were irradiated, and the means of the five aliquots were used to calculate the final results. These results are reported in Table 3.11 on a lipid basis.

Hexane was observed to have the best overall extraction efficiency for EOCl at 328 μg Cl g^{-1} lipid. The EOCl results for the remaining solvents ranged from 168 to 270 μg g^{-1} lipid. Hexane had a fairly low RSD of 12% while pet ether had the lowest of 8.1%. As mentioned previously, hexane also had one of the lowest procedural blanks (0.26 μg Cl g^{-1}).

Although the cyclohexane results for EOBr (11.4 μg g^{-1} lipid) and EOI (3.33 μg g^{-1} lipid) were higher than those of hexane (8.86 and 2.79, respectively), the relative standard deviations were higher, about 20%. For both iodine and bromine, hexane gives the next highest results with much lower RSD.

Table 3.11. Relative Extraction Efficiencies

Non-polar Solvent	Cl (RSD) $\mu g\ g^{-1}$ lipid	Br (RSD) $\mu g\ g^{-1}$ lipid	I (RSD) $\mu g\ g^{-1}$ lipid
cyclohexane-acetone	167 (16%)	11.4 (20%)	3.33 (21%)
hexane-acetone	328 (12%)	8.86 (2.0%)	2.79 (12%)
pet ether-acetone	270 (8.1%)	7.73 (13%)	1.74 (12%)
isooctane-acetone	216 (35%)	7.59 (5.5%)	2.88 (13%)

Isooctane and pet ether were approximately equivalent in their relative extraction efficiencies for OBr, 7.59 and 7.73 $\mu g\ g^{-1}$ lipid, respectively; but gave lower results than hexane. Pet ether yielded significantly higher results for EOCl, 270 $\mu g\ g^{-1}$ lipid than isooctane (216 $\mu g\ g^{-1}$). However, isooctane gave higher results for EOI, 2.88 $\mu g\ g^{-1}$ lipid compared to 1.74 for pet ether. These differences in extraction efficiency may be related to the type of compounds that are being extracted and again this hypothesis could be tested employing GC-MS. Based on these results, hexane-acetone is certainly the most efficient solvent mixture for the extraction of EOX from shrimp tissue.

3.11.6 Comparison of Efficiencies of Extraction by Polytron to Shaking

It was necessary to determine whether the Polytron method of extraction was more efficient than the leaching method using a shaking board as described by Martinsen *et al.* [37]. The method described by Martinsen *et al.* required that homogenized whole shrimp be combined with the solvent mixture and extracted on a shaking board for 2 h, the solids would then be re-extracted in the same manner with fresh solvent. Each

method was used to extract 4 similar sized samples of whole shrimp. The results are summarized in Fig. 3.19.

The Polytron proved to be much more effective in extracting OCl, probably because the Polytron homogenizes the sample, both mechanically and sonically. The sonication may also have assisted in disruption of cell membranes and further liberation of organochlorines. The results for EOBr and EOI were less conclusive. Organoiodine seemed to be better extracted by the Polytron, but the difference was not as marked as in the case of EOCl. When experimental errors were taken into consideration, no discernible difference was observed in the extraction of OBr or EOI.

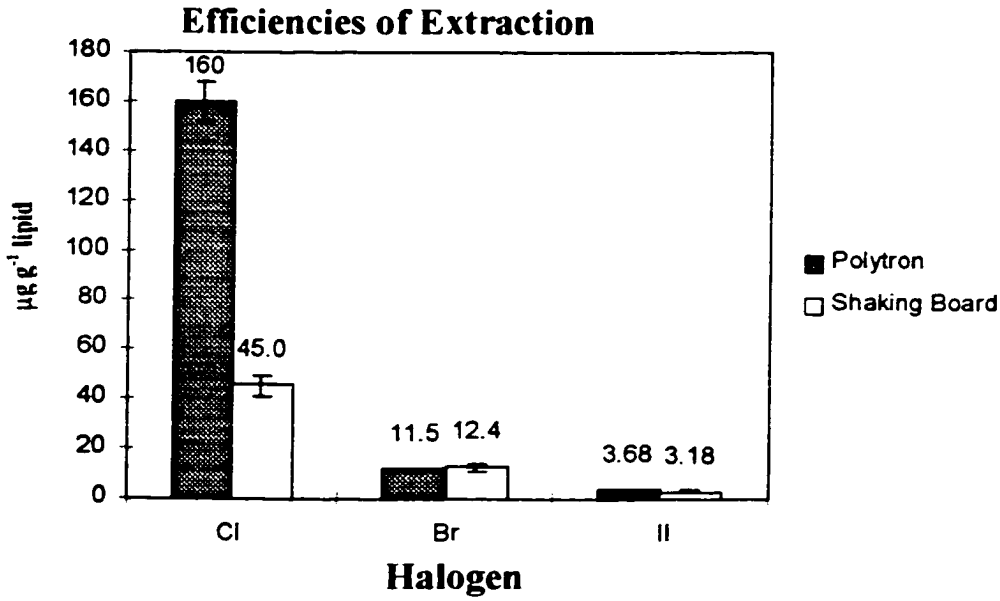


Fig. 3.19. Efficiencies of Extraction Apparatus

An additional experiment was performed using the shaking board to determine whether improvements could be made. The experiment was performed on three samples. The samples were extracted as described using the hexane-acetone mixture. The non-polar phase was removed, and then fresh solvent was added, and the mixture was placed

on the shaking board for 2 more h. This was repeated once more for a total of three extractions. The polar phases for each step were prepared and analyzed in the same manner as other samples.

The amounts of bromine and iodine measured in the second and third extraction steps were similar to those found in the procedural blank and so it was concluded that there was no improvement in extraction efficiency for these organohalogens. A small amount of additional chlorine was measured in the first repetition of the extraction with fresh solvent (0.43 μg g^{-1} shrimp homogenate, the first extraction accounted for 2.98 μg g^{-1}). No measurable amount was found in the extract from the second repetition. If this type of leaching method is used, it is recommended that the extraction be repeated using fresh solvent.

3.11.7 Washing Procedure

When the extracts were washed with DDW a tenacious emulsion formed which did not decompose completely upon centrifugation in a clinical centrifuge. One way this problem could be solved was to make the aqueous phase more polar. Martinsen *et al.* [37] described the use of a solution of 0.1% ammonium phosphate and 0.9% sodium nitrate as well as the use of pH 2 sulfuric acid for washing, however, their description of the method was not very clear. Moreover, since sodium can cause a high background in NAA (it worsens the detection limit), a modified method based on their model was developed in this work.

The "nitrate wash" solution was prepared by dissolving 0.5g of dibasic ammonium phosphate [$(NH_4)_2HPO_4$] and 4.5g of KNO_3 in DDW to 500 mL. An added

benefit of using the nitrate wash is that the ions may actually replace any chloride that may be occluded in the lipids. The pH 2 sulfuric acid (henceforth called "acid wash") was prepared using DDW and 0.7 mL of concentrated sulfuric acid. Many of the lipids (*e.g.* fatty acids and phospholipids) are ionized at normal body pH and so the acid was used to protonate any polar head groups and to ensure that any free fatty acids would be protonated and stay in the non-polar phase.

The non-polar extracts were first washed three times with the nitrate solution (30, 20, 20 mL), then three times with the acid solution (30, 20, 20 mL) in a separatory funnel. The aqueous washes were then combined and extracted with 10 mL of fresh non-polar solvent. This extract was washed and combined with the rest of the non-polar extracts and transferred to an Erlenmeyer flask. The separatory funnel was rinsed twice with 5 mL of non-polar solvent, and the rinses also were added to the Erlenmeyer flask.

Radiotracers are a valuable way to follow the distribution of an analyte of interest through an experimental procedure. This was employed to examine the efficiency of the above wash procedure. In this case a solution of a known concentration of potassium chloride was irradiated and then added to chicken egg yolk. The egg yolk was used because of its high lipid content, especially polar lipids that are expected to occlude chloride ions. The lipid was partitioned against hexane-acetone and the wash solutions and aliquots were taken from the organic layer before the wash and after each subsequent wash and counted for chlorine. This was continued until chlorine could no longer be detected.

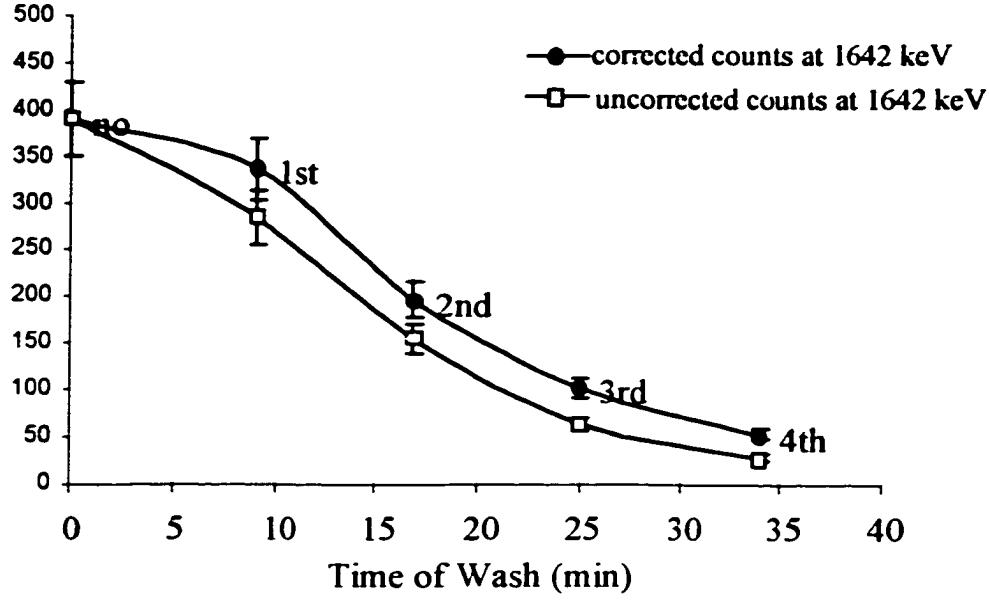


Fig. 3.20. Evaluation of Washing Procedure using ^{38}Cl Radiotracer

The results presented in Fig. 3.20 show that the wash seems to be effective, and by the fourth washing step more than 90% of the original chloride measured in the sample was removed. To evaluate whether the later washing steps are necessary one could use a longer-lived nuclide of a salt. Alternatively, an experiment was devised where a sample with a high content of polar lipids was spiked with sea salt and then extracted and submitted to the wash procedure. In this case, an egg yolk sample was mixed with 1 g of sea salt and submitted to the same procedure for extraction and washing. Chlorine, bromine and iodine were measured in the washes of each step.

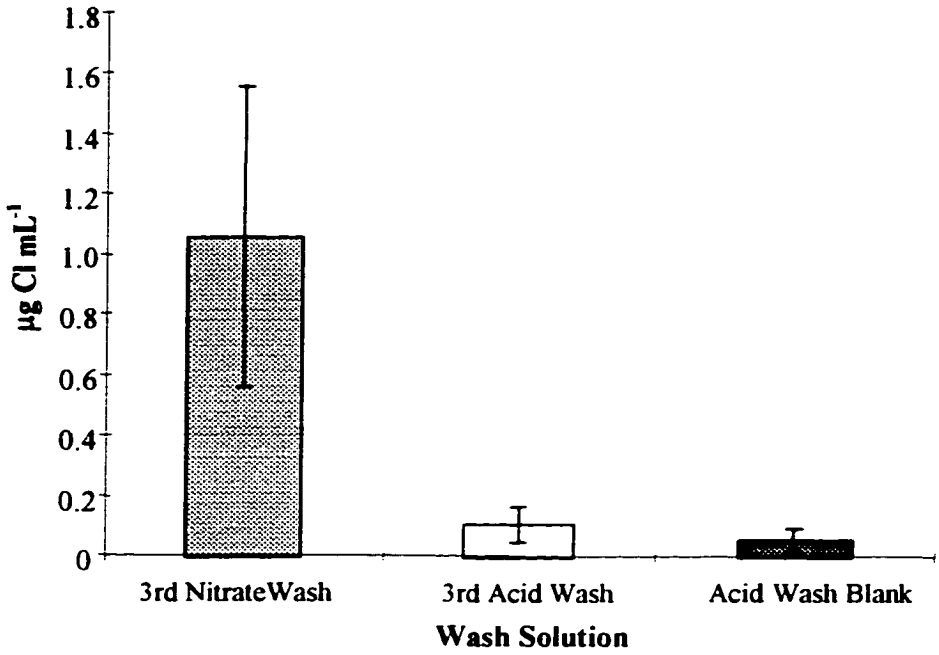


Fig. 3.21. Chlorine in Washes

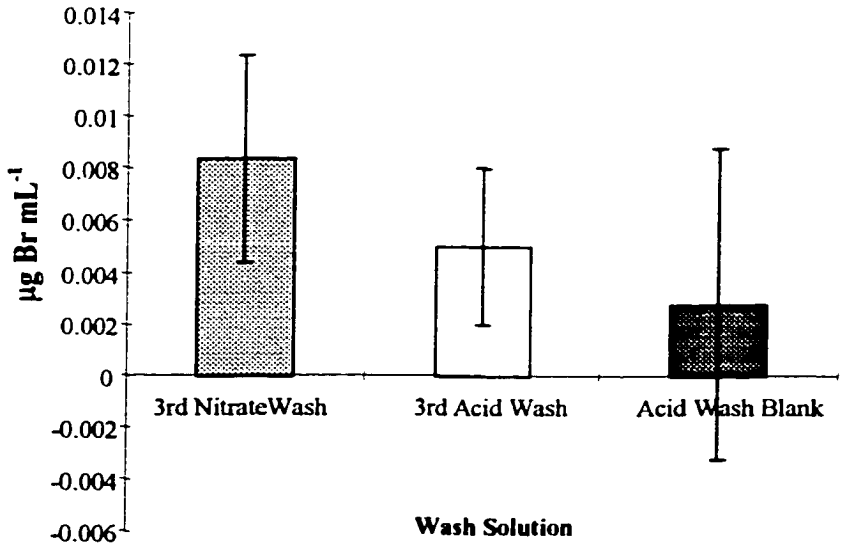


Fig. 3.22. Bromine in Washes

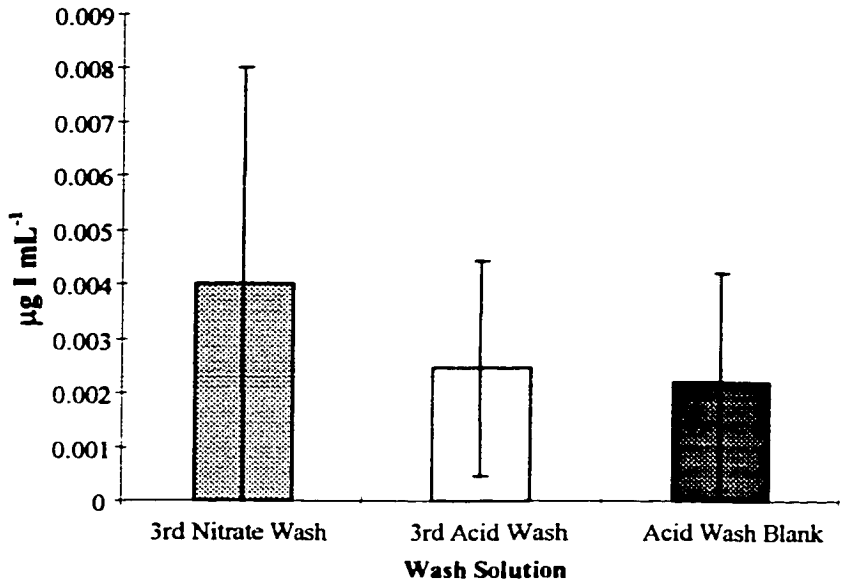


Fig. 3.23. Iodine in Washes

From the results shown in Figs. 3.21 through 3.23, one can see that the six washing steps are needed particularly for the removal of chloride. The third wash still contained significant amounts of chloride, but the amount of chloride in the sixth was indistinguishable from the blank. The use of the six washes also seems to be useful for the removal of bromide and iodide, although less conclusively.

3.11.8 Drying

Initially, anhydrous sodium sulfate (oven-dried for 24 h at 120°C, and stored in a desiccator) was used to dry non-polar extracts. In an attempt to further minimize background contribution by sodium, it was replaced with anhydrous magnesium sulfate (prepared and stored in the same manner). Magnesium sulfate was problematic because

some of the extracted material adsorbed, seemingly irreversibly, resulting in a lowered recovery.

The results of drying using the two different salts and a desiccator were compared. Drying in the desiccator was accomplished by placing an open Erlenmeyer flask containing the washed extract in a desiccator and the results are shown in Fig. 3.24. The recovery for chlorine using magnesium sulfate was poor. The results using the desiccator were comparable to those for the sodium sulfate for chlorine and bromine but seem to be low for iodine. It also took three days for all the water to be removed using the dessicator. As a result, anhydrous sodium sulfate was used in further experiments with the extracts dried over 2.5 g of sodium sulfate in an Erlenmeyer flask for 2 h. The extract was then filtered into a round-bottomed (r.b.) flask, and the sodium sulfate was washed twice with 10-mL aliquots of non-polar solvent, which were then filtered and combined with the rest of the non-polar extracts.

In terms of EOCl and EOBr, the best methods for drying are the use of anhydrous sodium sulfate or desiccation with no statistical difference between them. However the desiccation took more that 24 h; so it is preferable to use the sodium sulfate. The magnesium sulfate seem to yield better results for EOI but could not be used because of the results for EOCl and EOBr. The desiccation yielded poor results for EOI relative to the other methods and that may be due to losses related to evaporation of EOI compounds.

3.11.9 Evaporation

Extracts were concentrated using a rotary evaporator (Büchi Rotovapor-R) at 33°C and a Cole-Parmer vacuum pump. A vacuum was applied to the r.b. flask containing the extract, the flask was warmed slowly to 33°C, and the solvent was evaporated to 1-2 mL which was transferred to a 10-mL volumetric flask.

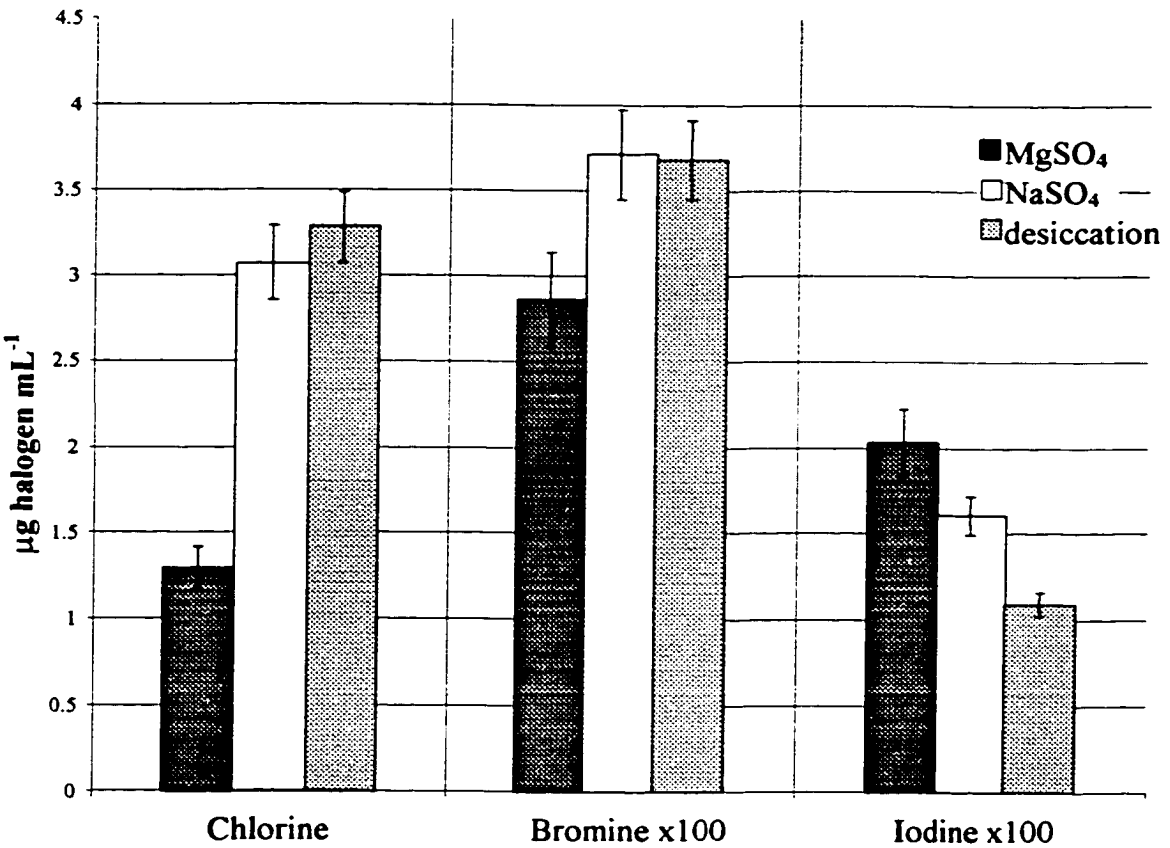


Fig. 3.24. Comparison of Drying Methods

The r.b. flask was washed 5 times with 1-mL aliquots of non-polar solvent (hexane when lower boiling point solvents were used for extraction) and the washings were also added to the volumetric flask. The solution in the volumetric flask was made

up to the mark with the same non-polar solvent and this solution was stored in a pre-cleaned vial with a Teflon-lined screw top.

Room temperature evaporation in a fumehood was also used but it was found to take more that 24 h to reduce the volume for each sample which made it time prohibitive. Warm nitrogen blow-down was also used, but this also took an excessive amount of time to achieve adequate volume reduction. The recoveries were also not significantly improved. The results for the nitrogen blow-down were 102%, 105% and 98% respectively for chlorine, bromine and iodine compared to the results for the rotary evaporator. This is likely within experimental error, and so it was used for all further experiments.

3.12 Conclusions

The experiments performed in this chapter illustrate the need for thorough investigation of all variables related to a given method. In terms of extraction of lipid and overall extraction of EOX from whole shrimp and especially shrimp muscle, the best solvent system for this work is acetone-hexane. This system also shows good precision and low procedural blanks.

Section 3.11.7 indicated that inorganic halides are effectively removed by the washing procedure described therein. The most efficient extraction and throughput of samples are achieved using the Polytron for extraction, sodium sulfate for drying and the rotary evaporator for solvent removal.

The QA program demonstrates that the results produced are reliable and under statistical control.

4. ORGANOHALOGEN DISTRIBUTION IN SHRIMP MUSCLE AND ROE

4.1 Introduction

An important part of this thesis work has been to measure EOX levels in shrimp muscle and roe from a number of commercial fishery locations. The results were analyzed for trends in distribution. Measurements of EOX in shrimp may be particularly useful for a few reasons. First, they are consumed directly by humans. Secondly, they are a valuable commercial fishery. Thirdly, they are a food source for other fish species such as cod that are also harvested commercially. Finally, they are a good indicator of recent input of EOX into the environment as their average age is around 4 years and they typically live a maximum of only 8 years.

The optimized methods described in Chapter 3 were used for the extraction of lipid and EOX from shrimp muscle and roe. Organohalogens were then determined in these extracts using NAA as described in Chapter 3. A few studies reported research on spatial trends in OCl levels in other species, such as harbour porpoises in Scandinavia [256]. One of the objectives of this study has been to determine total EOX in shrimp from several locations from which they are commercially harvested, and to determine if any spatial trends exist.

In addition to the spatial distribution pattern, there would also likely be some differences in the distribution of EOX between muscle and roe. These might be related to differences in lipid types and concentrations in the two compartments or maybe related to the depuration of EOX by the shrimp to its eggs.

4.2 Experimental

4.2.1 Sample Collection and Handling

The staff members at Fisheries and Oceans Canada in St. John's, Newfoundland collected all samples from the Labrador Coast that were used in this work. The samples from the Canso Hole, Nova Scotia were collected by the staff at the Bedford Institute of Oceanography. The samples from Maine, USA, were collected by local fisheries personnel. The latitudes and longitudes for each of the sampling locations are given in Table 4.1 and the locations are also given on the map in Fig. 4.1.

All samples were collected, frozen and shipped on dry ice when required. After the samples were received at our laboratory, they were stored at –25°C until they could be prepared and analyzed.

Table 4.1 Latitudinal and Longitudinal Co-ordinates for Location of Sampling

Location Number	Location		Date Collected
	Latitude (N)	Longitude(W)	
1	61° 05'	64° 31'	1997 August
2	60° 51'	61° 36'	1995 December
3	60° 22'	61° 24'	1995 December
4	54° 45'	53° 35'	1996 February
5	53° 12'	54° 08'	1995 January
6	53° 03'	52° 06'	1997 October
7	45° 09'	61° 15'	1996 June
8	43° 30'	70° 00'	1996 August

4.2.2 Sample Preparation and Extraction Procedure

Approximately 1 kg of shrimp collected from each of the locations described in Table 1 were dissected, yielding pools of muscle and roe for each area. Shrimp from Canso Hole did not carry eggs, likely due to the time of year they were caught and so only pools of muscle were available for this location. To ensure some homogeneity of the samples taken from the pooled muscle, the muscle was homogenized using dry ice as described in section 3.4. The shrimp roe was not submitted to this procedure, but the thawed roe was mixed manually, using a glass stirring rod. It was then weighed into ~10 g samples and refrozen for storage.

All chemicals used were the same as those described in section 3.1. The procedure used in cleaning the glassware can be found in section 3.2.

The optimized solvent extraction procedure used here is described in Section 3.11.1 employing a Brinkman polytron (Section 3.11.6) and the acetone-hexane (1:1) solvent mixture (Section 3.11.3, 4, 5). Samples between 10 and 20 g (depending on the estimated lipid content) were extracted.

4.2.3 Washing Procedure

The method used to remove the inorganic halides from the hexane extract was the same as that described in section 3.11.7 using the nitrate wash (30, 20, 20 mL) followed by the acid wash (30, 20, 20 mL). The combined extracts were dried using anhydrous sodium sulfate as described in section 3.11.8.

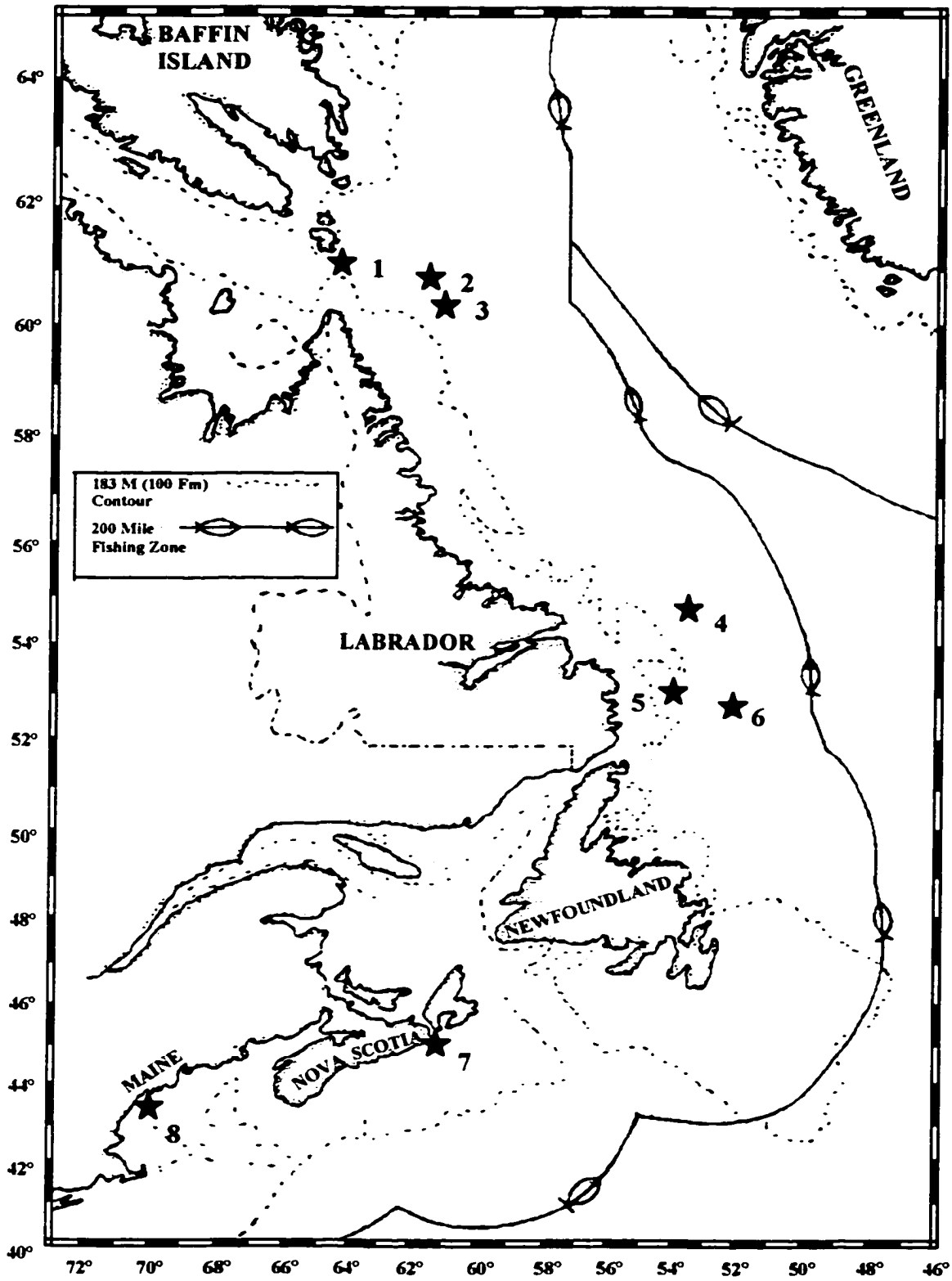


Fig. 4.1. Map of Sampling Locations: Sample Locations Shown with Stars (★)

The extracts were concentrated using a rotary evaporator apparatus in a water bath at 33°C as described in Section 3.11.9. Evaporation to dryness was avoided as it might result in significant losses of the more volatile compounds. The extract was stored in a clean glass vial with a Teflon lined screw top.

4.2.4 Lipid Determination

The concentration of lipid was determined gravimetrically as described in Section 3.5. The sample was left for no more than 24 h because of the possibility of oxidation of unsaturated lipids, which could potentially lead to artificially elevated results.

4.2.5 Elemental Standards, Sample Irradiation, and Detection

Elemental comparator samples were prepared as described in Section 3.6. The results of irradiations of the comparator standards were used to calculate the concentrations of EOX in the extracts.

All samples were prepared as given in Section 3.7, and irradiated and transferred in the same manner as described in Section 3.8. Samples were counted on both Ge(Li) #1 and Ge(Li) #2 for higher throughput. The results were tabulated using comparator standards counted on each of the systems; the sensitivities of the standards were calculated as given in section 3.9.

4.3 Results and Discussion

It is generally accepted that the levels of persistent organopollutants (POPs) like EOX are closely linked to the amount of lipid, especially triacylglycerols, extracted from

marine biota [257]. For this reason, it is important to measure the amount of lipid present to determine if the levels of EOX are related to the lipid concentration in the sample and not to metabolic differences in the tissues. The percent lipid found in the muscle and roe samples from the various locations are summarized in Table 4.2.

The levels of lipid found in the shrimp were consistent with those reported in the literature [157, 241, 252]; however, the seasonal fluctuations that have been reported by other authors [157, 252] are not obvious in our study. This may be partly due to the fact that the larger, older shrimp do not experience as much variation and the other studies examined shrimp that were 4 years or younger and of mixed gender, while samples used in the present work were all egg-bearing female shrimp. Some of the peaks in lipid levels that are often seen late in summer would also be minimized by the fact that all the shrimp carried eggs and a proportion of the lipid that was accumulated through the summer months would have been transferred to the roe. The shrimp collected in 1996 June from the Canso Hole region did not carry eggs (these are noted as NR in the tables) but the size was consistent with female shrimp. It should be noted that June is 2 to 3 months before spawning normally takes place. Some of the variation in lipid may also be due to the location where the shrimp were caught. This could be partly due to metabolic differences related to water temperature or to the availability and type of food in the area.

The results for the determinations of EOCl, EOBr, and EOI in shrimp muscle and roe are reported in Tables 4.3, 4.4 and 4.5, respectively. The reported results are the means of 5 muscle samples and 4 roe samples. The standard deviations for these measurements are also reported as 1σ. Five aliquots of each sample extract were analyzed for halogen levels by NAA and the averages of these were used to calculate the final results.

Table 4.2. Percent Lipid in Muscle and Roe in Samples from Several Locations

Location	% Lipid in Muscle ± 1σ	% Lipid in Roe ± 1σ	Ratio %lipid Roe/Muscle
1	1.17% ± 0.06%	8.66% ± 0.22%	7.6
2	1.23% ± 0.02%	10.1% ± 0.60%	8.2
3	1.49% ± 0.03%	10.4% ± 0.10%	7.0
4	1.25% ± 0.08%	8.66% ± 0.13%	6.9
5	1.34% ± 0.08	8.48% ± 0.01%	6.3
6	1.31% ± 0.05%	10.7% ± 0.50%	7.8
7	1.45% ± 0.14%	NR	
8	1.27% ± 0.13%	5.99% ± 0.72%	4.7

NR – no roe

In general the highest levels of all organohalogens, per unit wet weight of sample, are found in the shrimp roe (Figs. 4.2, 4.3, and 4.4). This is largely due to the association of organohalogens with lipid and the higher proportion of lipid in the roe (~8-10%) as compared to the amount in muscle (1.2–1.5%). This is particularly true for EOBr and EOI. In the case of EOCl, there is one exception to this trend: shrimp from Location 2 had higher levels of EOCl in the muscle than found in the roe (6.19 $\mu g\ g^{-1}$ versus 4.19 $\mu g\ g^{-1}$).

To minimize the effect of lipid content on the differences between EOX levels in the muscle and roe, it is useful to examine these levels relative to lipid (*i.e.* μg halogen g^{-1} of lipid). In this way it can be determined whether differences in levels are simply due to differing amounts of lipid or to some other factors. Once again the comparative levels of EOCl differ from those of EOBr and EOI. All samples show higher lipid-basis levels of

OCl in muscle. If the deviation at the 1σ level is considered in these comparisons there is actually no measurable difference between the level of EOCl in muscle and roe from Location 8. There are no significant differences between lipid-basis EOBr levels for Locations 1, 2, 3 and 6. For the samples from Locations 4, 5, and 8, higher lipid-basis levels are found in roe. The distribution shown for EOBr is mirrored in the results for EOI; the only exception being Location 6, which has higher levels of EOI in roe than that found in muscle.

Table 4.3 Organochlorine in Shrimp

Area	Muscle		Roe	
	μg Cl g^{-1} ± 1σ (wet weight)	μg Cl g^{-1} ± 1σ (lipid basis)	μg Cl g^{-1} ± 1σ (wet weight)	μg Cl g^{-1} ± 1σ (lipid basis)
1	1.86 ± 0.63	160 ± 63	3.98 ± 1.70	45.7 ± 19.3
2	6.19 ± 0.53	498 ± 4	4.19 ± 0.13	41.4 ± 1.3
3	1.93 ± 0.79	130 ± 52	6.21 ± 0.19	57.4 ± 1.4
4	1.79 ± 0.24	142 ± 11	7.56 ± 0.39	86 4 ± 4.5
5	5.57 ± 0.77	420 ± 79	12.3 ± 8.6	145 ± 102
6	1.39 ± 0.60	105 ± 42	4.14 ± 0.35	39.0 ± 2.3
7	2.49 ± 0.25	162 ± 12	NR	NR
8	2.13 ± 0.37	168 ± 41	8.25 ± 4.00	147 ± 77

NR - no roe

Table 4.4 Organobromine in Shrimp

Area	Muscle		Roe	
	$\mu g\ Br\ g^{-1} \pm 1\sigma$ (wet weight)	$\mu g\ Br\ g^{-1} \pm 1\sigma$ (lipid basis)	$\mu g\ Br\ g^{-1} \pm 1\sigma$ (wet weight)	$\mu g\ Br\ g^{-1} \pm 1\sigma$ (lipid basis)
1	0.105 ± 0.013	9.18 ± 0.61	0.659 ± 0.033	7.61 ± 0.31
2	0.117 ± 0.024	9.31 ± 1.33	0.707 ± 0.009	6.96 ± 0.09
3	0.130 ± 0.011	8.88 ± 0.70	0.850 ± 0.210	8.34 ± 1.90
4	0.061 ± 0.011	4.85 ± 0.29	0.933 ± 0.055	11.0 ± 0.7
5	0.065 ± 0.013	4.74 ± 0.58	0.981 ± 0.029	11.6± 0.3
6	0.139 ± 0.021	10.5 ± 0.6	1.030 ± 0.059	9.73 ± 0.83
7	0.081 ± 0.011	5.38 ± 0.38	NR	NR
8	0.116 ± 0.013	9.25 ± 1.08	0.814 ± 0.085	13.6± 0.4

NR - no roe

The results indicate that concentration trends might be related to the location in which the shrimp was harvested. The data from EOX in roe reveals a spatial trend along the Labrador coast that increases as one moves south (Figs. 4.2, 4.3, and 4.4). Data analysis was performed for correlation between location and EOX for Locations 1 through 6. The results showed that there was a strong negative correlation between latitude and EOX in roe, as well as longitude and EOX in roe. The correlation coefficient, R for location and EOBr was -0.925 for latitude and -0.940 for longitude. Similar high R-values were found between location and EOI, *i.e.* -0.959 for latitude and -0.935 for longitude. A

negative correlation was also calculated for location and EOCl in roe, however the results weren't as conclusive, -0.553 for latitude, and -0.463 for longitude. The R-values for EOX in muscle and location were low.

Table 4.5. Organoiodine in Shrimp

Area	Muscle		Roe	
	$\mu g\ I\ g^{-1} \pm 1\sigma$ (wet weight)	$\mu g\ I\ g^{-1} \pm 1\sigma$ (lipid basis)	$\mu g\ I\ g^{-1} \pm 1\sigma$ (wet weight)	$\mu g\ I\ g^{-1} \pm 1\sigma$ (lipid basis)
1	0.0166 ± 0.0004	1.43 ± 0.12	0.123 ± 0.007	1.42 ± 0.08
2	0.0212 ± 0.0086	1.72 ± 0.24	0.159 ± 0.006	1.56 ± 0.06
3	0.0217 ± 0.0045	1.61 ± 0.28	0.154 ± 0.020	1.52 ± 0.23
4	0.0181 ± 0.0025	1.44 ± 0.04	0.306 ± 0.049	3.58 ± 0.60
5	0.0139 ± 0.0034	1.03 ± 0.17	0.349 ± 0.013	4.11 ± 0.11
6	0.0232 ± 0.0056	1.76 ± 0.17	0.281 ± 0.009	2.65 ± 0.11
7	0.0227 ± 0.0028	1.50 ± 0.19	NR	NR
8	0.0166 ± 0.0035	1.33 ± 0.28	0.195 ± 0.026	3.26 ± 0.16

NR - no roe

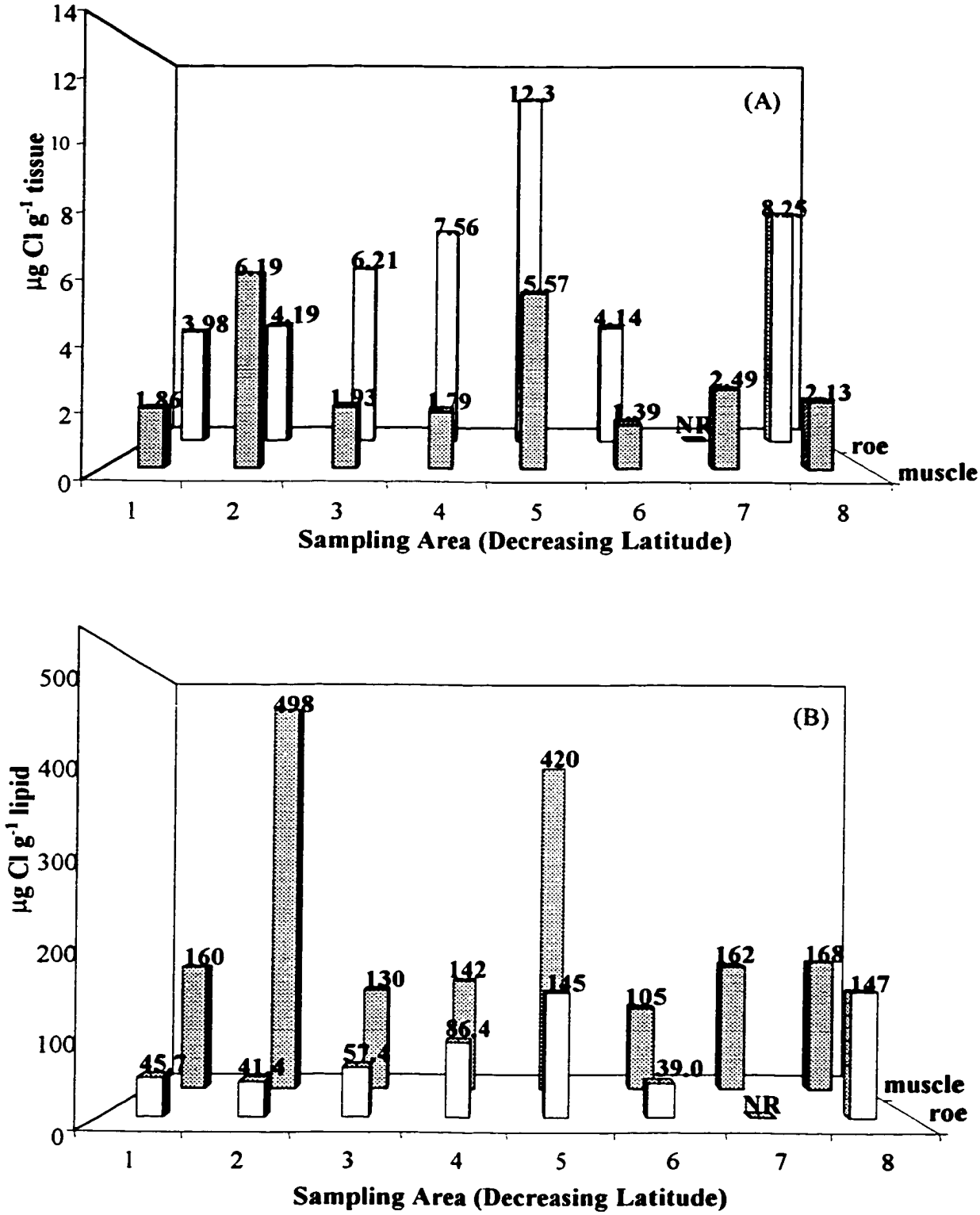


Fig. 4.2. EOCl in Shrimp Muscle and Roe (A) wet weight basis, (B) lipid basis (NR – no roe)

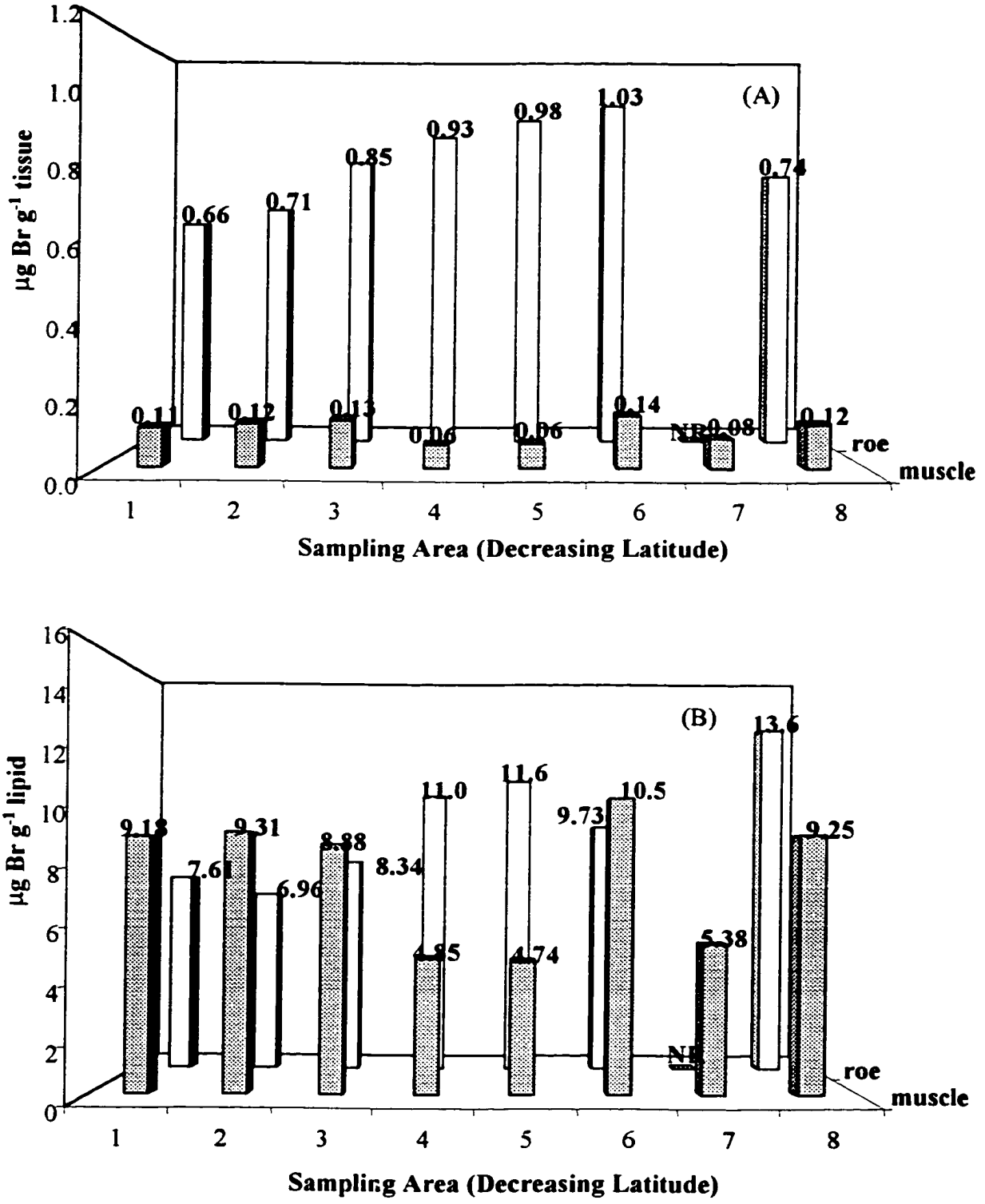
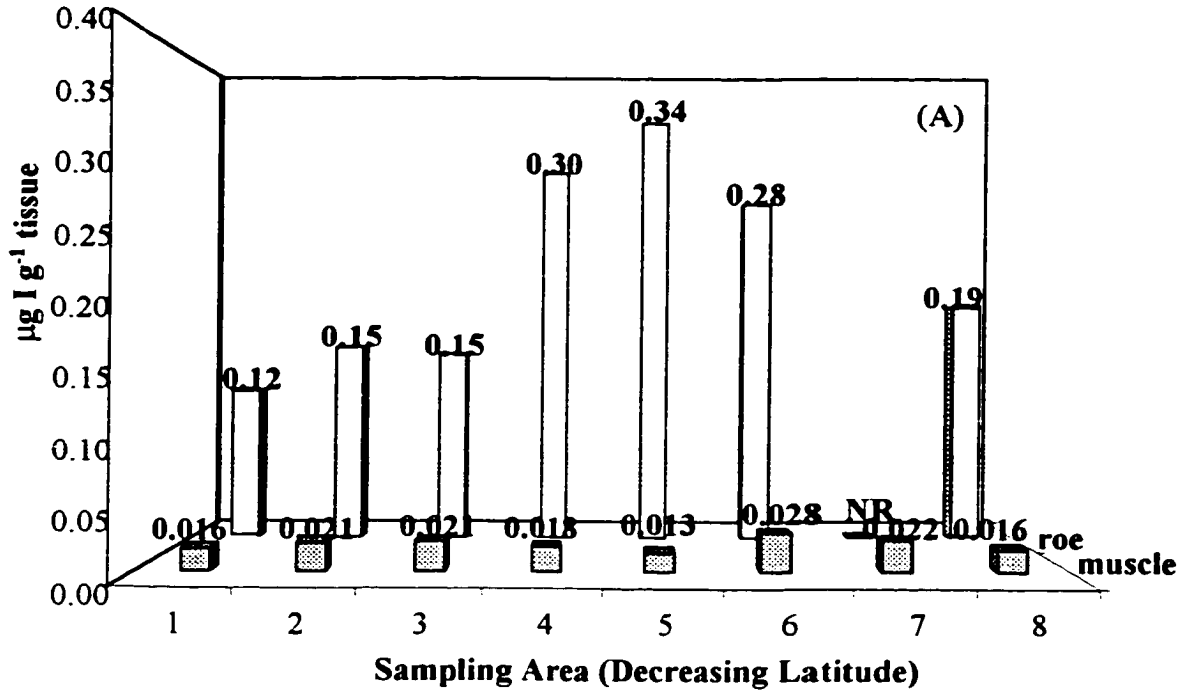


Fig. 4.3. EOBr in Shrimp Muscle and Roe (A) wet weight basis, (B) lipid weight basis (NR – no roe)



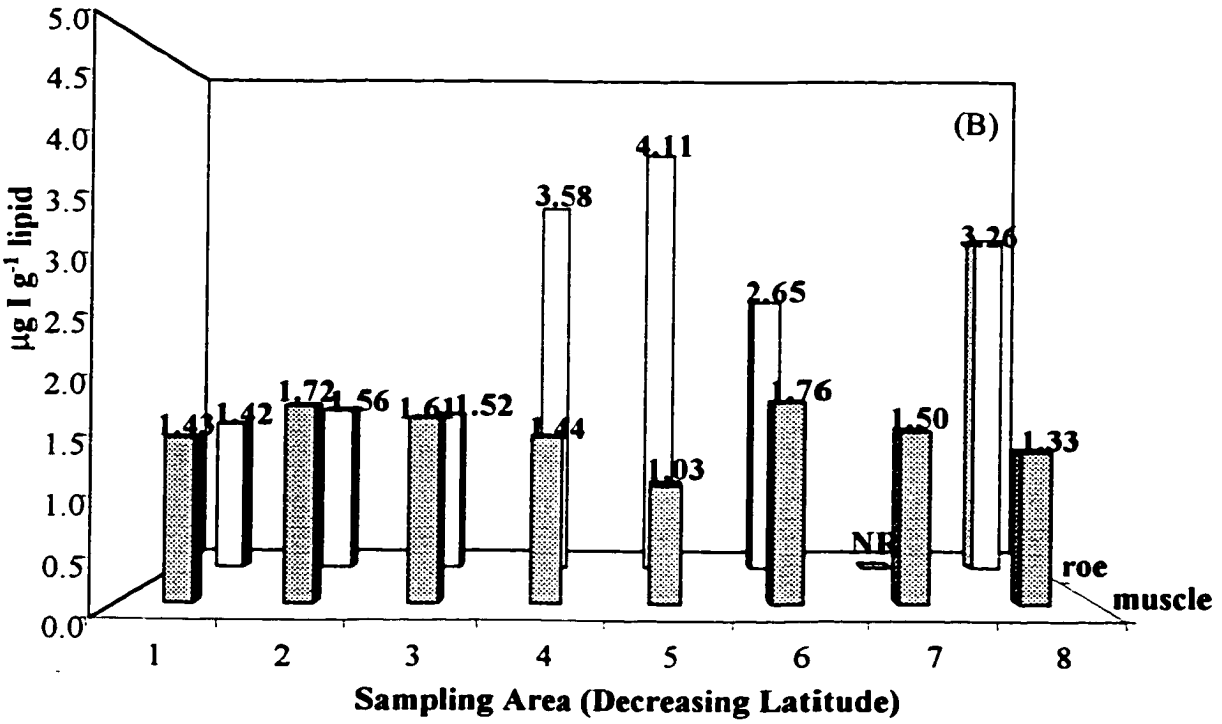


Fig. 4.4. EOI in Shrimp Muscle and Roe (A) wet weight basis, (B) lipid weight basis (NR – no roe)

When the levels of EOX are plotted against one another additional trends can be seen as illustrated in Figs. 4.5 through 4.10. The EOCl levels in muscle (Figs.4.5 and 4.7) seem to fall into two groups. Locations 2 and 5 are at one end of the spectrum with high amounts of EOCl (6.19 and 5.55 $\mu g\ g^{-1}$ tissue, respectively) while the remaining locations with lower levels (1.39-2.49 $\mu g\ g^{-1}$ tissue) are clustered together. These differences dominate the plots relating EOCl to EOBr and EOI in muscle. The same two subgroups of samples appear when EOCl is expressed on a lipid basis, where samples 5 and 2 are high (420 and 498 $\mu g\ Cl\ g^{-1}$ lipid, respectively) and the remaining samples are lower (105-168 $\mu g\ Cl\ g^{-1}$ lipid). The grouping does not appear to be related to location or to the time of year that the shrimp were caught, since the sample 2 was collected in the same month and very near to that of sample 3, and yet the samples are remarkably different. Additionally, the date the shrimp with the high levels were sampled is separated by nearly one year (1995 January and 1995 December). The differences between the two groups might be attributed to the age of the shrimp in the samples, where it is possible that older shrimp would have a longer time to accumulate higher levels of EOX. It is important to note that the locations in which the shrimp are caught are not entirely random, the shrimp themselves have many preferences for water temperature, depth, available food, *etc.* which would vary with location.

The plots for EOCl versus EOBr and EOI in roe show a positive correlation especially when the values are expressed on a lipid basis. The R-value for EOCl and EOBr in roe on a lipid basis is 0.879. The R-values for EOCl and EOI in roe are 0.79 for a wet weight basis, and 0.80 for a lipid basis. The plots of EOBr *vs.* EOI (Figs. 4.10 and 4.11) show significant positive correlation in all graphs, and the most marked correlation is once

again seen for the roe, where the R-value is 0.856 for the levels on a wet weight basis and 0.858 for the levels on a lipid basis. The relationships between the EOX values in the roe may be due to some similarity in the types of compounds the adult shrimp from all locations are transferring to its roe. For example, halogenated fatty acids may be transferred to the young in phospholipids and triacylglycerols. In areas where the diet is rich in these compounds, more would be transferred to roe. The fact that there is a less pronounced trend in the distribution of EOCl may indicate some differences between its origin and the origin of EOBr and EOI.

The geographical trends seen along the coast could be related to oceanic currents that are present in the area as well as atmospheric modes of deposition of halogenated compounds. In the case of atmospheric modes of deposition, one would expect an increase in concentrations as the sampling location came closer to a source of industrial input of halogenated organics. Since there are no known major anthropogenic sources on the Labrador coast, one might infer that the source could be to the south of Labrador. There is more human habitation as one moves down the coast of Labrador and also south east of Labrador (the Maritimes and Eastern Seaboard of the United States), and so there may also be some increased input of organohalogens related to human activities in these areas. The trade winds could conceivably transport significant amounts of anthropogenic organohalogens north from these heavily industrialized and densely populated areas [30, 159, 161]. Oceans in northern regions are more susceptible to this input, partly because of the high ratio of ocean to land in the polar regions.

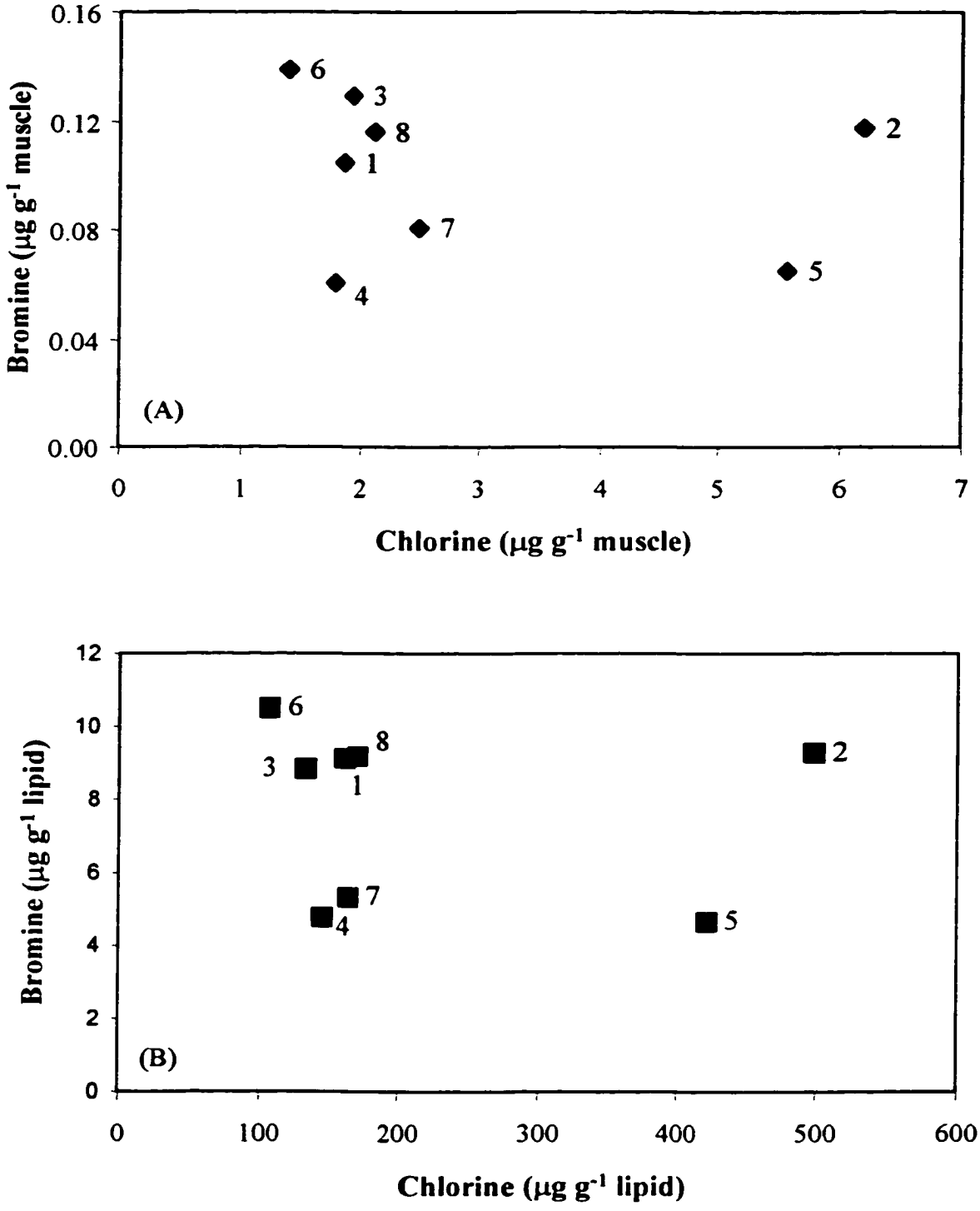


Fig. 4.5. EOBr *vs.* EOCl in Muscle (A) Wet Weight and (B) Lipid Weight Basis

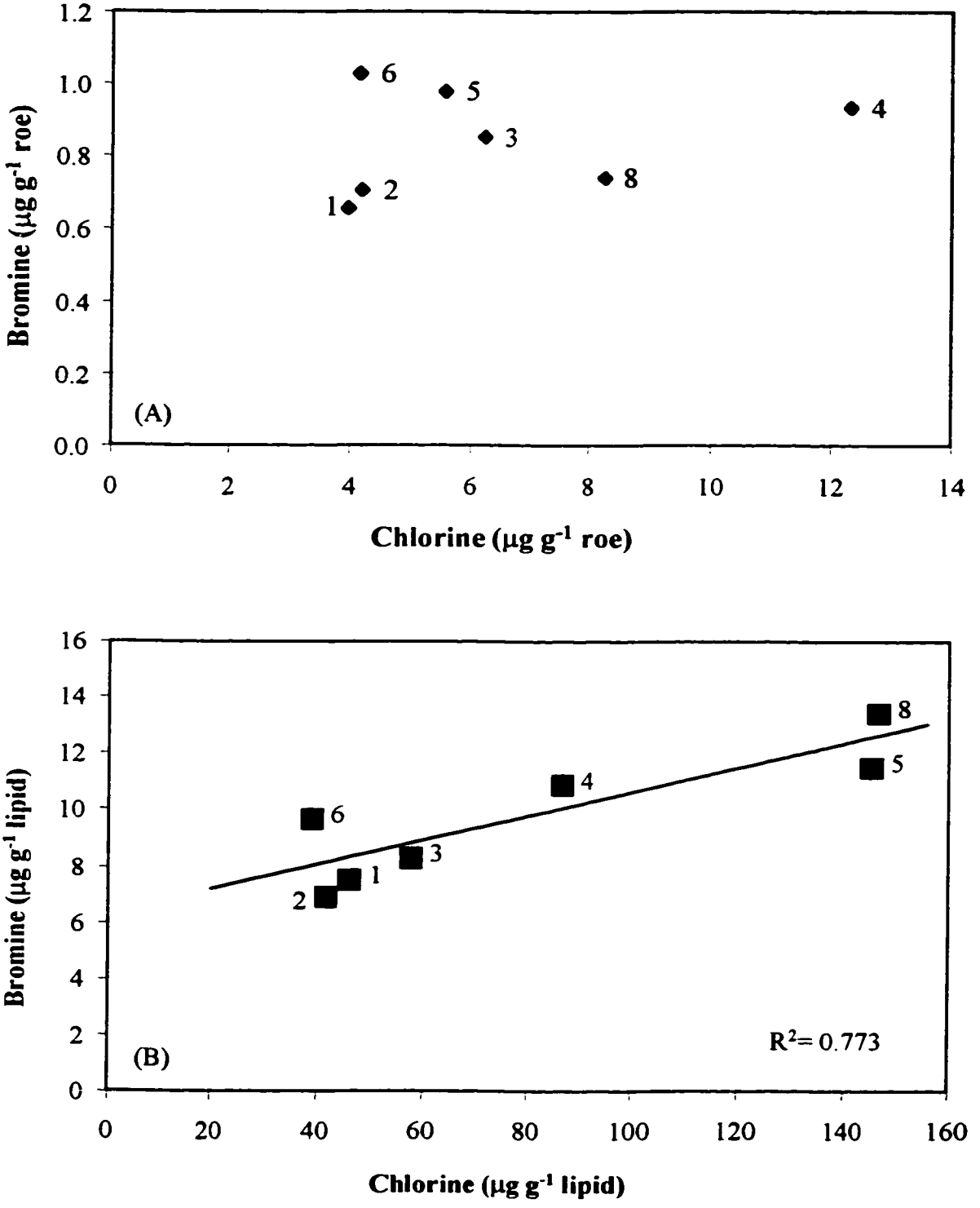


Fig. 4.6. EOBr *vs.* EOCl in Roe (A)Wet Weight and (B) Lipid Weight Basis

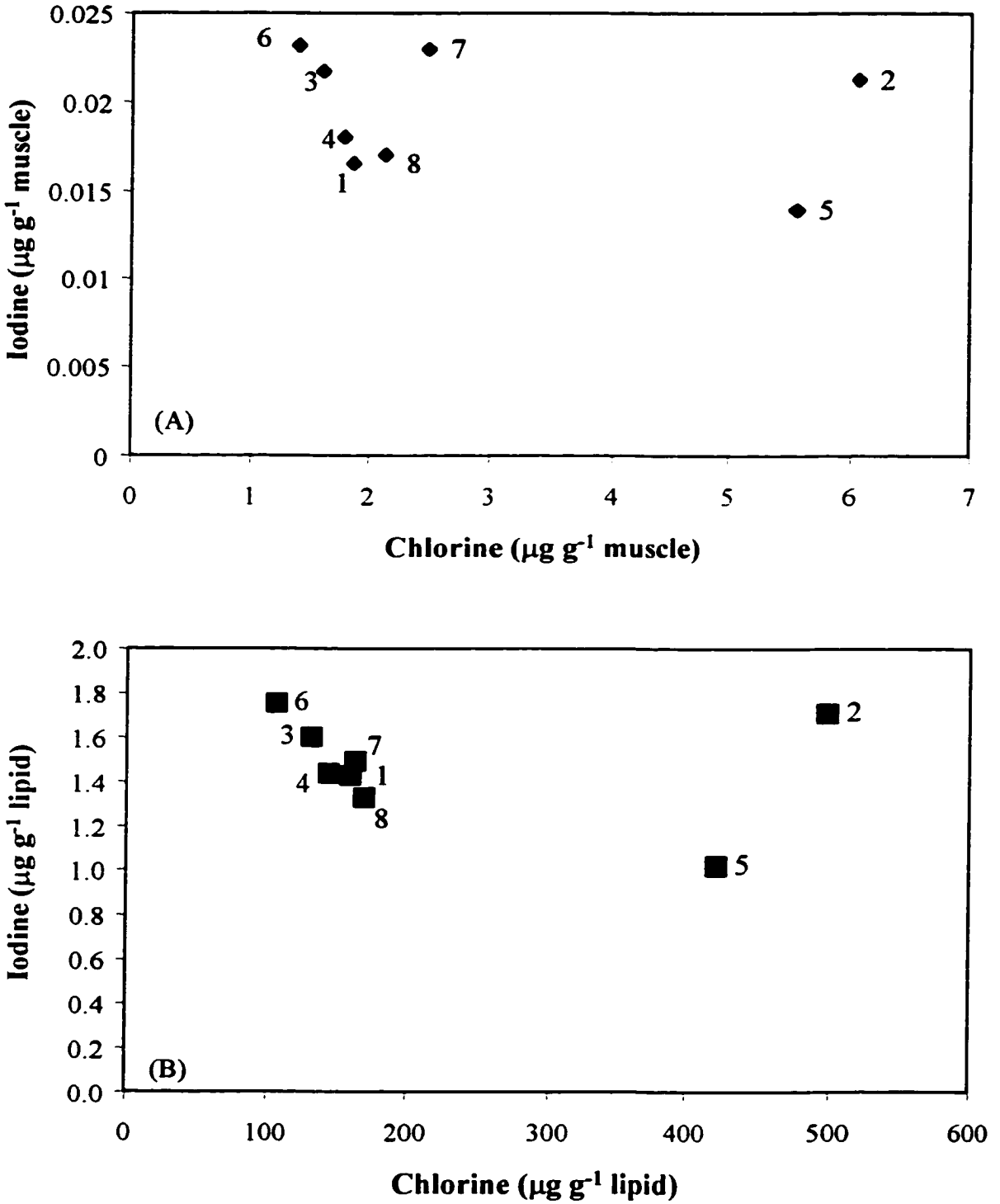


Fig. 4.7. EOI *vs.* EOCl in Muscle (A)Wet Weight (B)Lipid Weight Basis

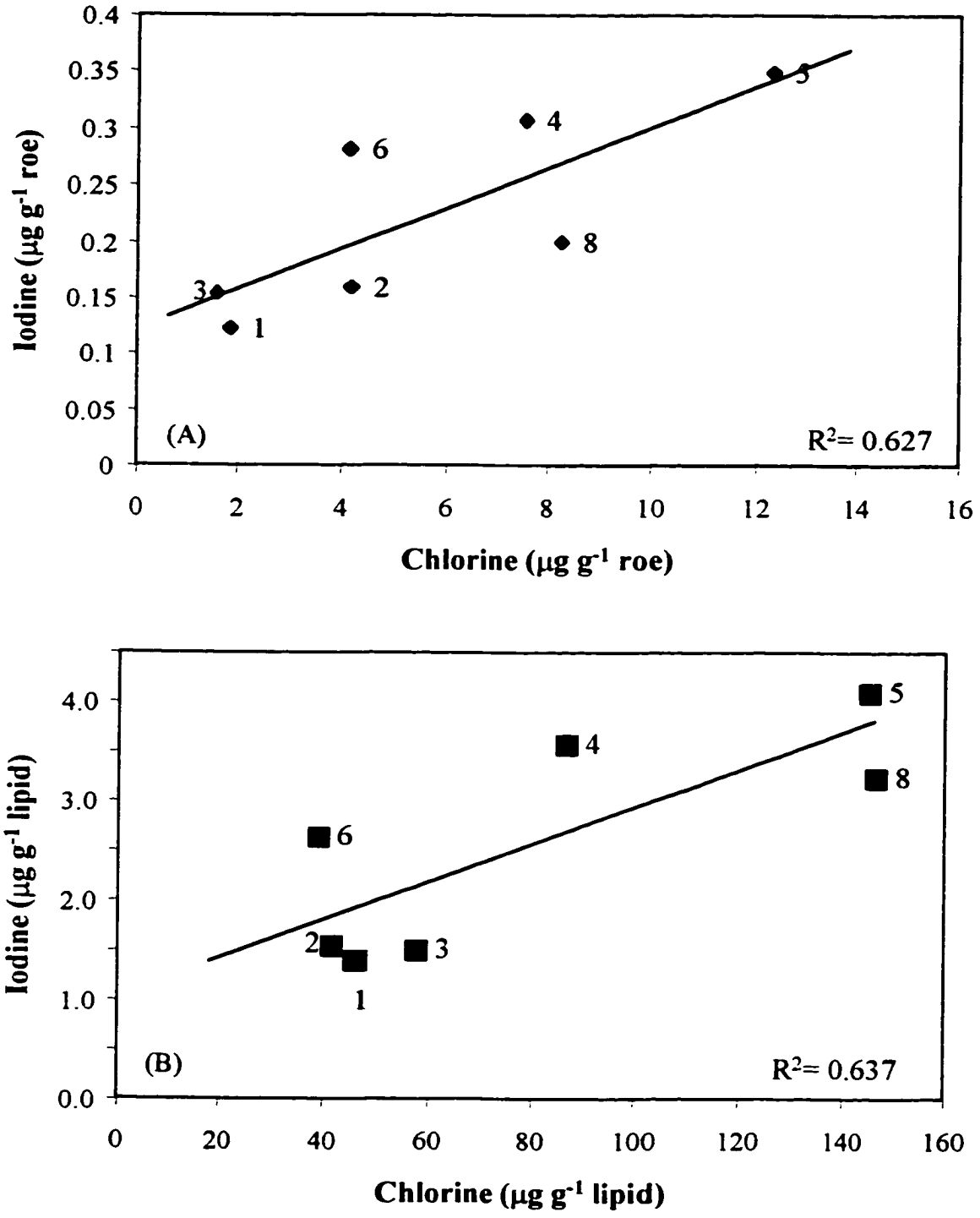


Fig. 4.8. EOI *vs.* EOCl in Roe (A) Wet Weight and (B) Lipid Weight Basis

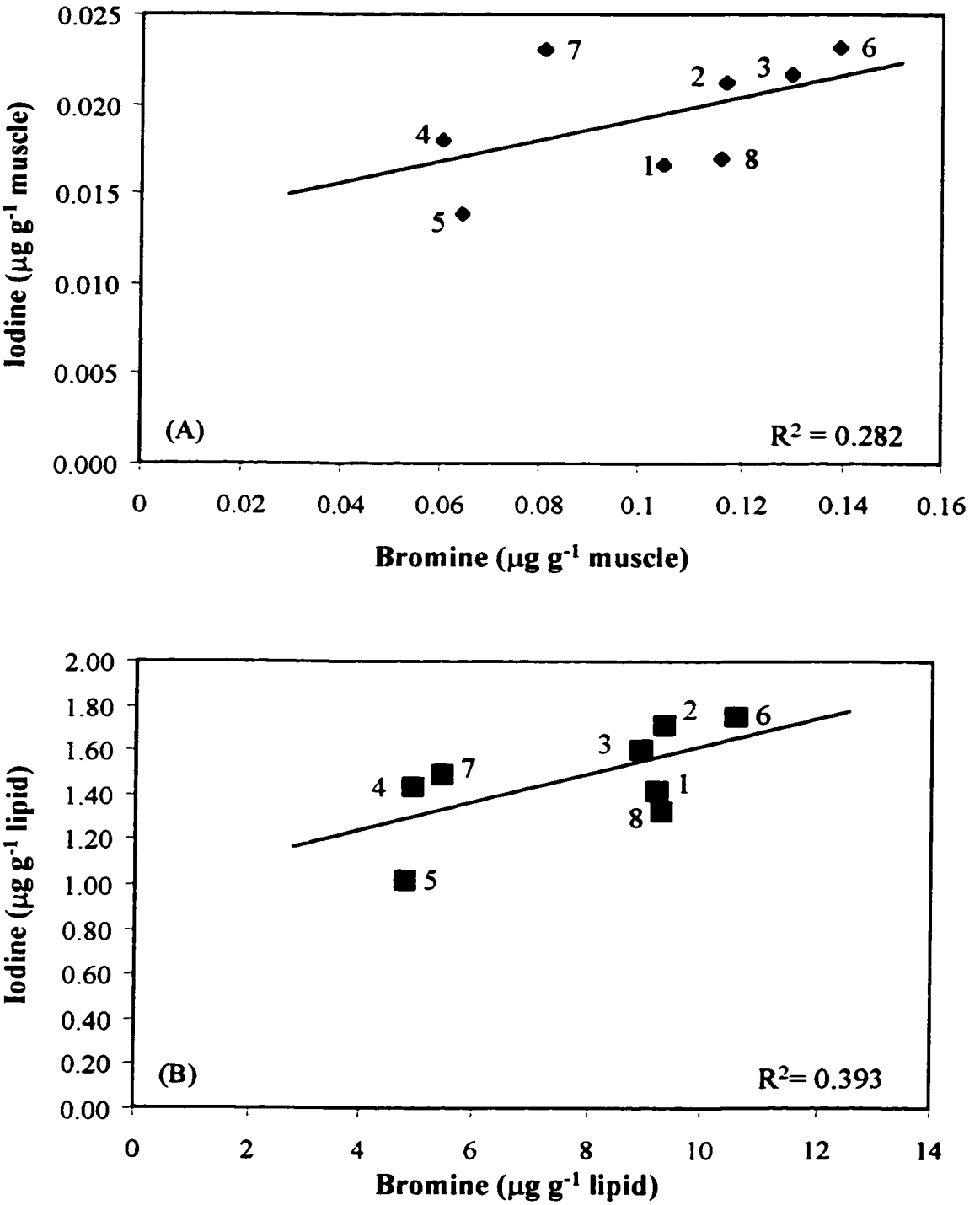


Fig. 4.9. EOI *vs.* EOBr in Muscle (A) Wet Weight and (B) Lipid Weight Basis

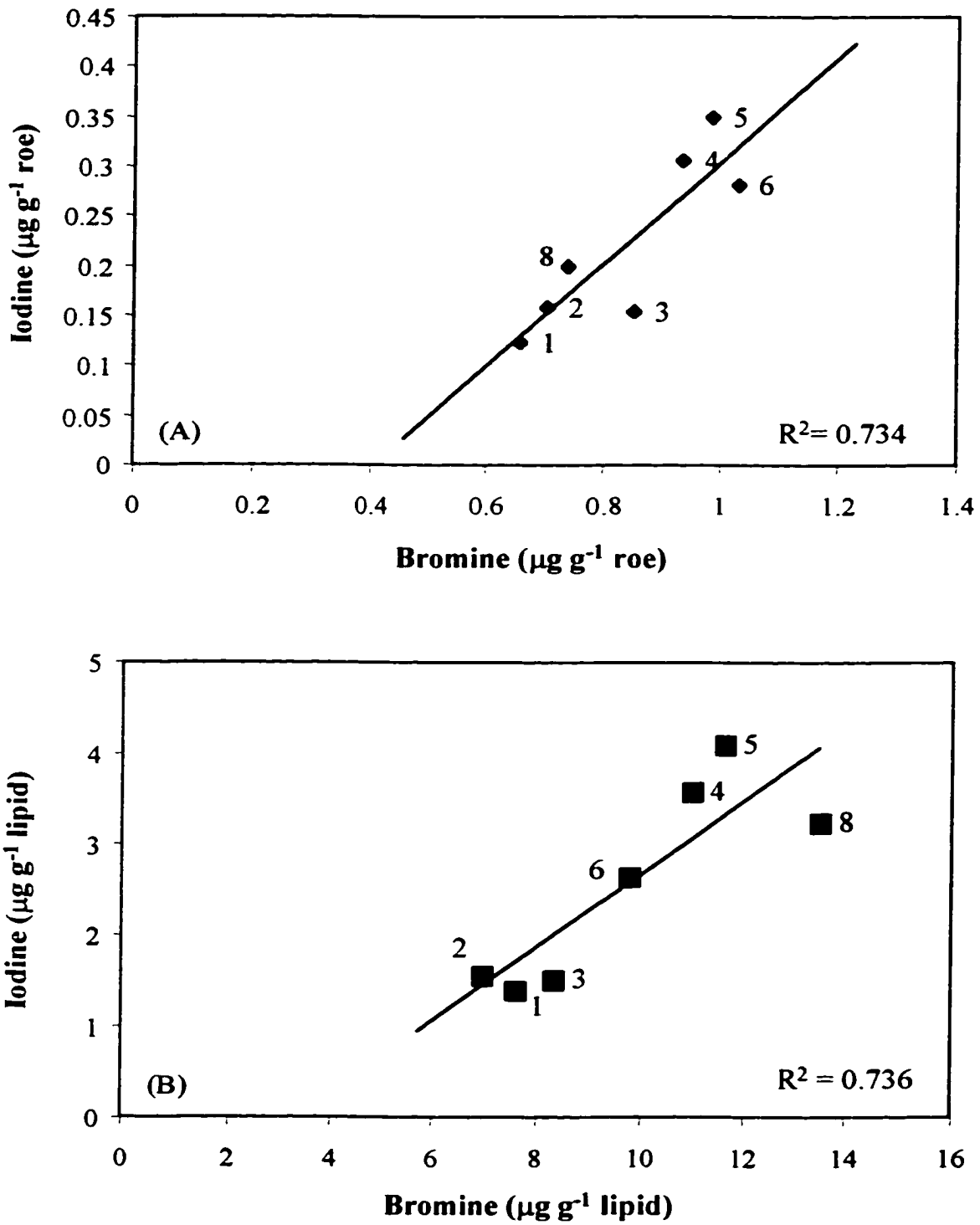


Fig. 4.10. EOI *vs.* EOBr in Roe (A) Wet Weight and (B) Lipid Weight Basis

Also the lower temperatures make the deposition of volatile organohalogens more favourable, as the rate of evaporation is lower than that of condensation and the rate of decomposition slower [147, 252, 257]. Wania and Mackay [258] have suggested that global fractionation of volatile compounds is also occurring during atmospheric transport.

The movement of shrimp may be an important factor in determining whether the concentration differences reflect real trends in organohalogen levels in the environment of a specific area or are due to other factors. It is known that *P. borealis* prefers areas that have soft mud where there is a high organic content, and it has been shown that shrimp occur in higher abundance in areas that display these features. It is conceivable that this would also be the location where natural organohalogens would be synthesized [93] and also a location where there would be high levels of organic particulate material, which might adsorb organohalogens and thus produce deposits of anthropogenic compounds. When in the larval stage, the shrimp are subject to the currents to which they are exposed. In the adult stage, the movement appears to be limited to a daily cycle of movement up and down the water column. There is also seasonal migration between the inshore and offshore related to developmental stage. Larvae and juveniles spend the majority of their lives in the inshore and as they mature to adult males, they migrate to the offshore where they live and function for more than a year. They then transform to female and remain in the offshore until they spawn. Ovigerous females will spend most of their lives offshore but will migrate inshore in winter to spawn.

Latitudinal movements are reported to be limited [241]. One can infer from the migration of this species that levels of EOX found in the shrimp would primarily be related to their exposure to such compounds at the latitude at which they are caught. The reported

correlation between longitude and EOX would likely be due to the fact that the samples that are further West are also further North, and the decrease in levels is more likely due to the differences in latitude. All the samples from along the coast of Labrador are approximately the same distance from the coast.

Studies of EOX in *P. borealis* are fairly limited, although the species has a substantial commercial fishery [36]. The levels of EOX found in shrimp in areas where there is an active fishery are significant, but no comparable studies have been found for this species so it is difficult to assess whether these levels are changing. Hellou *et al.* [152] have quantified a selection of anthropogenic organochlorines for shrimp from the same general area as this work. Their results show significant levels of chlorinated pesticides, but these amounts are trivial when compared to the levels of total EOCl. The concentrations of EOCl found in this work ranged from 1.39-12.3 μg Cl g^{-1} of tissue, whereas the total amounts of chlorinated pesticides reported by Hellou were less than 50 ng g^{-1} of tissue. Organochlorine compounds at these levels have been reportedly correlated to detrimental effects on reproduction and on the viability of the offspring [10] while high EOCl in the diet of fish has been reported to affect the reproduction of fish [261]. Shrimp are relatively low on the food chain and the relatively higher levels found in Arctic beluga [183] and much higher levels in tissue lipids of cod [252] suggests bioaccumulation of EOCl. The consequences of these body burdens of EOCl are not known. However, since the accumulations are in critical compartments, such as brain and eggs of cod [262] and kidney and brain of belugas [183], there is reason for concern.

No information could be found on EOBr or EOI in shrimp but research has shown that many brominated anthropogenics have toxic effects [15, 48] and so the levels shown in this work could have some impact on the species.

4.4 Conclusions

It is evident that there is an emerging spatial trend in concentration for EOX levels in the roe of *P. borealis*, although none is apparent for EOX in muscle. The fact that levels of EOBr are highly correlated to the level of EOI might indicate that EOBr and EOI could be composed of similar compounds. These compounds may be related to natural components found in the food of the shrimp or may be produced *in vivo* by the shrimp. The difference in the distribution of EOCl indicates a source that is different than that for EOBr and EOI. Further study of EOX in the marine environment is necessary to determine whether there is any threat to the species in this area.

Trends reported in shrimp may also be found in other species at higher trophic levels, especially those which consume the shrimp such as cod. If this were so, shrimp may be useful as an indicator species for many trends in EOX because of its intermediate position in the food chain, life-span (5-7 years), wide geographical distribution, and lack of migration in a north-south direction.

5. CHARACTERIZATION OF EXTRACTABLE ORGANOHALOGENS

5.1 Introduction

There exists a need to separate and characterize organohalogens that actually comprise EOX. Different properties of the analytes such as size, polarity, aromaticity, acidity, *etc.* can be used to accomplish the separations. In this work, size has been used to separate the compounds.

Gel permeation chromatography, more aptly called size exclusion chromatography (SEC) separates compounds based on their ability to permeate the pores of the column packing. The smaller compounds (also moderately sized linear compounds) are most able to do this and so are retained longest while the larger, globular compounds are not retained very well and so are eluted the fastest (*i.e.* the smallest elution volume).

Many reports in the literature appear to indicate that a high percentage of EOX has not been identified. Additionally, there is some indication that a large amount of the unidentified material is found in the lipid fractions [25, 183, 262]. It follows that the fractions from SEC which contain higher molecular size compounds and which also contain the highest amounts of organohalogens would be of the most interest. In each fraction there may be a significant number of different compounds eluted, the number of which would vary depending on the size of the fraction and the resolution of the column employed. These fractions can be analyzed as is or can be submitted to another method of separation producing a less complex fraction for analysis.

In practical terms, the secondary method of separation should use a parameter that is different than the original to yield a sample pure enough to allow for characterization. One such alternative criterion is polarity, where the fractions are separated using normal-phase chromatography in which the solid support is highly polar (such as silica gel). The separation may be achieved using either column chromatography or thin layer chromatography (TLC).

The methods of analysis employed here for characterization were NMR and MS. Fluorescence, which is particularly valuable for the study of aromatic compounds, would not be practical for work with organohalogens as halogens quench fluorescence. Organobromines and organoiodines are present in very low levels even relative to lipid and so would be difficult to isolate and characterize using any of the methods described. Although the levels of EOCl are also only present in ppm levels, their levels are considerably higher than that the levels of EOBr and EOI so the focus will be primarily on organochlorines.

5.2 Experimental

Fig. 5.1 shows a flow chart of the steps taken to characterize the EOX in shrimp. This section describes the chemicals and apparatus used as well as the pertinent procedures involved in the work.

5.2.1 Chemicals

The same solvents were used for this work as described in Chapter 3. Azulene, used in the size exclusion chromatography as an indicator for reproducibility of retention volume and a molecular weight standard, was purchased from Aldrich Chemical Co.

Other standards used in this work included: myristic acid and lauric acid from Aldrich Chemical Co.; oleic acid from J.T. Baker Chemical Co.; palmitic acid from Fisher Scientific Co., stearic acid from Sigma Chemical Co.; and linoleic acid, trimyristin and tripalmitin from Eastman Organic Chemicals.

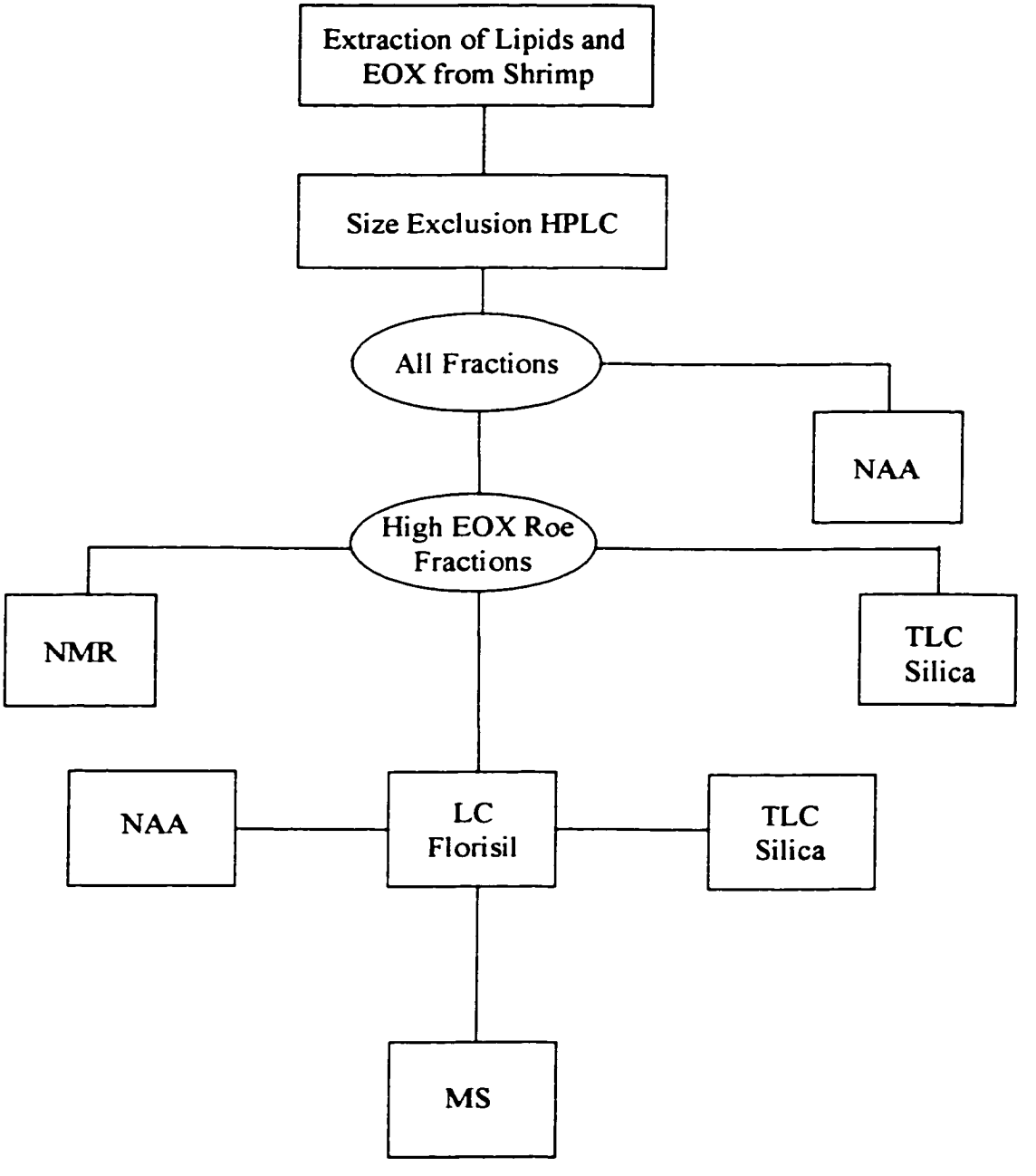


Fig. 5.1 Separation and Characterization of EOX

5.2.2 Sample Sources

A large amount of lipid was required for the work done in this chapter and since roe were lipid-rich and high in EOX, they were chosen to be the source of the lipid. A new sample of ~300 egg-bearing shrimp caught on 1997 September 29 at 53°44' N, 52°50'W (Shrimp Fishing Area 6, SFA6) were obtained from Fisheries and Oceans Canada in Newfoundland. The eggs and muscle were separated and extracted as described in Chapter 4. Most of the work was carried out using the roe extract, however, for comparison size exclusion chromatography was carried out on the muscle extract from SFA6 and roe extract from Location 1 (described in Chapter 4).

5.1.3 Size Exclusion Chromatography

Lipid samples, dissolved in hexane-acetone (1:1), were injected onto a Phenomenex Phenogel (polymerized styrene-divinylbenzene) 10μm MXL bed column. The injections of 0.9-1mL were made with a Hamilton syringe into a ~1.1-mL injection loop attached to a Rheodyne valve. The column configuration consisted of a pre-column, 60 x 21.2 mm i.d., followed by two 300 x 21.2 mm i.d. primary columns in tandem. The eluting solvent was the same mixture of acetone and hexane. The solvent was pumped through the column at a rate of 2.5mL min^{-1} by a Perkin –Elmer Series 100 HPLC pump. The samples were collected in glass scintillation vials using an Eldex Universal fraction collector. The first 100mL of solvent were collected in 20-mL fractions; the following 200 mL were collected in 5-mL fractions. The last 200 mL were collected in 20-mL fractions. All fractions from SEC were analyzed for halogens using NAA as described in Chapter 3.

This particular column was reported by the manufacturer to provide linear separation from 10^2 to 10^5 daltons for a given series. To give an indication of the molecular masses in the sample fractions, the standards used were trilaurin, trimyristin, stearic acid and myristic acid.

5.2.4 Thin Layer Chromatography

Thin layer chromatography was used on the fractions generated from SEC that contained the highest levels of EOX. These fractions also contained the highest amount of lipid.

Pre-coated, silica gel on glass, acid washed, 20x20 cm, plates were purchased from Aldrich Chemical Co. and were used without further treatment. The plates were spotted manually with 5 μL of sample, air-dried, placed in the developing tank and developed for 90 min with 10% acetone in hexane (v/v). Spots containing unsaturated lipids, fatty acids, sterols, *etc.*, were revealed using 10% phosphomolybdic acid in ethanol spray reagent (purchased from Sigma). The plates were sprayed evenly with the reagent and then placed in an oven at 120°C for 1-2 min resulting in blue spots on a yellow background. The standards which contained saturated fatty acids could not be revealed in this manner and so an alternative method was used, in which the plate was sprayed with 3M sulfuric acid and was placed in an oven at 150°C for 15 minutes. The remaining compounds showed as dark-brown spots on a lighter brown background. The mixed standard used for the TLC contained equal parts of trilaurin, trimyristin, and stearic acid.

5.2.5 Florisil Column

Separation using Supelco ENVI Florisil solid phase extraction tubes (1g packing, 6mL tube) with stainless steel frits were used for further clean-up and separation of two fractions from SEC roe from SFA6. The 135-mL and 140-mL fractions (named SEC135 and SEC140, respectively) were used in this work. The tubes were washed with 10 mL of hexane and the excess hexane was blown off with air. Fractions from SEC were dried and then made up in hexane. In the case where the sample was not completely soluble in hexane, up to 30% acetone was added to dissolve the residue. One mL of the sample (in hexane) was added dropwise to the tube and all eluates were collected from this point onward. Six mL of hexane were added dropwise to the tube to elute the less polar compounds and the fractions were collected in 1-mL increments labelled as SEC135-1 through -6 and SEC140-1 through 6. The more polar compounds were eluted in one-mL fractions with 4 mL of acetone-hexane (1:1). Four more fractions were collected using acetone as the eluent and these were designated as SEC135-7,8,9,10 and SEC140-7,8,9,10. All the fractions were analyzed by NAA, and further fractionated by TLC.

5.2.6 Nuclear Magnetic Resonance

Experiments using ^{1}H NMR (250.1 MHz), ^{31}P NMR (101.3 MHz) and ^{13}C NMR (62.9 MHz) were performed by the Atlantic Region Magnetic Resonance Centre on fractions SEC135 and SEC140 (described above). All NMR measurements were carried out at 22°C with a Bruker AC 250F NMR Spectrometer. All samples were dissolved in 0.3% trimethylsilane in $CDCl_3$.

5.2.7 Mass Spectrometry

Mass spectrometry was performed on fractions of SEC135 and SEC140 (Section 5.2.5) from the Florisil treatment that contained the highest amounts of EOCl as determined by NAA. All MS data were acquired with a VG Quattro quadrupole mass spectrometer. The samples were injected in 50-μL aliquots using flow injection driven by an HPLC pump at a flow rate of 200 μL min^{-1}. The source temperature was 120° and the probe was at 350°C. The samples were ionized using atmospheric pressure chemical ionization (APCI) with in both positive and negative ion mode, with a corona voltage of 3.75 kV and variety of negative and positive cone voltages. Nitrogen was used as the nebulizing gas. The flow rates were 300 standard litres per hour (slph) for the bath and sheath gas and 30 slph for the nebulizer. If chlorine is present in a sample, negative ion APCI is particularly useful as it allows one to "see" chloride ions which are not seen when positive ion APCI is used. All spectra were smoothed using a Savitzky-Golay filter.

5.3 Results and Discussion

5.3.1 Size Exclusion Chromatography

Size exclusion chromatography using the Phenogel column provided the most effective method of bulk separation with the most information compared to the other methods used in this work. For example, an effort was made to use dialysis for separation of the smaller components from the larger components of the lipid extract. While this might theoretically yield large samples of partially purified extract, unfortunately the membrane employed (polyethylene, 1.5 inch diameter) was not robust

enough to withstand the solvent for more that a few days, at which point the membrane would begin leaking and the separation would be compromised. Size exclusion chromatography was preferable since the Phenogel column offered good resolution and the large column allowed the separation of relatively large samples (~0.25 g).

Lipids extracted from the roe and muscle taken from SFA6 were submitted to SEC, and the results are presented in Figs. 5.2, 5.3, 5.4, and 5.5. Lipids extracted from shrimp roe from Location 1 (Chapter 4) were also separated by SEC, and the results are presented in Figs. 5.6 and 5.7. In all cases the values for amounts in each fraction are presented in terms of percent of the total amount injected. The results for all points are shown in terms of the total amount found in 5 mL and in the fractions where 20 mL were collected, the total amounts were divided by 4 to yield a comparable value for the point. The recoveries of lipid and EOX from the extracts compared to the total amounts injected were also calculated in this process and they ranged from 94-112% of the total.

For each sample, the majority of the lipid was found to elute at 130 mL (Figs. 5.2A, 5.4A and 5.6A). After removal of the solvent, crystalline material was observed at 155 mL in all samples which is likely sterols or may possibly be saturated fatty acids that are solid at room temperature.

Most of the chlorine was found in the 140-mL fractions for the roe lipid from SFA6. Similarly, the highest amount of the chlorine was found in the 150-mL fraction of the muscle. In the case of the lipid from Location 1 roe, the highest amount of the chlorine was found in the 250-mL fraction, although a significant peak was also found at 135-mL. Significant chlorine peaks were also found in the roe extract from SFA6 at 285-mL and in roe extract from Location 1 at 290-mL. The EOCl in the SFA6 roe sample is

distributed over a wider range than for the muscle; the fractions containing the highest levels of EOCl are only approximately 2.5% of the total amount injected. In the case of the muscle, the highest fractions contained nearly 7% of the total while the remaining EOCl was distributed throughout the remaining fractions.

For EOI and EOBr, the highest levels were found in the high molecular weight fractions for all samples, typically around 135 mL and more than 20% of the EOBr found in the roe from SFA6 were found in this fraction. The muscle extract differed slightly from the roe extracts as the highest levels of EOBr and EOI were found at 155-mL and 145-mL respectively. However, EOI and EOBr peaks with retention volumes analogous to those found in the roe extracts of SFA6 are found in the muscle extracts

Data analysis was performed on the results of the SEC for each of the samples. They were first analyzed for correlation between the EOX in each fraction within the results for a given sample (Table 5.1). The correlation between samples for the levels of each halogen in the fractions was also calculated and the results are presented in Table 5.2.

The results from the correlation analysis show that there is low correlation between the distribution of EOCl and that of EOBr or EOI in each fraction (Table 5.1), especially for the roe from Location 1 (R= 0.243). The strongest relationship is for the distribution of bromine to that of iodine in both roe samples (R=0.906 and 0.894). While the value for the correlation between the distribution of bromine and iodine in the muscle is fairly low at 0.665, it is consistent with the other correlation coefficients calculated for the muscle extract (0.673 and 0.671).

Table 5.1 Intra-sample Correlation for Distribution of Halogens

Sample	Correlation Coefficients (R) for the Halogens in SEC Fractions		
	Cl/Br	Cl/I	Br/I
Roe SFA6	0.575	0.519	0.906
Muscle SFA6	0.673	0.671	0.665
Roe Location 1	0.243	0.130	0.894

Table 5.2. Correlation between Halogen Distribution and Sample Origin

Halogen	Correlation Coefficients (R) for Fraction/Fraction Comparison		
	Roe/Muscle SFA6	Roe SFA6/Roe Location1	Muscle SFA6/Roe Location1
Cl	0.533	0.319	-0.010
Br	0.401	0.955	0.361
I	0.648	0.925	0.794

In the comparison of distribution of EOCl in the muscle and roe samples, low values were found. The highest correlation was found between the roe and muscle from SFA6, and no correlation was found between the muscle from SFA6 and the roe from Location 1. A strong positive correlation was found between the distribution of EOBr in

both roe samples (R= 0.955) and a similar strong relationship was found for EOI in the roe from both locations (R= 0.925). For EOI, the comparison of the two roe samples to the muscle indicated some positive correlation.

These results indicate that EOCl must be somewhat different than EOI and EOBr and the differences in distribution of the EOCl compared to EOBr illustrate this. This could be related to the origin of EOCl, *i.e.* natural or anthropogenic or to the levels found in the local food source. The fact that the strongest correlation for EOCl distribution is between the roe and shrimp from the same location also supports this idea, inasmuch as all the EOX in the roe must originate in adult shrimp. The correlation coefficients calculated for EOI distribution between the three samples are high and seems to indicate that EOI may serve some natural function or are simply more evenly distributed in the ocean. The fairly constant relationship between EOI and EOBr, especially in the roe samples, also seems to indicate the same status for EOBr. The relationship for EOBr between the individual samples is not as strong as the one found for EOI and so this may suggest some mixed origin for EOBr.

The HPLC column used in this work is rated to be linear over 4 orders of magnitude; however, this is only applicable for compounds of a homologous series. A calibration curve was produced (Fig. 5.8) using trimyristin, trilaurin, stearic acid, myristic acid, azulene and vanillin; the absence of linearity suggests that molecular mass could not be accurately assessed from this graph. The steep slope of the linear portion of the curve also indicates that each fraction would have a fairly broad mixture of molecular weights.

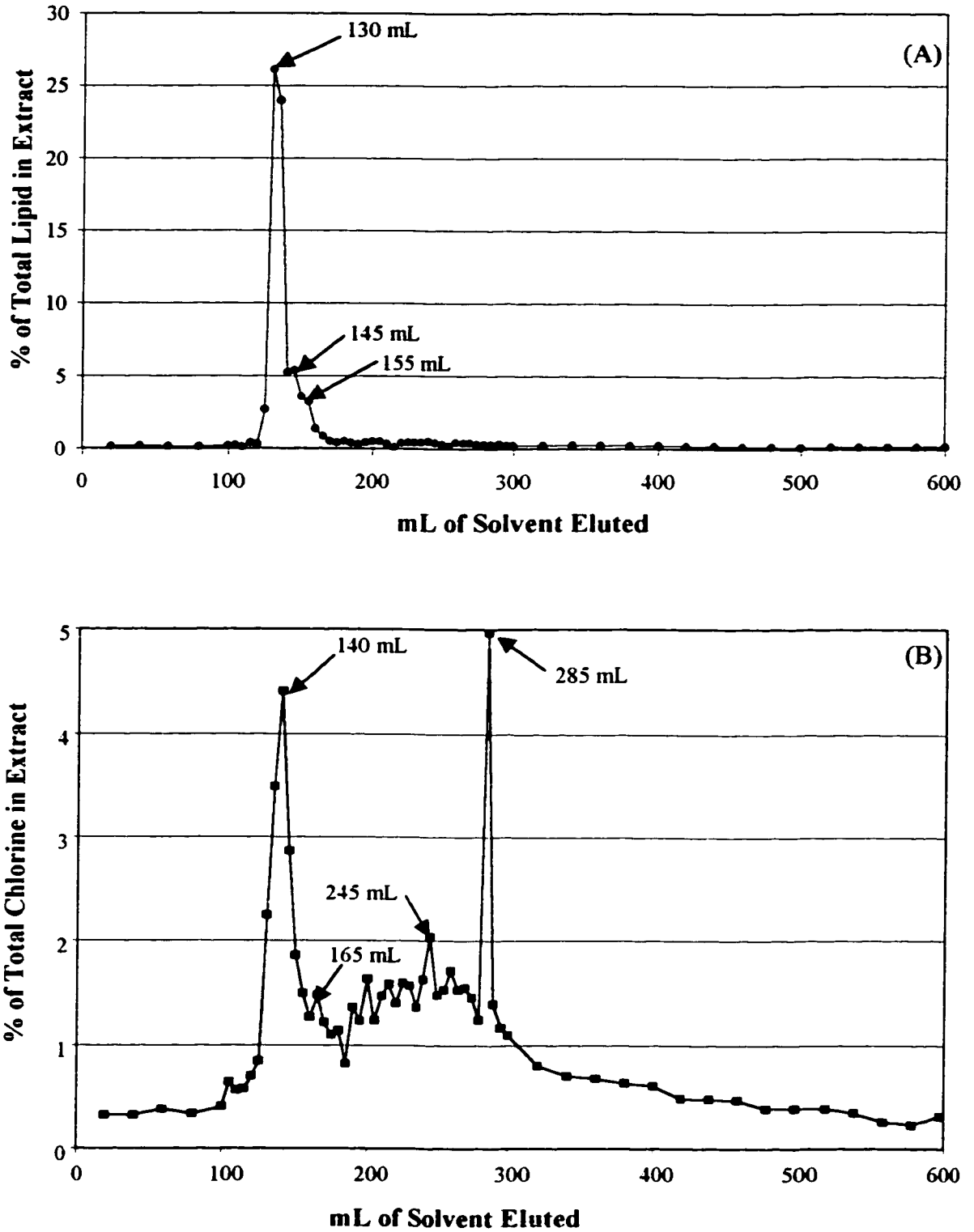
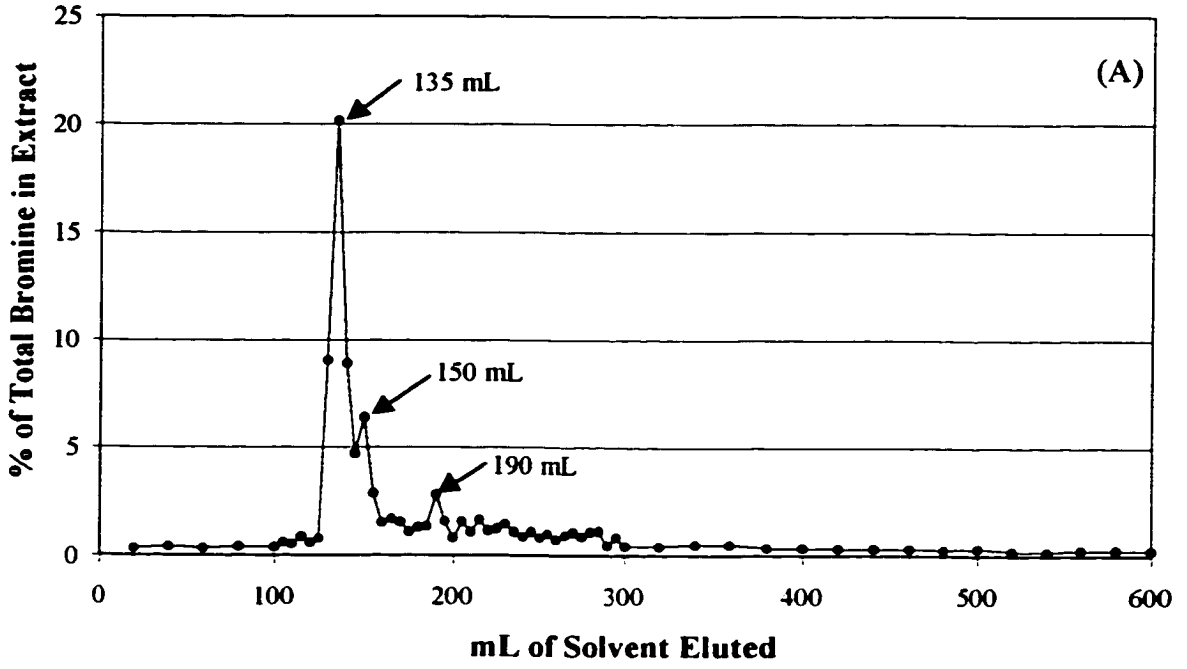


Fig. 5.2 Size Exclusion Chromatography of Roe Extract SFA6,
(A) lipid in fractions, (B) chlorine in fractions



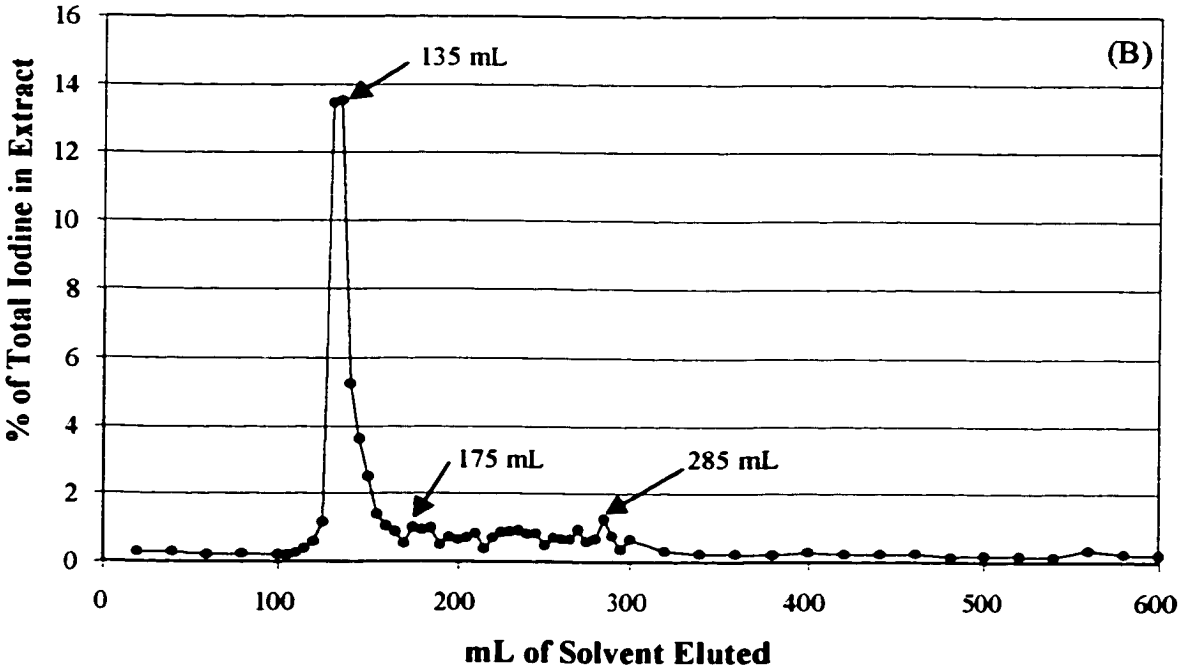


Fig. 5.3 Size Exclusion Chromatography of Roe Extract SFA6,
(A) bromine in fractions, (B) iodine in fractions

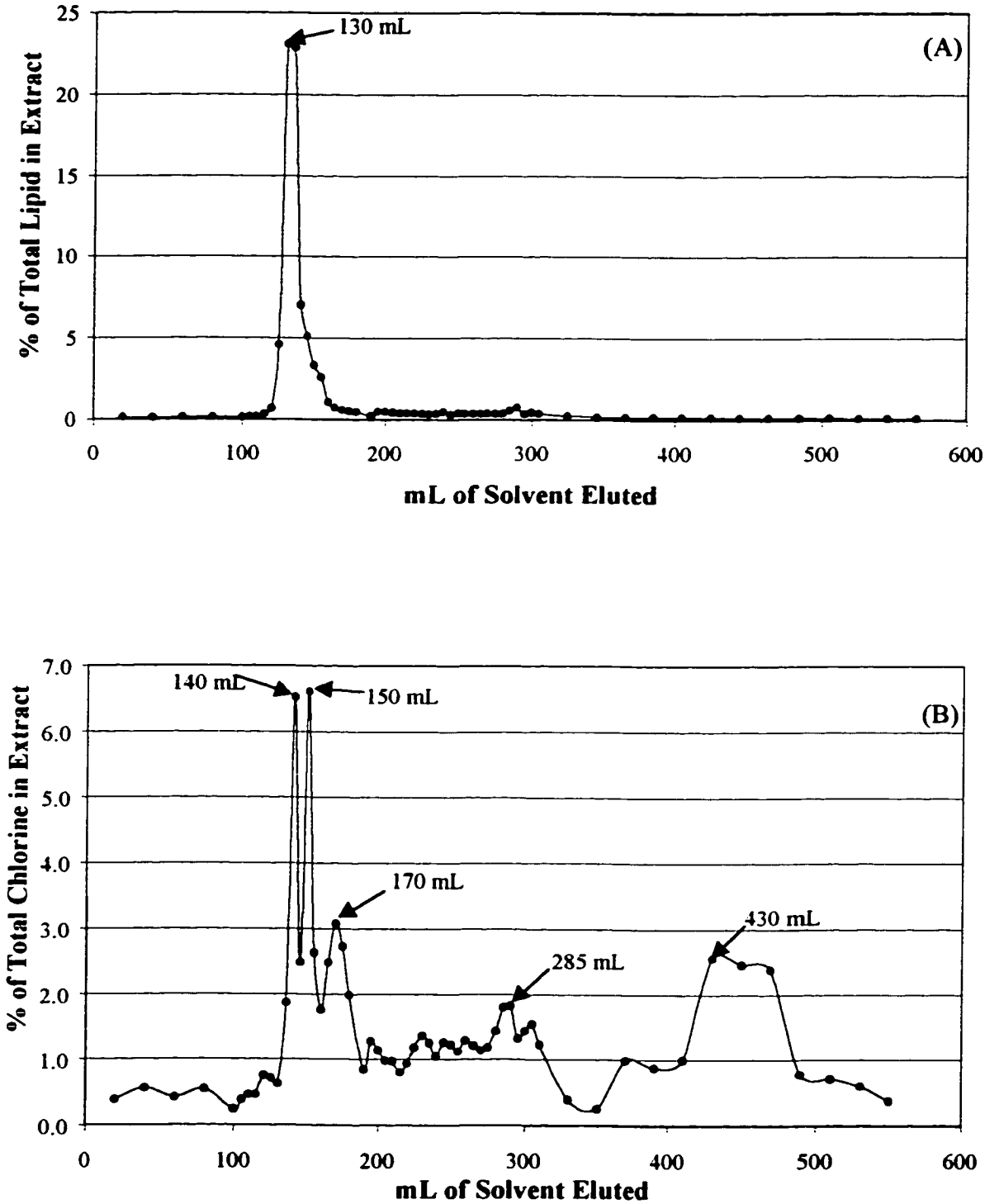
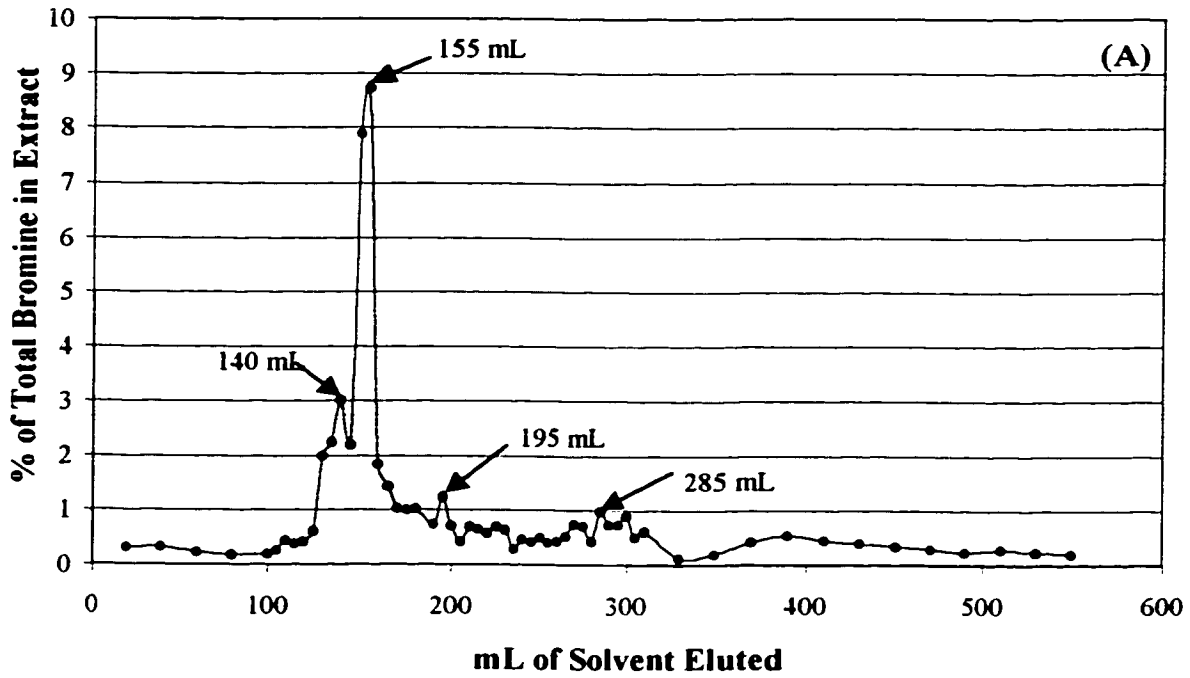


Fig. 5.4 Size Exclusion Chromatography of Muscle Extract SFA6,
(A) lipid in fractions, (B) chlorine in fractions



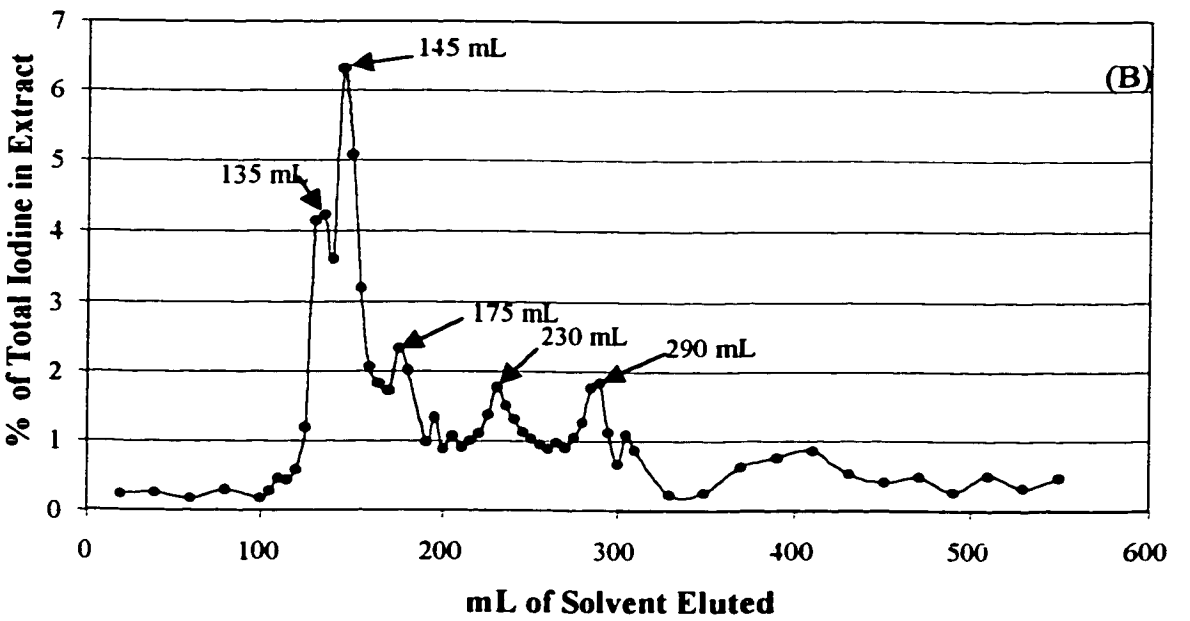
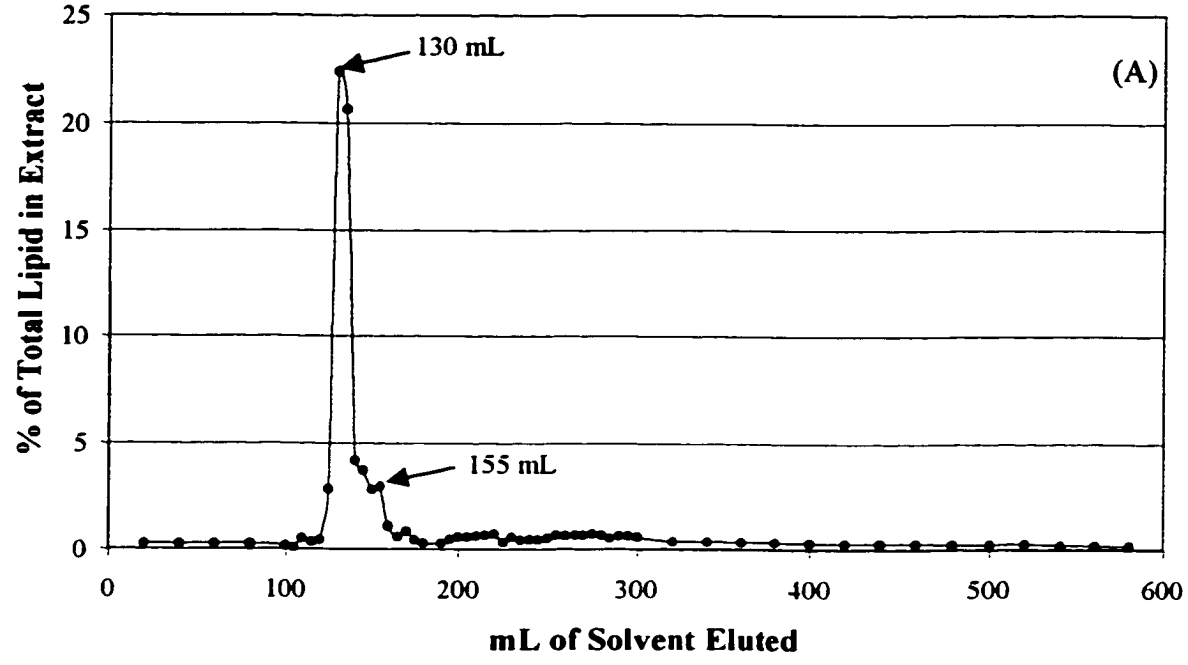


Fig. 5.5 Size Exclusion Chromatography of Muscle Extract SFA6,
(A) bromine in fractions, (B) iodine in fractions



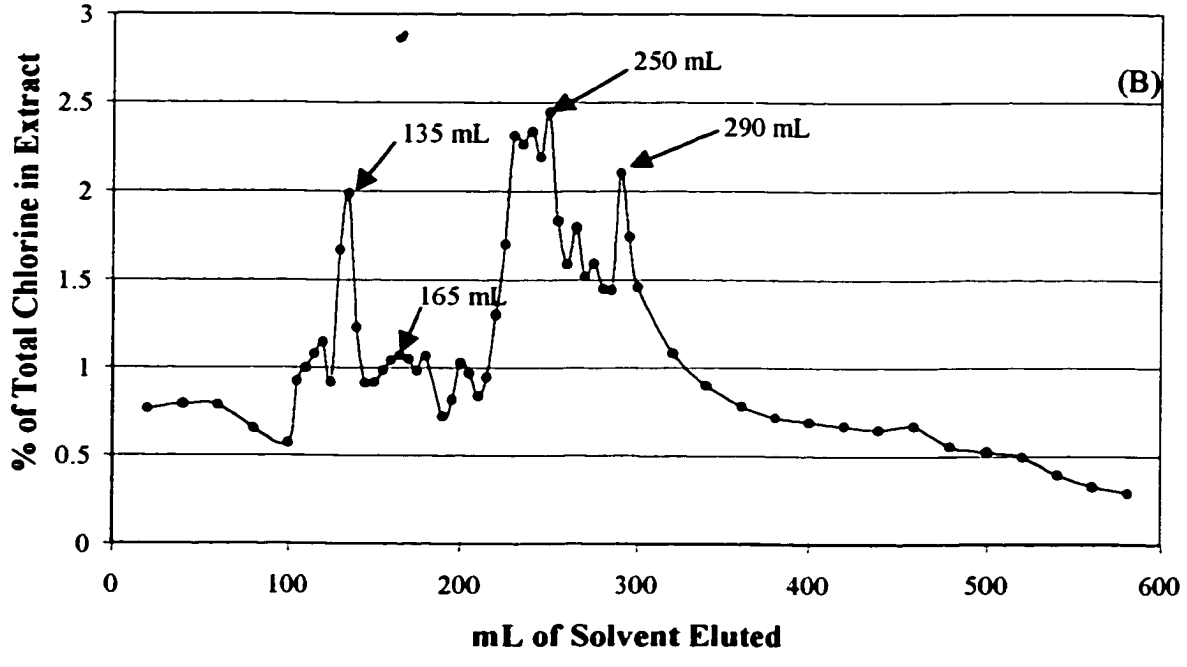
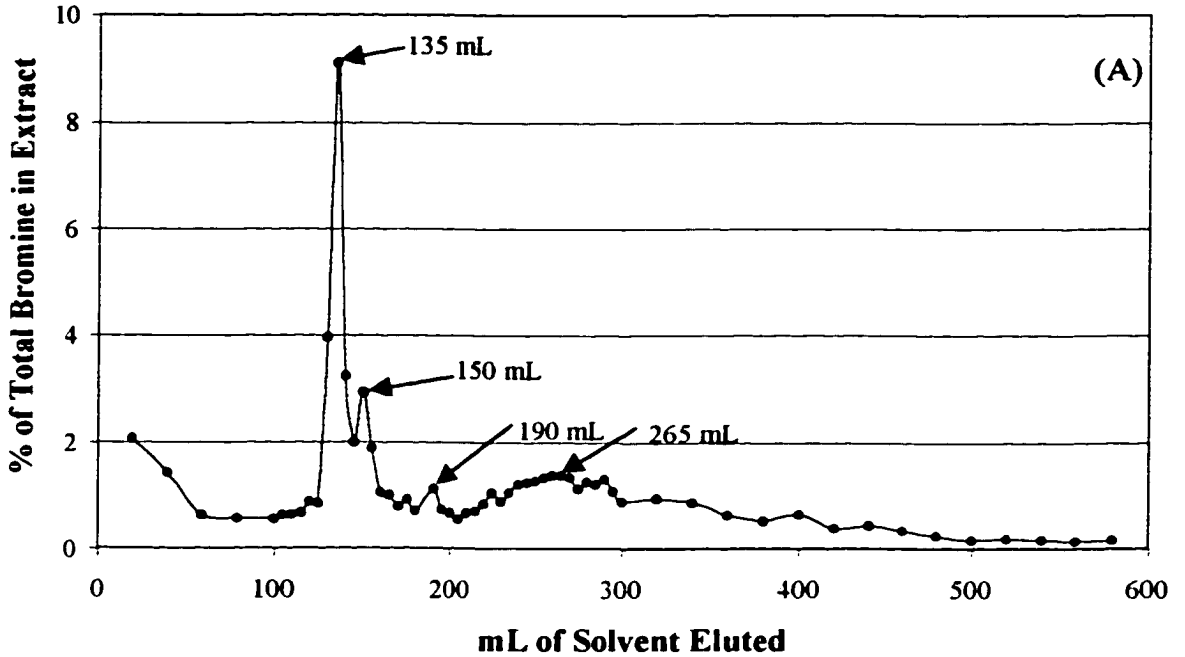


Fig. 5.6 Size Exclusion Chromatography of Roe Extract Location 1, (A) lipid in fractions, (B) chlorine in fractions



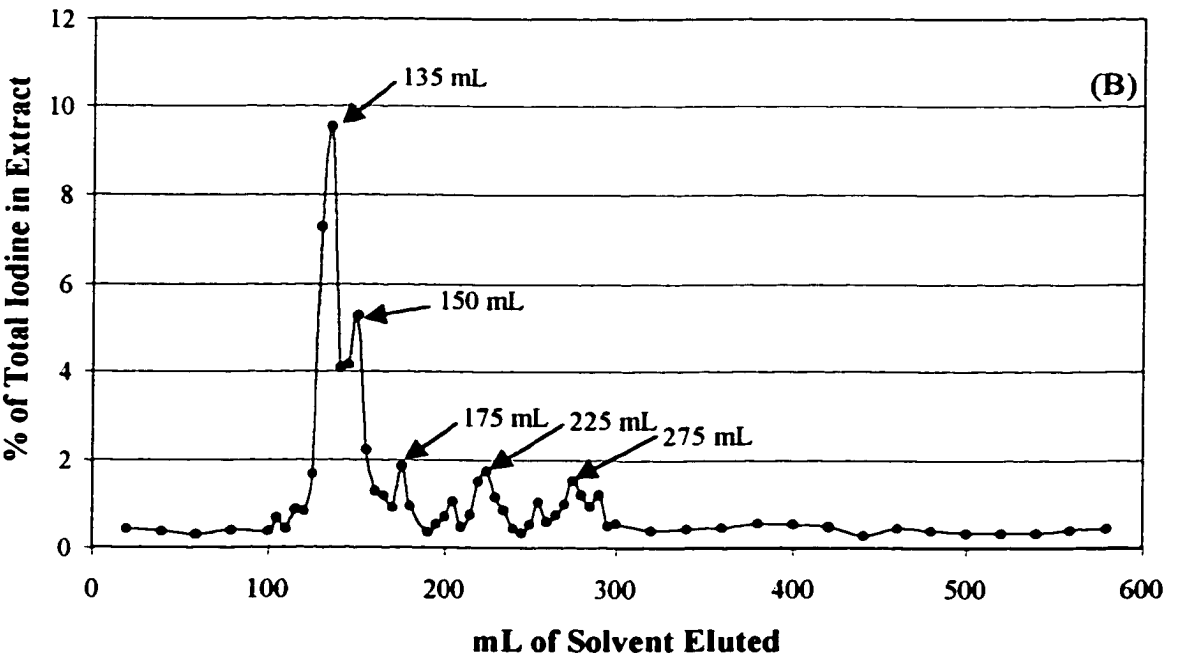


Fig. 5.7 Size Exclusion Chromatography of Roe Extract Location 1,
(A) bromine in fractions, (B) iodine in fractions

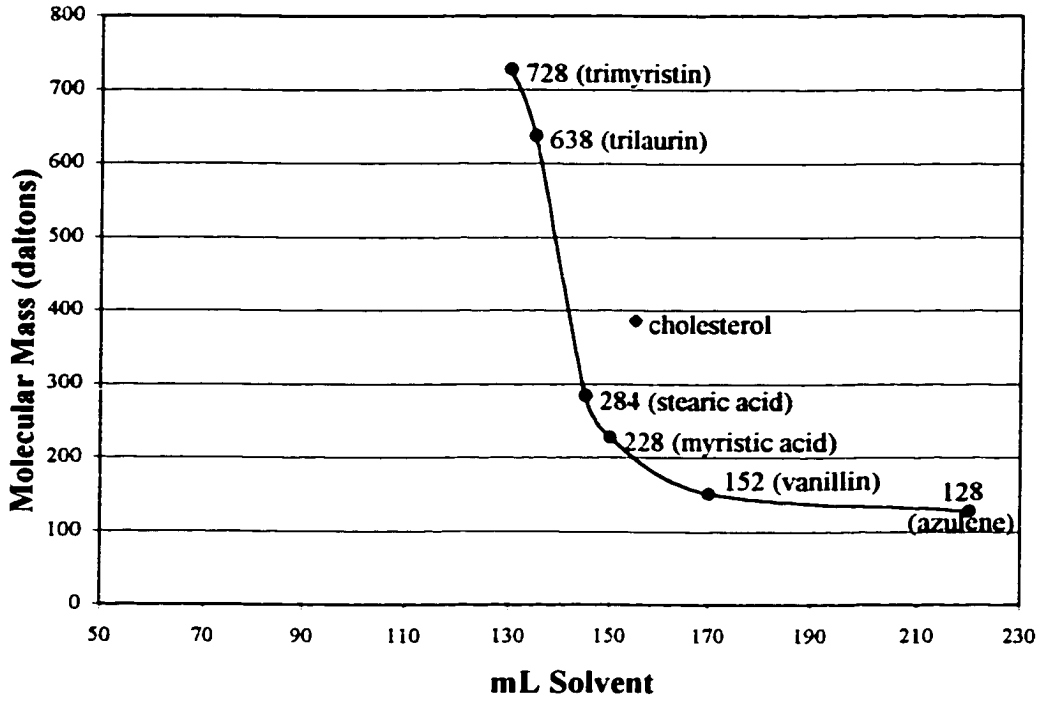


Fig. 5.8 SEC Calibration for Molecular Mass

5.3.2 Nuclear Magnetic Resonance

The results from ^{31}P NMR indicated that the two fractions SEC135 and SEC140 contained some phospholipids as was evident from the signals at −1.22 ppm (Fig. 5.9) and −1.26 ppm (Fig. 5.10); no other significant phosphorus signals were present. From the chemical shift of the signal in the ^{31}P NMR spectra, it appears that the fraction contains phosphotidylcholine. The literature [263] indicates that it is usually the most abundant of the phospholipids and the chemical shift is consistent with this supposition.

The ^{1}H (Figs. 5.11 and 5.12) and ^{13}C NMR (Figs. 5.13 and 5.14) do not show any evidence of phospholipids, the spectra are dominated by what appear to be signals from triacylglycerols. Both ^{1}H spectra clearly show a pattern of eight lines at 4.22 ppm associated with an ABX system, which corresponds to the protons on the C-1 and C-3

carbons of the glycerol portion of the triacylglycerol. The signal associated with the C-2 carbon of the glycerol can be found downfield at 5.27 ppm. The signals due to the olefinic protons of an unsaturated fatty acid can be seen at ~5.34 ppm. This signal is particularly well resolved in SEC135, whereas the analogous signal in the spectrum for SEC140 is more complex, which indicates that the unsaturation found in that sample is mixed, *i.e.* more than one double bond in a fatty acid or a variety of mono-unsaturated fatty acids.

The ^{13}C spectra of both fractions have signals at 130.0 and 129.7 ppm that would correspond to olefinic carbons which have slightly different environments; smaller additional signals at 128.1 and 127.0 ppm are present in the spectrum of SEC140 (Fig. 5.14).

The triplet at 2.31 ppm in the ^{1}H spectra is caused by the protons of a methylene group of a fatty acid which is α to the carboxyl group of the fatty acid. Similarly, the signal at 1.61 ppm results from the methylene protons β to the carboxyl group. The signal at 1.69 ppm is related to the methylene that is β to a double bonded carbon. The lack of resolution in some signals such as the one at 1.26 ppm indicates that the sample is a mixture of chain-lengths. The mixture of chain lengths is more evident in the spectrum for SEC140, where an additional triplet caused by a terminal methyl on a shorter chain can be seen at 0.97 ppm. The NMR spectra clearly show that these fractions are a mixture of triacylglycerols and, in the case of SEC140, also less substituted glycerols such as diacylglycerols. From the spectrum of SEC135 the average chain length of the fatty acid was calculated to be around 16 carbons.

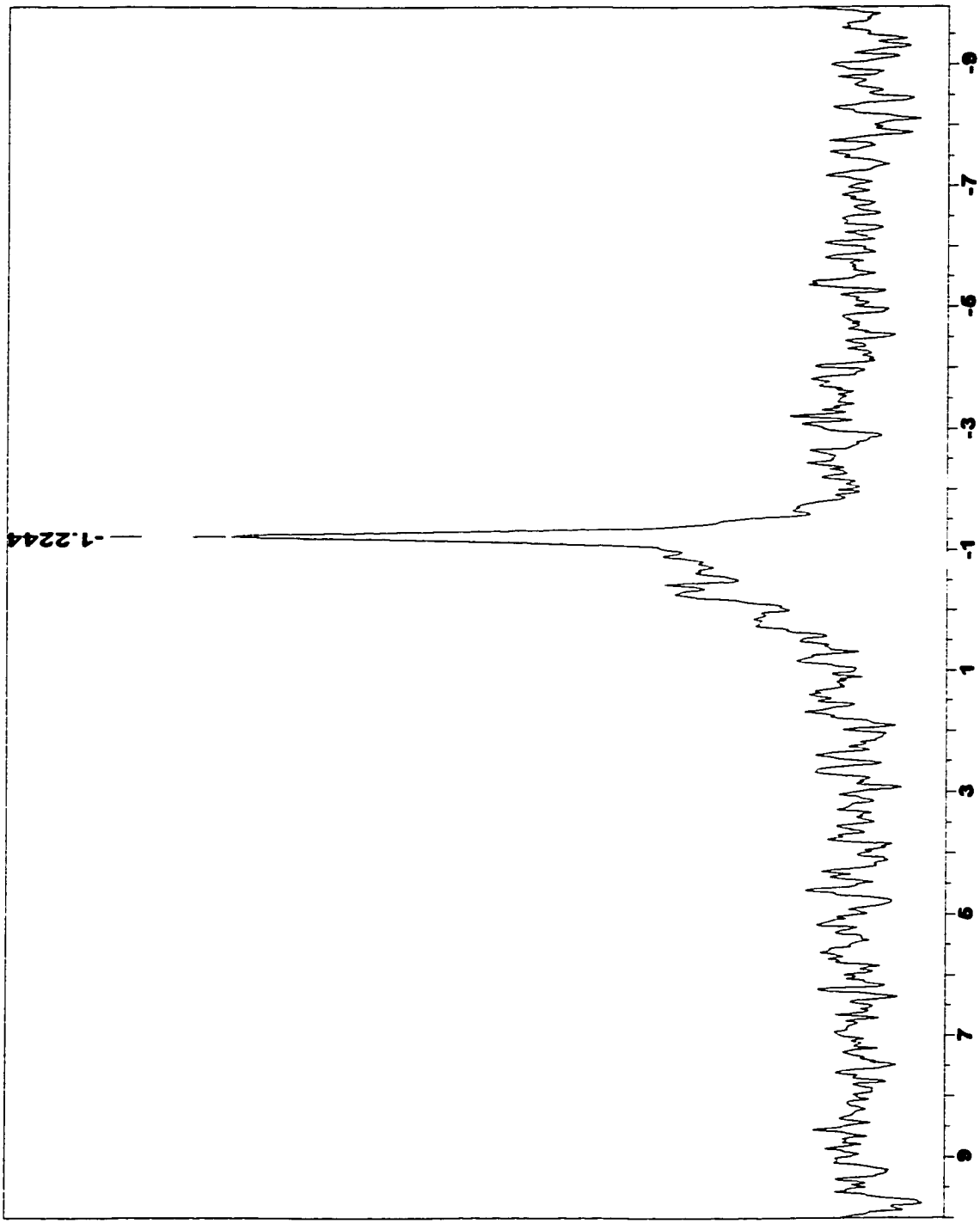


Fig. 5.9. ^{31}P NMR of SEC135

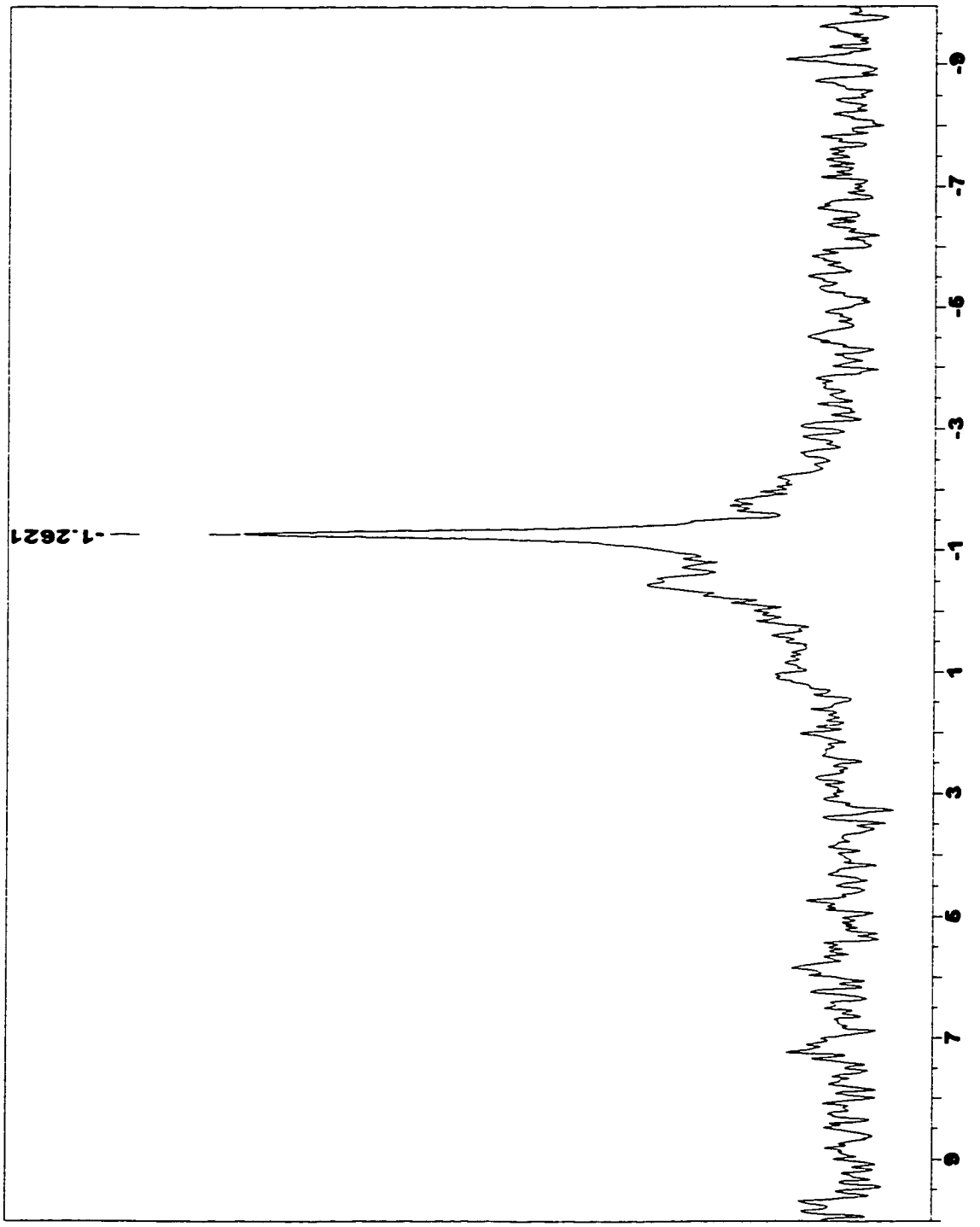


Fig. 5.10. ^{31}P NMR of SEC140

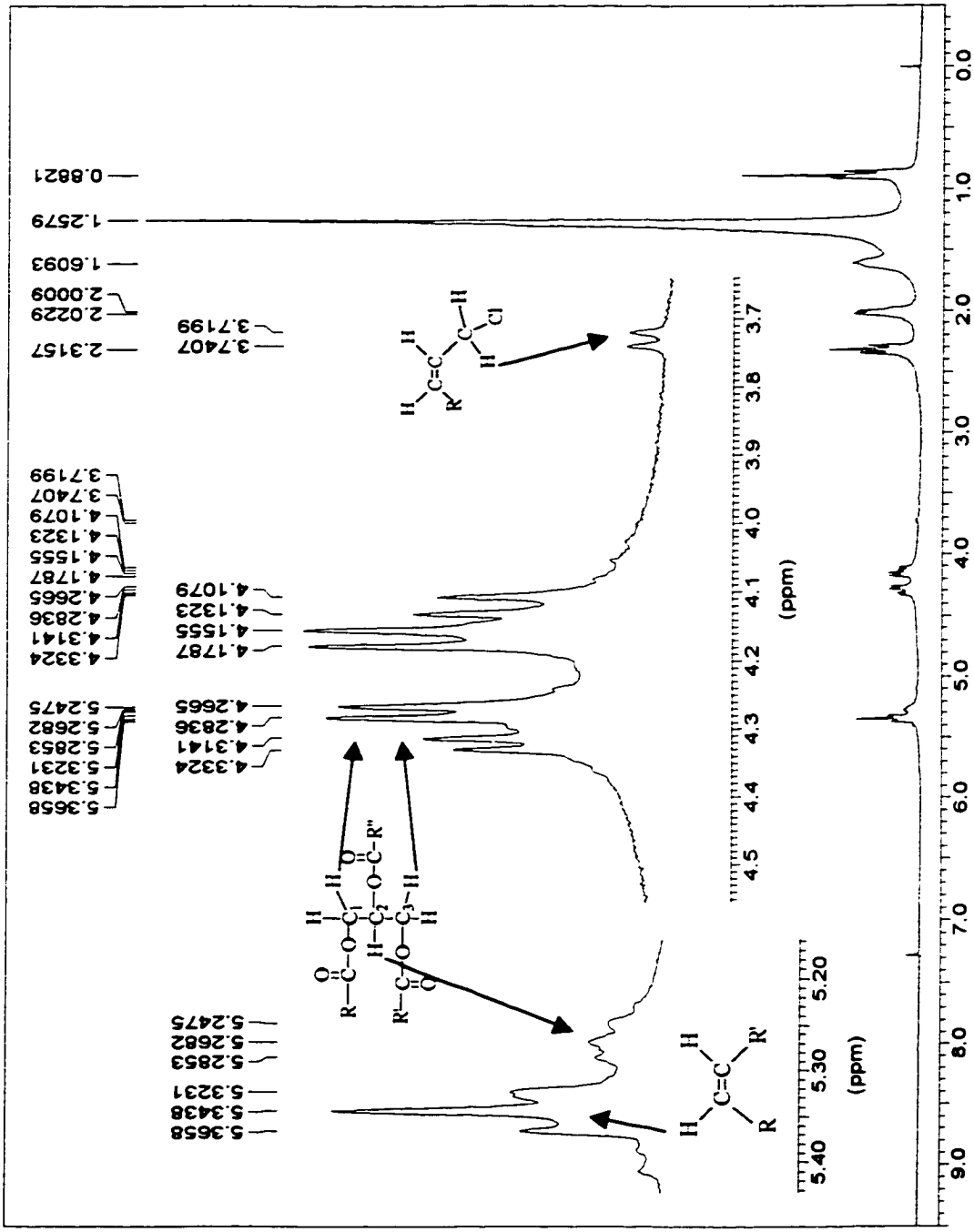


Fig. 5.11. ^{1}H NMR of SEC135

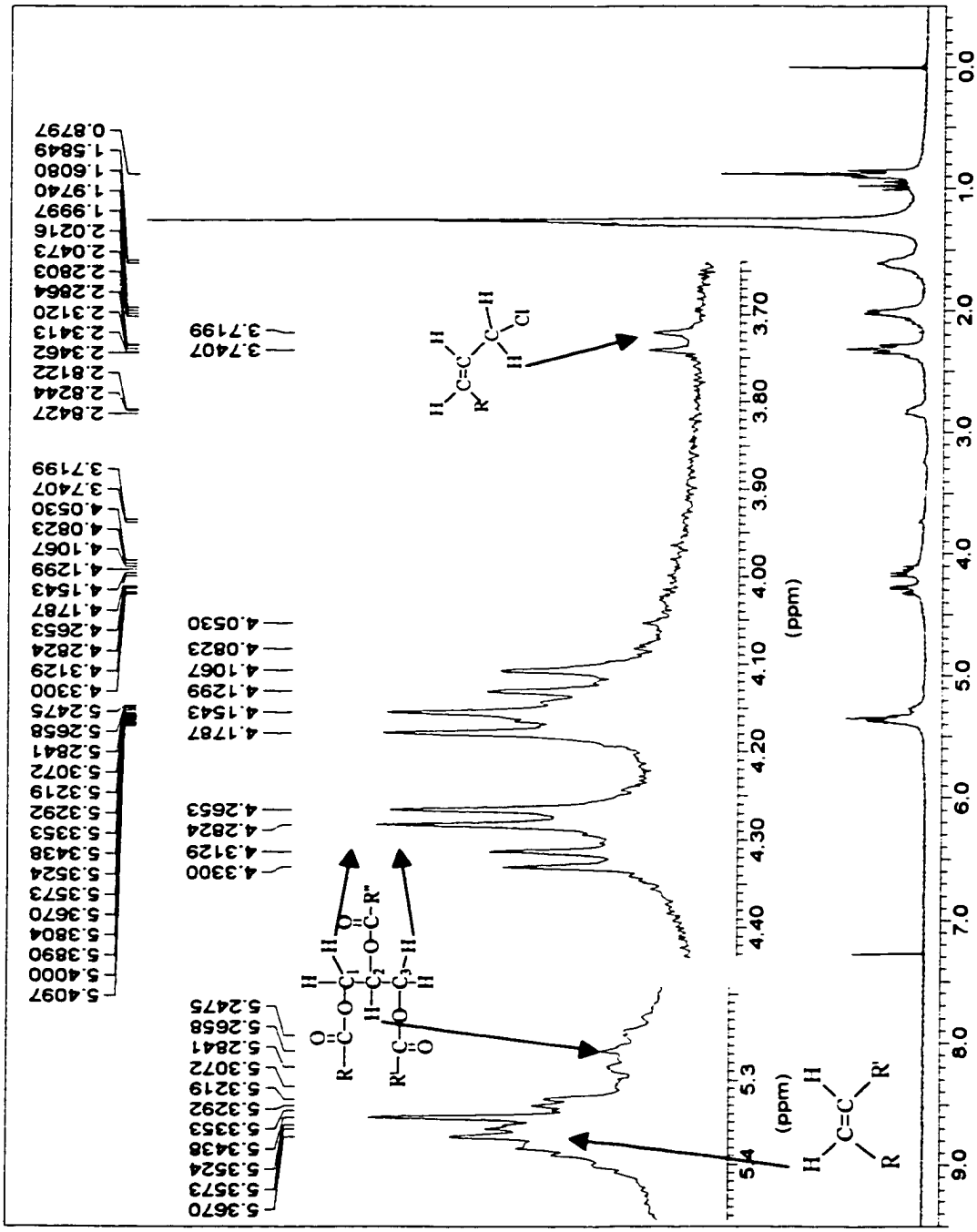


Fig. 5.12. ^{1}H NMR of SEC140

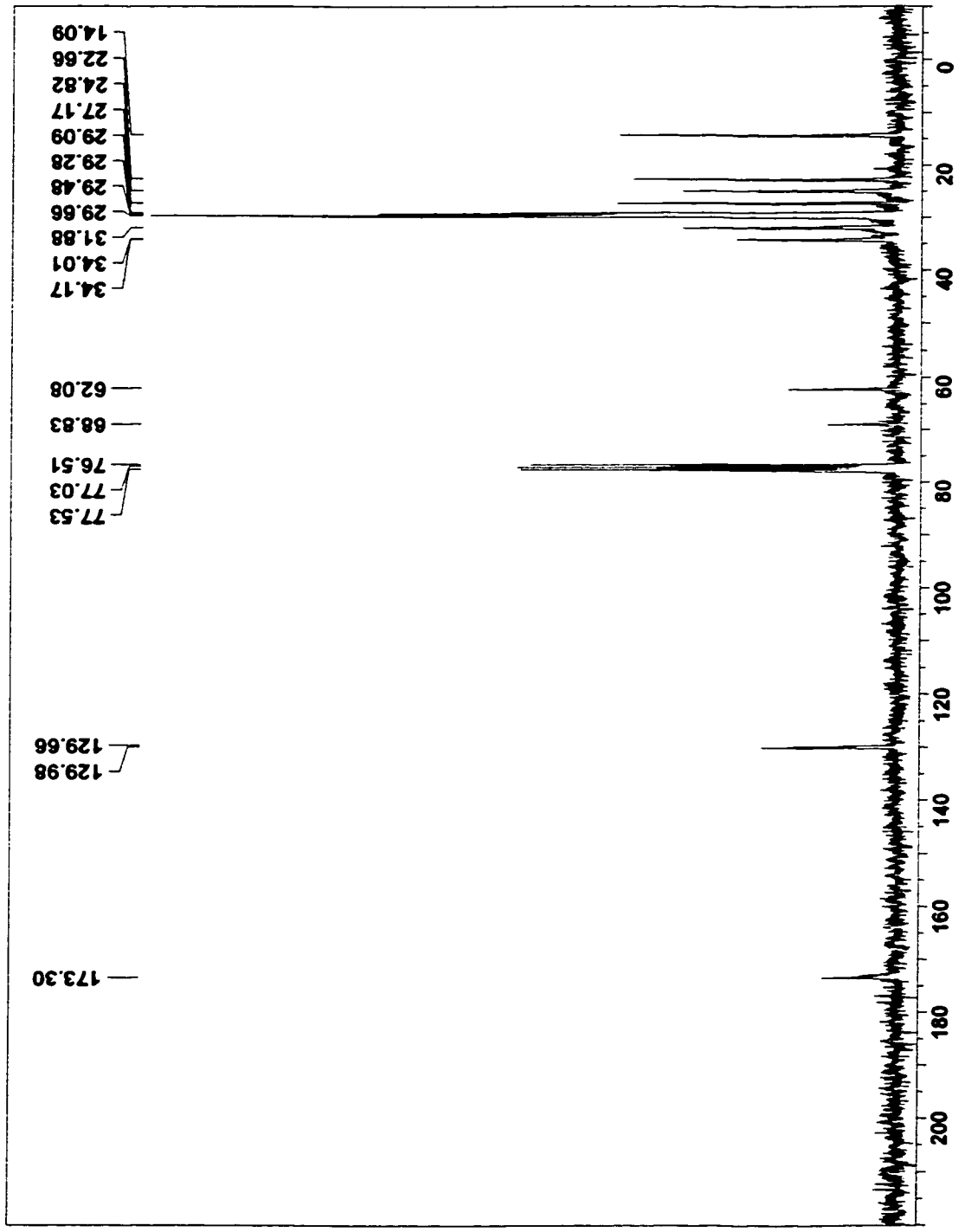


Fig. 5.13. ^{13}C NMR of SEC135

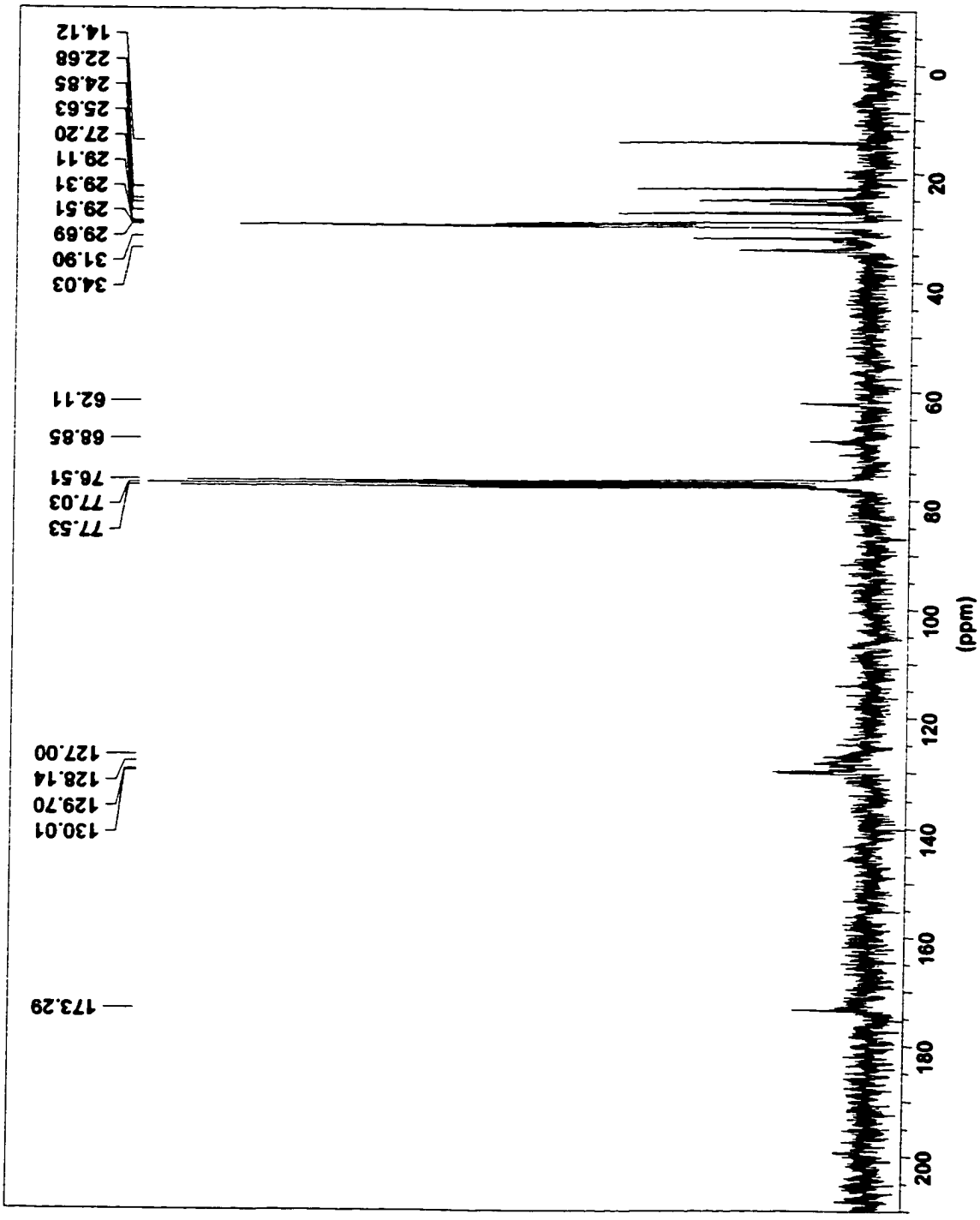


Fig. 5.14. ^{13}C NMR of SEC140

The most interesting feature of the ^{1}H spectra is definitely the doublet at 3.73 ppm. The proposed origin of this signal is shown in Figs. 5.11 and 5.12. This chemical shift and splitting is consistent with those reported in the literature for the same configuration [264]. The doublet is consistent with the single proton on the neighboring carbon. Since there are no other unexplainable signals common to both spectra, it may be concluded that this compound is a chlorinated unsaturated fatty acid. If this were the case, it could be distributed over a wide molecular mass range in the form of glycerol esters and as free fatty acid. It is difficult to find other configurations that would fit the criteria for chemical shift and coupling and one that would also not cause other unexplainable signals in the spectra. It should also be pointed out that this signal is not an artifact since it was found in the spectra of both fractions which were acquired weeks apart. This signal is probably most unusual because it seems to correspond to ω-2 (or n-2) fatty acids. Omega-3 fatty acids (n-3) are well known and reported for shrimp [246] and other species [265], perhaps the proposed structure above is due to a fatty acid that is found naturally in only trace amounts.

Gribble's review [15] of naturally produced organohalogens reported several structures that might offer support for the proposed structure including a brominated fatty acid isolated from marine sponges that has a terminal bromine substituent and an ω-1 (n-1) double bond *i.e.* a vinyl bromide. Although no chlorinated fatty acids were reported in Gribble's review that are consistent with the structure proposed here, a 2,2-chloro-1-bromo-1-iodo-*cis*-1-propene produced by red algae has been reported. In this compound, the position of the chlorine relative to the double bond is similar to that of the structure suggested in this work. It is unlikely that the compound found in the shrimp extract has a

terminal vinyl chloride, as one would expect the associated proton to be significantly more deshielded than the protons seen here. Nevertheless, these compounds suggest that it is possible that chlorinated fatty acids consistent with the NMR spectra could be found naturally. Further investigation is required.

5.3.3 Thin Layer Chromatography

Thin layer chromatography revealed that the fractions from SEC135 and SEC140 did contain several components of varying functionality as indicated by NMR. An illustration of the results can be seen in Fig. 5.15. The standards, which contained equal parts of trilaurin, trimyristin, and stearic acid, and the fractions are shown in this figure. It was quite clear from the TLC results that components that were least polar and moved the farthest from the origin (*i.e.* had the highest retardation factor R_F) were the triacylglycerols. The unsaturation of the fatty acid components of these lipids could also be elucidated from the fact that they could be revealed using the phosphomolybdic acid and this unsaturation is consistent with the results of the NMR.

The TLC results also indicated that there may be some proportion of free unsaturated fatty acid which has a similiar R_F to the stearic acid in the mixed standard. There were some components that did not move from the origin using the current developing solvent and increasing the acetone concentration did not change this result. These compounds might be pigments that are found in the shrimp tissue and may include some phospholipids. Commercial phosphotidyl choline from chicken egg was tested on the plate and it also showed no movement from the origin.

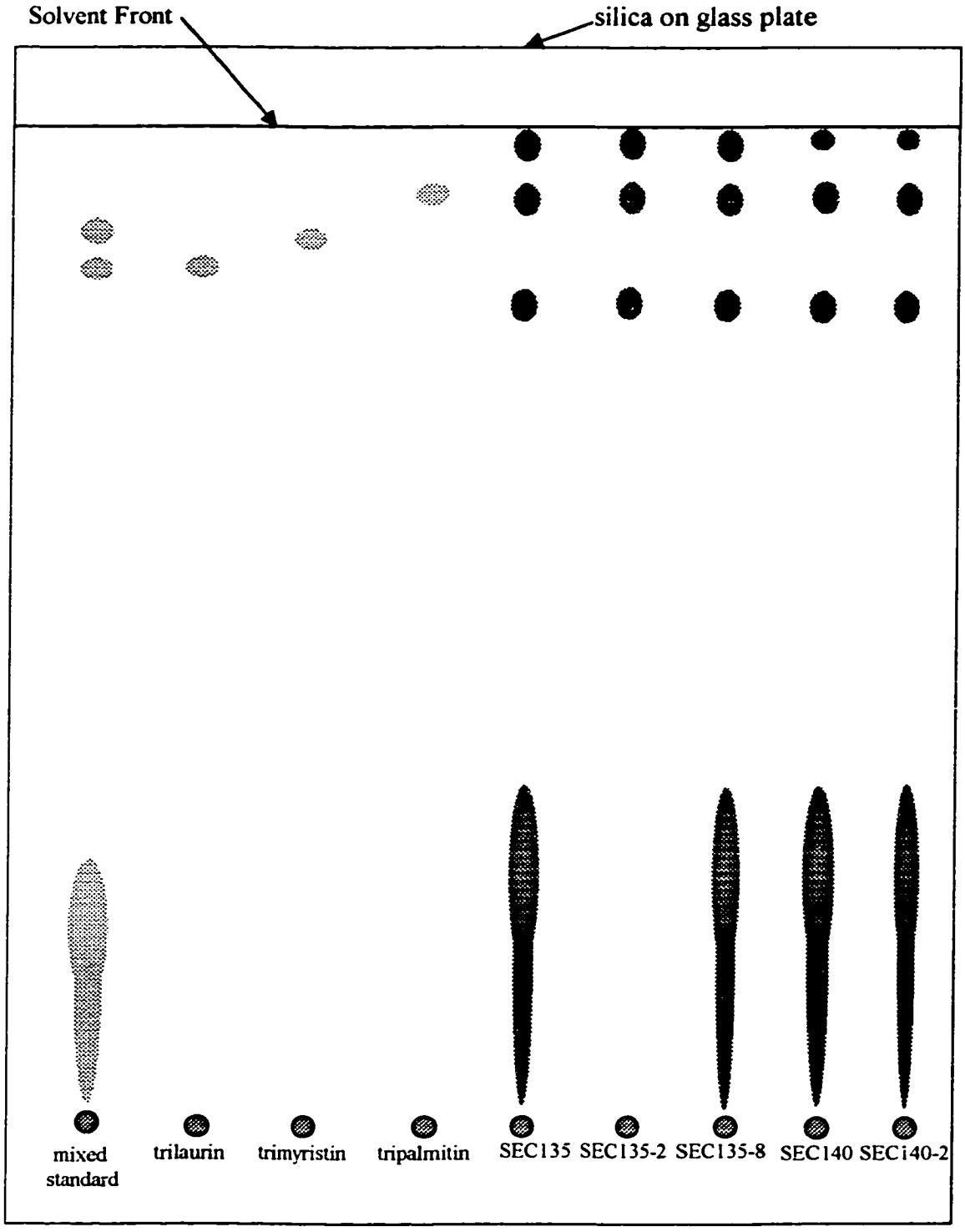


Fig. 5.15. Diagram of TLC Results

The TLC results for the fractions separated on Florisil (SEC135-2, SEC135-8 and SEC140-2) are also shown in Fig. 5.15. The results from the TLC verify recovery of components found in the original fraction. The TLC results also show that some of the triacylglycerols have been separated from SEC135 and these are found in fraction SEC135-2 which is free of other components.

5.3.4 Separation on Florisil

Acid washed Florisil was selected for the next separation step as it performs the same function as the silica gel on the TLC plate and it is available in clean pre-packed SPE columns that were suitable for use with small amounts of material. The cues for the solvent mixture used were taken from the results of the TLC. The sub-fractions from the Florisil separation were submitted to TLC and NAA to see if there was effective separation and also to discover which fractions contained more chlorine. The sub-fractions containing the most chlorine were submitted to MS.

SEC135 was readily soluble in hexane and added to the column in a 1-mL aliquot. The 6-mL of hexane were used to elute the least polar components of the fraction. These components (SEC135-2) had the same R_F on the TLC plate as the triacylglycerols. The hexane-acetone solution seems to have eluted more of these same triacylglycerols as well as other components that would be consistent with fatty acids, and other less substituted acylglycerols. The fraction corresponding to the second addition of acetone-hexane (SEC135-8) had the highest proportion of chlorine as determined by NAA.

SEC140 was less soluble in hexane and so could not be separated on the column in the same manner as the other fraction. It was added to the column in a solution of

70:30 hexane-acetone. This fraction was eluted with hexane (SEC14O-2), however the additional polarity from the acetone added meant that only the most polar compounds were retained. No additional components were eluted using the 50:50 acetone-hexane mixture.

Some coloured material was retained on the column for both fractions that likely corresponded to pigments and possibly may also have included phospholipids. Methanol elution, which would elute phospholipids, did not remove the coloured substance(s).

5.3.5 Mass Spectrometry

Attempts to find molecular ions that had chlorine substituents met with no success. There are several reason for this, not the least of which is the fact that the chlorinated component was present in very low concentrations. Chlorine was present in all the samples analyzed which was illustrated by the peaks at m/z 35 and 37 (Figs. 5.17, 5.19, 5.21) which exhibited the characteristic 3:1 (m/z 35:37) ratio of intensities related to the abundance of the isotopes of chlorine. This was also verified using analysis of total ion chromatograms, which monitored for the ions of chlorine at m/z 35 and m/z 37. The expected ratio of ~3 was found in all samples. The best results of TIC were found when a cone voltage of 75V was used (other lower cone voltages were also used) which would cause the highest degree of fragmentation and thus the most chloride ions. The signal to noise ratios ranged from 13.47 to 45.6, which is significantly higher than 3 (which is generally accepted as the limit of detection).

Table 5.3. Some Typical Fatty Acids and Triacylglycerols

Compound	Chain-Length:Unsaturation	MW (rounded to nearest integer)
myristic acid (Fig. 5.18)	14:0	228
palmitic acid (Fig. 5.19)	16:0	256
palmitoleic acid	16:1	254
stearic acid	18:0	284
oleic acid (Fig. 5.21)	18:1	282
linoleic acid (Fig. 5.20)	18:2	280
linolenic	18:3	278
arachidic acid	20:0	312
	20:1	310
behenic acid	22:0	340
	22:1	338
trimyristin (Figs. 5.22, 5.23)	saturated	723
tripalmitin (Figs. 5.24, 5.25)	saturated	807

Although no information could be found regarding the structure or composition of the chlorinated compounds, the results from APCI-MS provide significant information about the composition of the extracts. The spectra of the samples (Figs. 5.16-5.21) clearly showed the presence of several saturated and unsaturated fatty acids and acylglycerols, consistent with the results from NMR. Their presence was confirmed by comparison to standards analyzed under the same conditions (Figs. 5.22-5.29). The compositions of the prominent ions found in the spectra are listed in Tables 5.4 and 5.5. All spectra shown were obtained at a cone voltage of 15V; other cone voltages used were

55V and 75V. The spectra for SEC135-2 are cleaner, *i.e.* the sample is more pure. This agrees with the result of the TLC which suggest that this fraction consists primarily of triacylglycerols.

The negative ion APCI of the fatty acid standards confirmed the presence of myristic acid (m/z 227), palmitoleic (m/z 253), palmitic (m/z 255), mono-unsaturated analogues of arachidic acid (m/z 309) and behenic acid (m/z 337) in all the fractions. The SEC140-2 fraction also showed a high proportion of the C-22 fatty acid containing 2 double bonds (m/z 335).

Table 5.4. Common Ions Observed in Negative Ion APCI Mass Spectrometry for Fractions of SEC135 and SEC140

m/z	Assignment
32	$[CH_3OH]^{-\cdot}$
35	$[^{35}Cl]^-$
37	$[^{37}Cl]^-$
42	$[CH_2CO]^{-\cdot}$
46	$[HCOOH]^{-\cdot}$
61	$[CH_3CHOOH]^-$
71	$[CH_3CH{=}CHCH_2O]^-$
99	$[CH_3CH{=}CHCH_2CO_2]^-$
111	$[CH_2{=}CHCH{=}CHCH_2CO_2]^-$
113	$[CH_3CH_2CH{=}CHCH_2CO_2]^-$
227	$[CH_3(CH_2)_{12}CO_2]^-$
253	$[CH_3(CH_2)_5CH{=}CH(CH_2)_7CO_2]^-$
255	$[CH_3(CH_2)_{14}CO_2]^-$
281	$[CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2]^-$
309	$[CH_3(CH_2)_9CH{=}CH(CH_2)_7CO_2]^-$
335	$[CH_3(CH_2)_8CH{=}CHCH_2CH{=}CH(CH_2)_7CO_2]^-$
337	$[CH_3(CH_2)_{11}CH{=}CH(CH_2)_7CO_2]^-$

It should be noted that in the cases where unsaturation shown in the ion assignments, the unsaturation might occur in positions other than those shown.

The positive ion APCI produced the most information about the molecular ions, without actually showing a likely molecular ion. It is important to note that if the acylglycerols are composed of only 5 different fatty acids there could be as much as fifty different combinations of tri-, di- and monoacylglycerols. Thus, it would be difficult to find standards that would correspond to all the compounds in the fractions. With this in mind, the fragmentation pattern was examined for the standards (Figs. 5.26 and 5.28) and it was found that the primary ion formed at a 15V cone voltage was diacylglycerol (the triglycerol minus myristate in trimyristin or palmitate in tripalmitin). The next major peak (m/z 285 or m/z 313 in Figs. 5.26 and 5.28 respectively) corresponds to the diacylglycerol minus the fatty acid, plus mass equal to water (18). This fragmentation pattern was found for trilaurin, trimyristin, and tripalmitin. The monoacylglycerol fragments of the triacylglycerols explain the unusual pattern of peaks found around m/z 300 in the positive ion APCI of all the fractions. For example the major peak at m/z 311 (Figs. 5.16, 5.18, and 5.20) could be formed from the component at m/z 577 minus oleate plus 17 (OH). This process can be performed for all the peaks in the spectra. The more pronounced peak in the positive ion APCI of SEC140-2 is m/z 313, which likely corresponds to the loss of the linoleate ion from the peak at m/z 577.

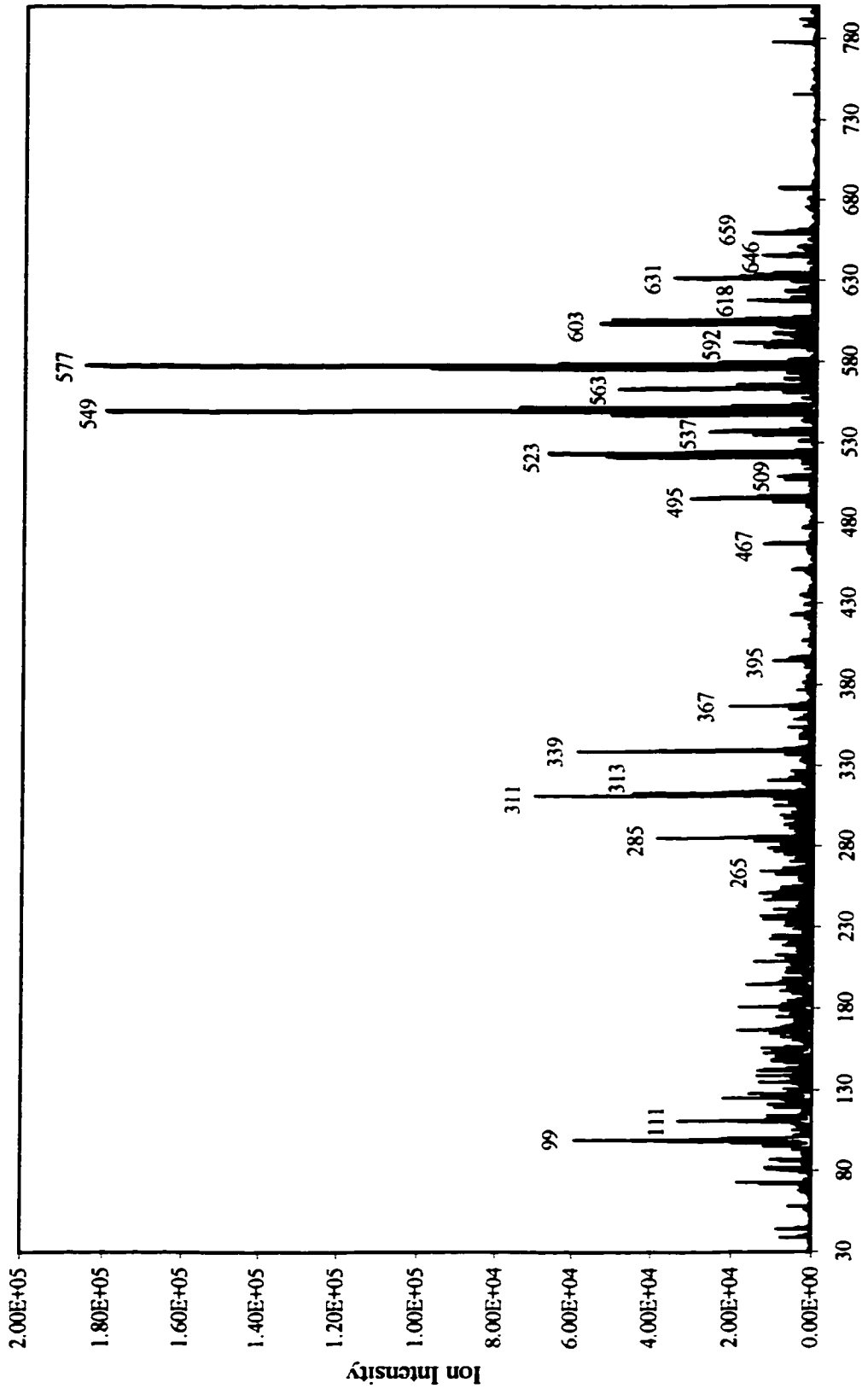


Fig. 5.16. SEC135-2, 15V, APCI+

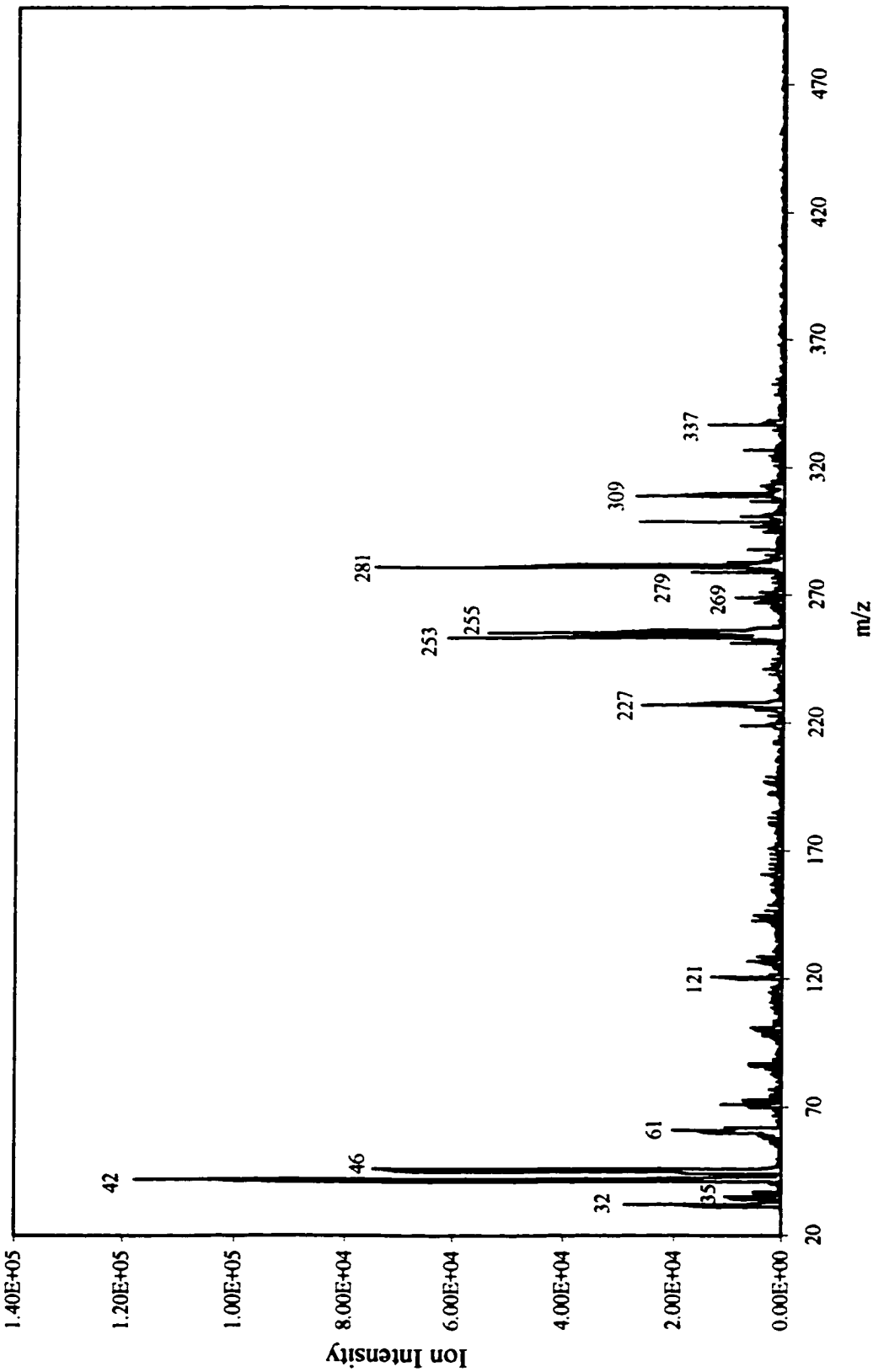


Fig. 5.17. SEC135-2, 15V, APCI-

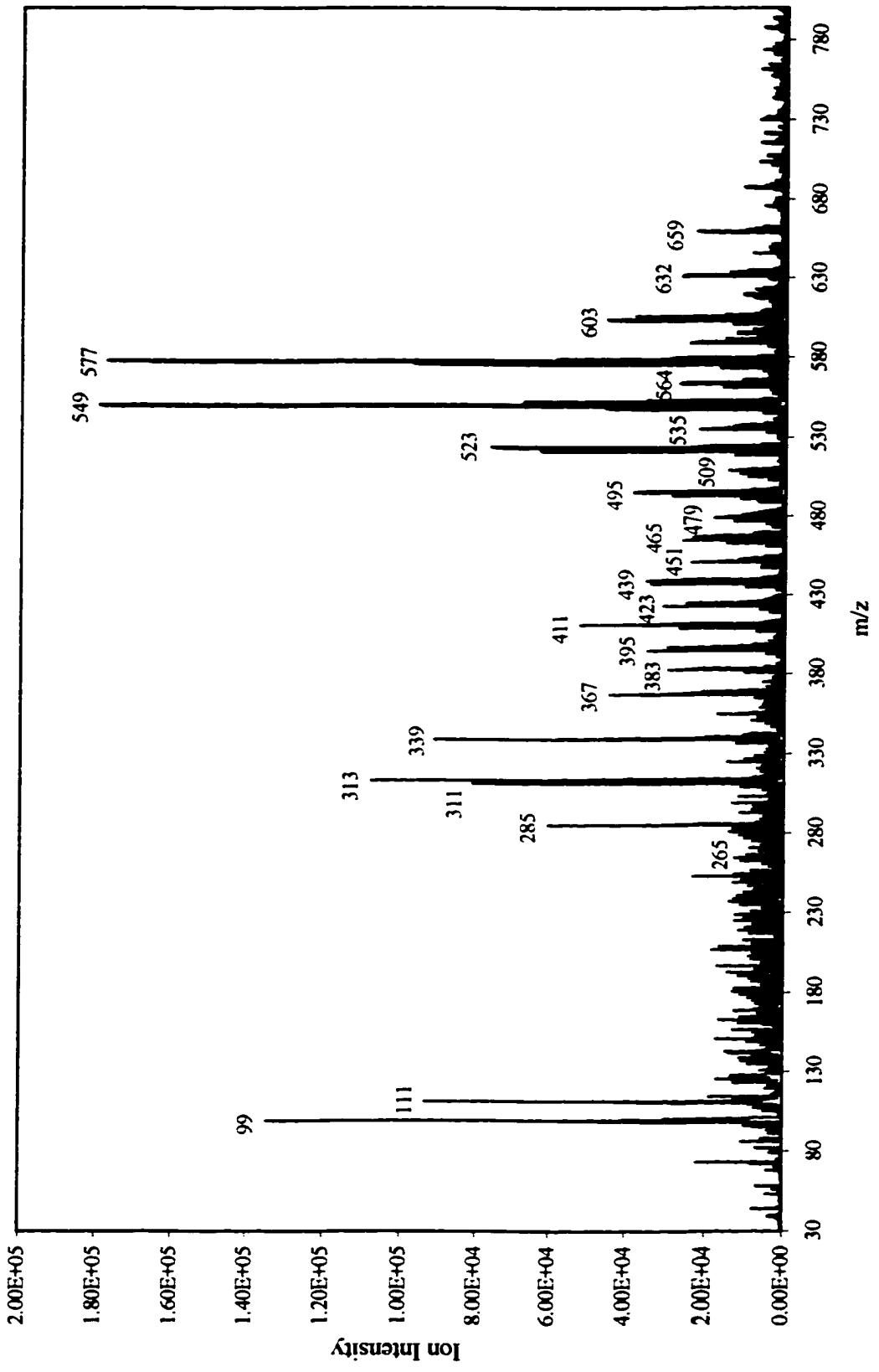


Fig. 5.18. SEC135-8, 15V, APCI+

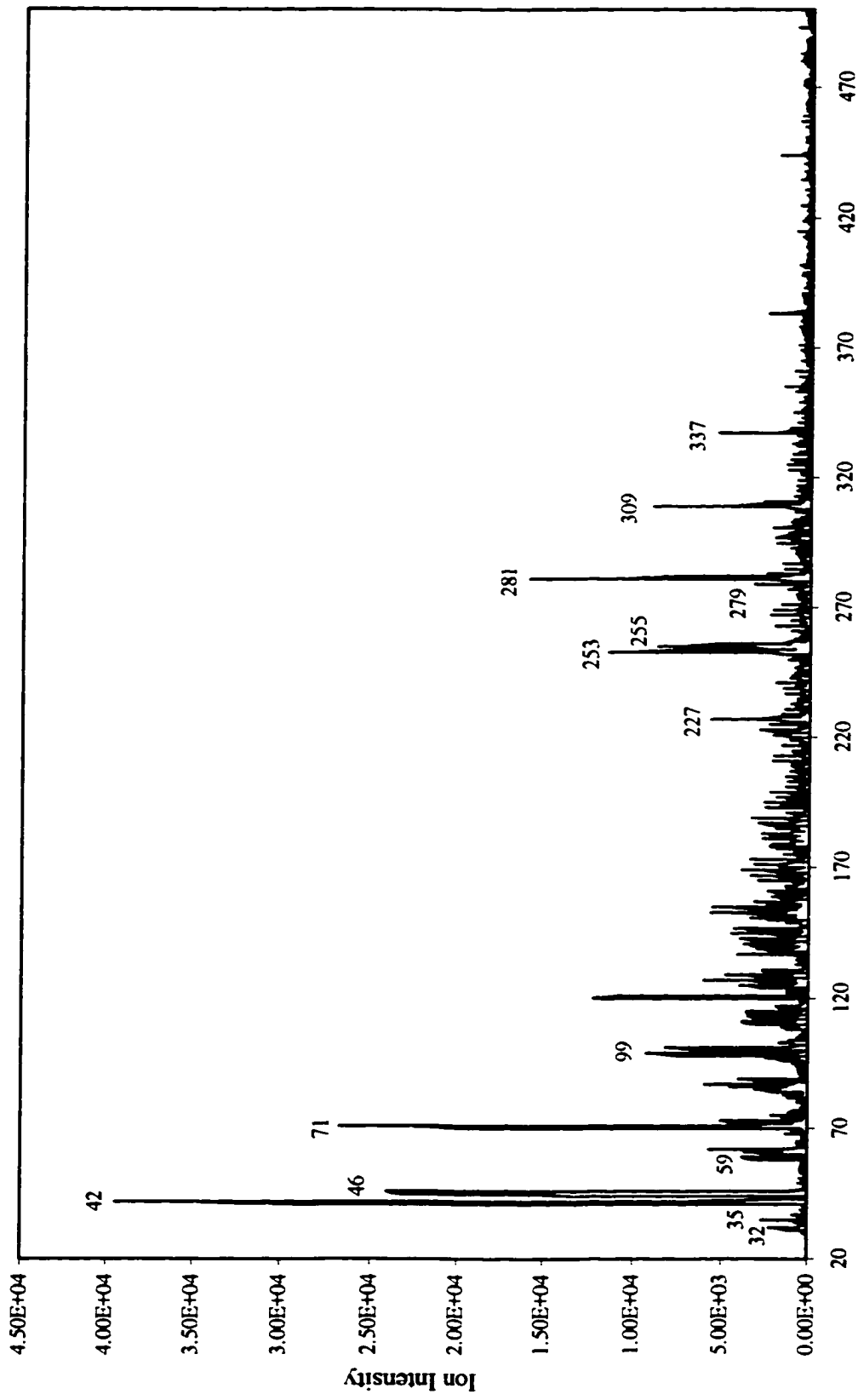


Fig. 5.19. SEC135-8, 15V, APCI-

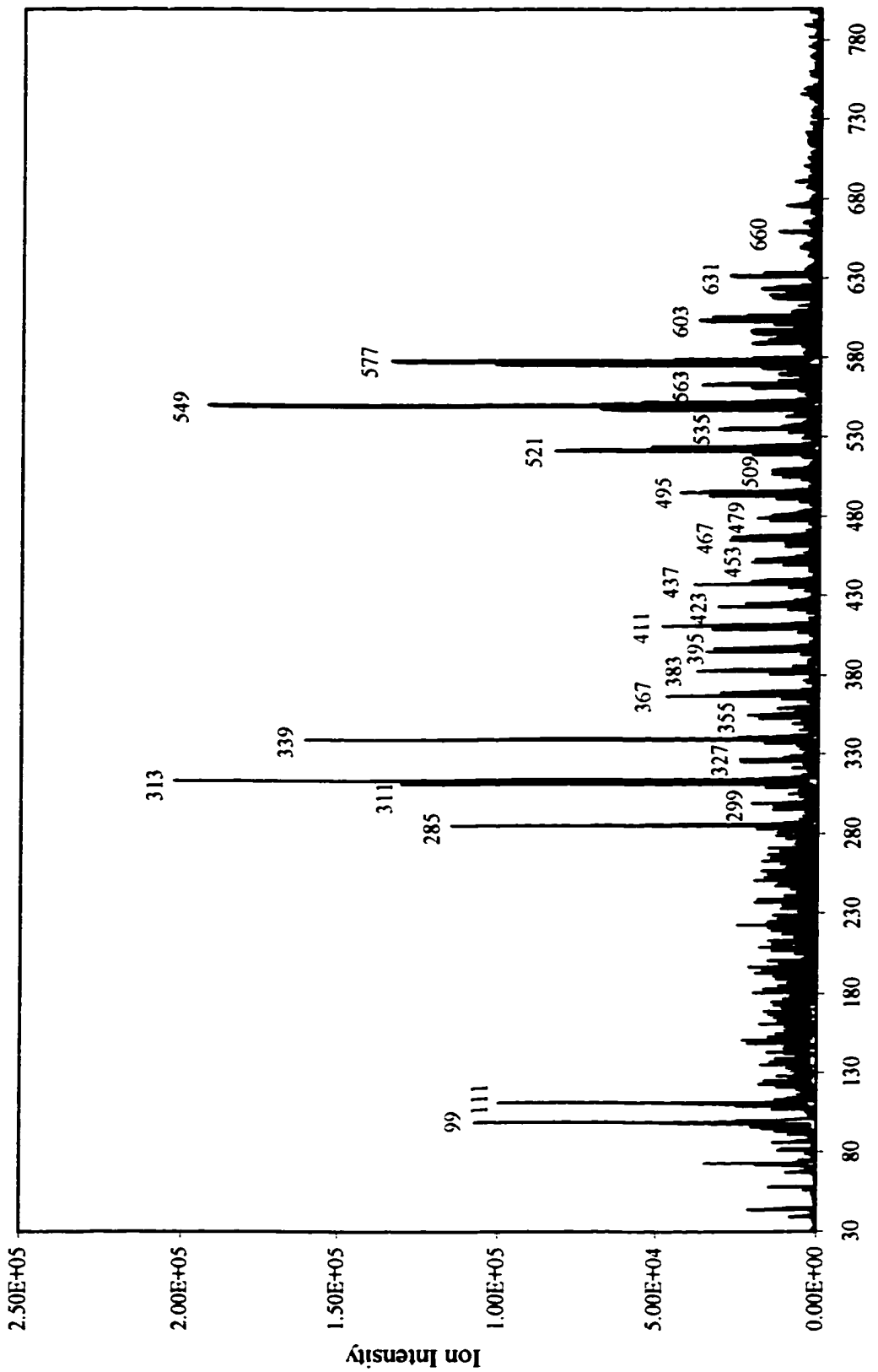


Fig. 5.20. SEC140-2, 15V, APCI+

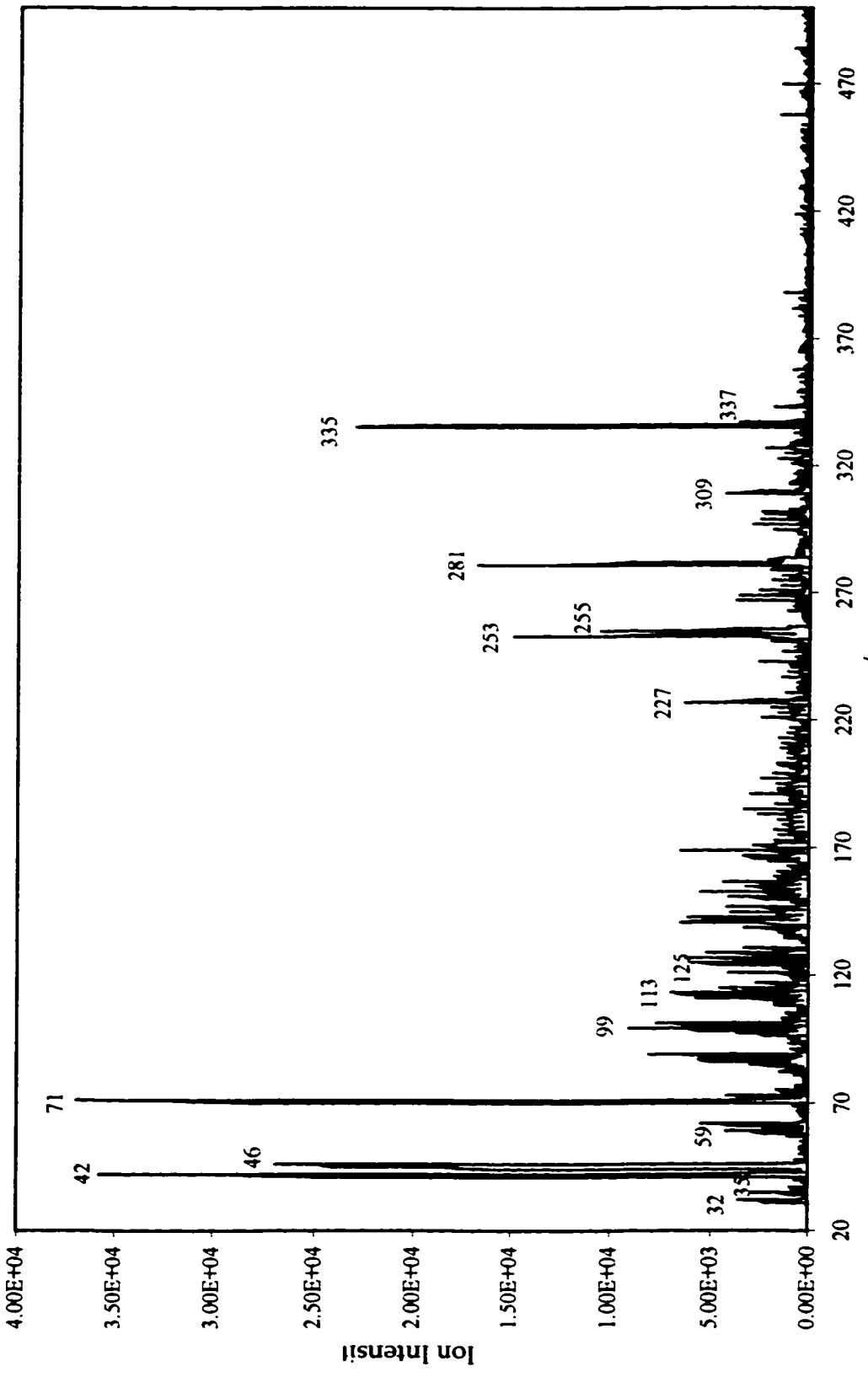


Fig. 5.21. SEC140-2, 15V, APCI-

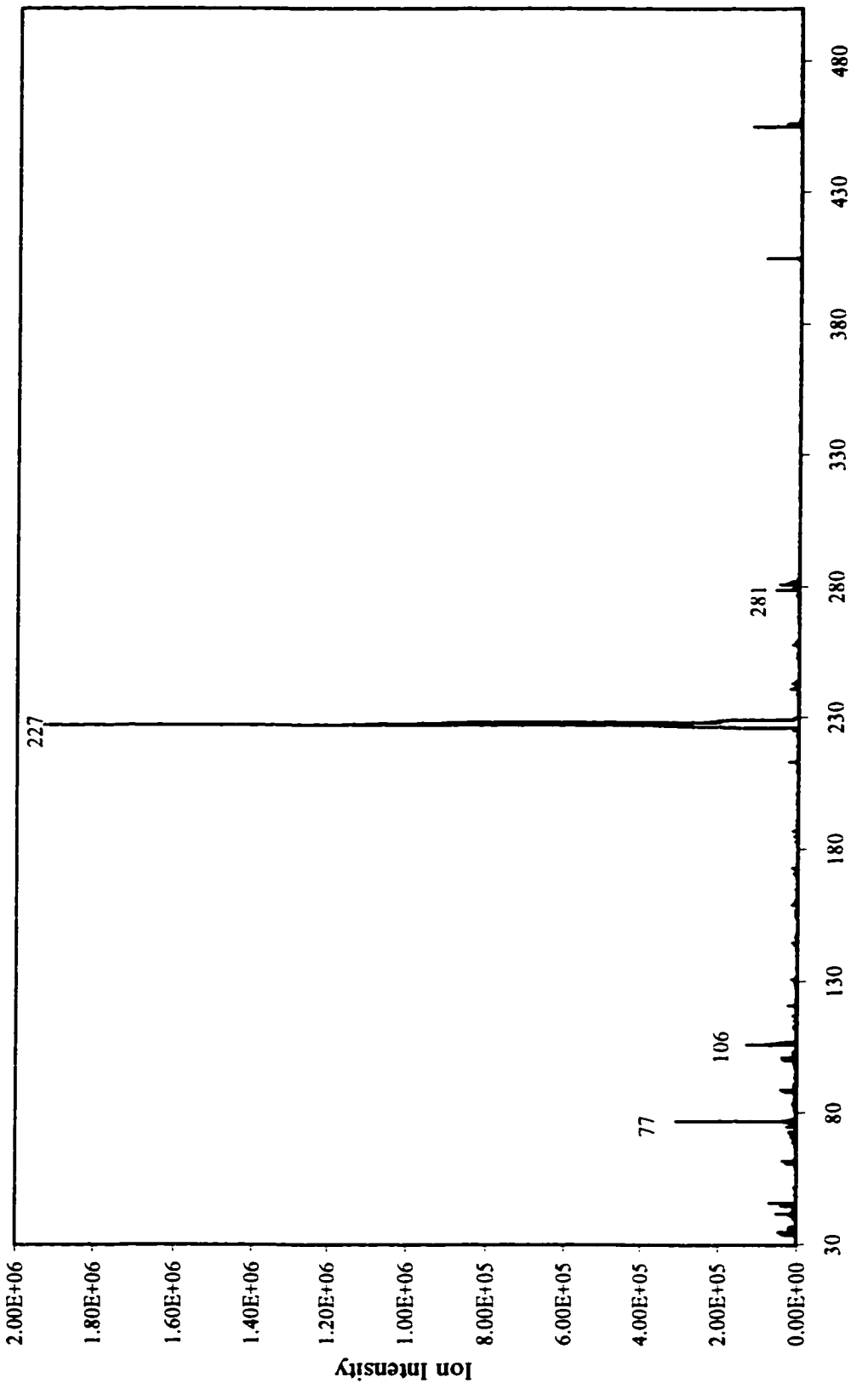


Fig. 5.22 APCI-, 15V, myristic acid

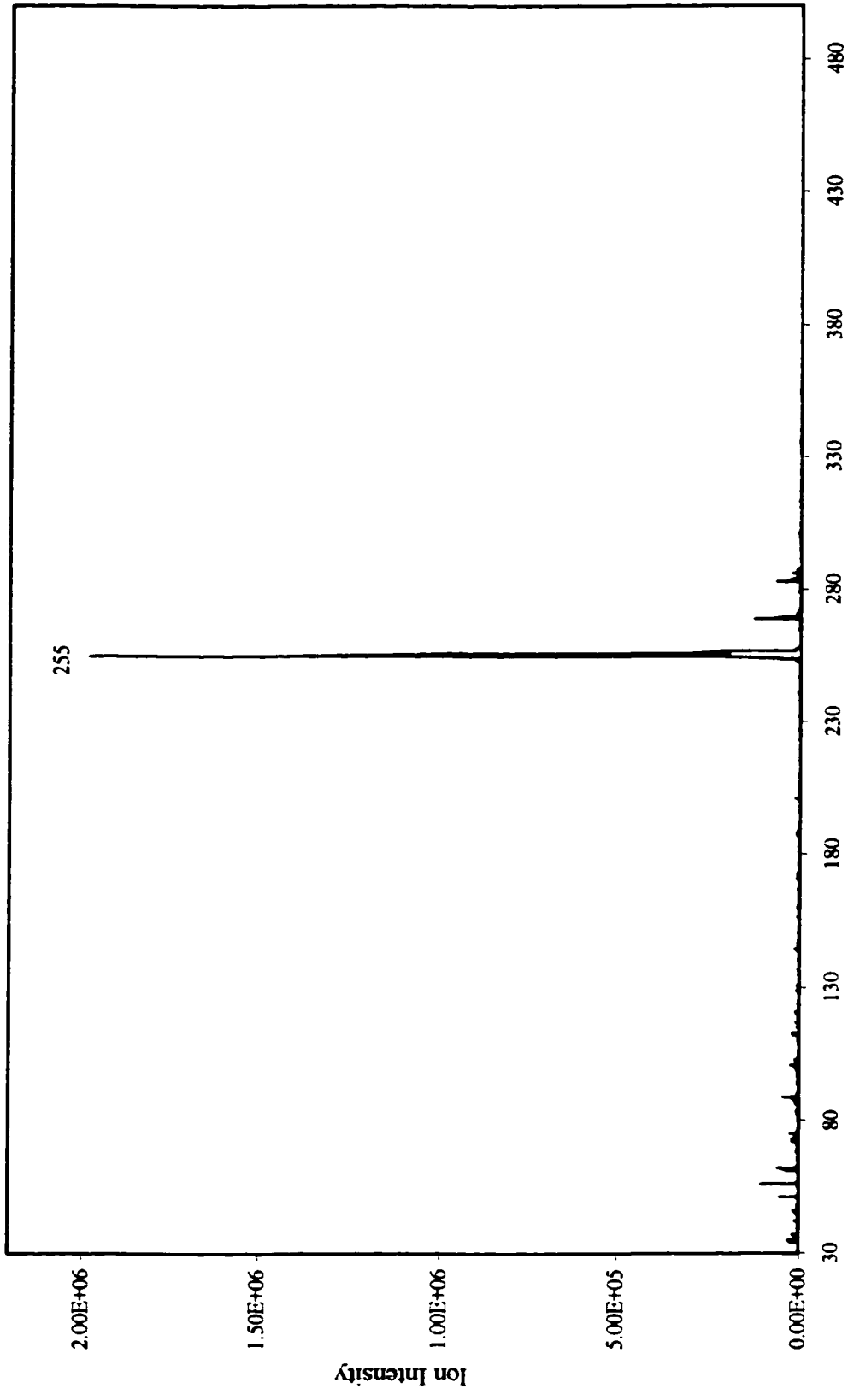


Fig. 5.23. APCI-, 15V, palmitic acid

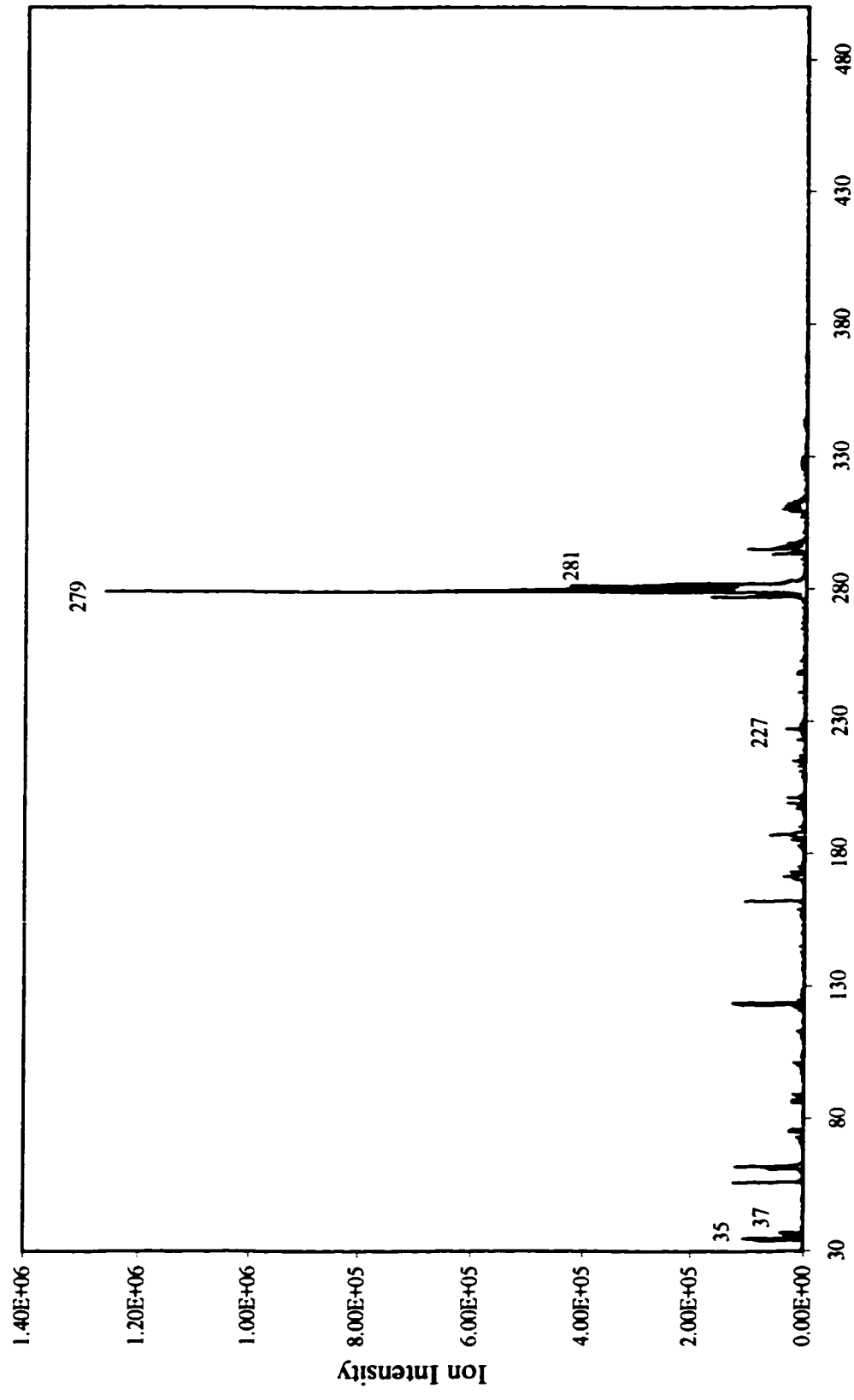


Fig. 5.24. APCI-, 15V, linoleic acid

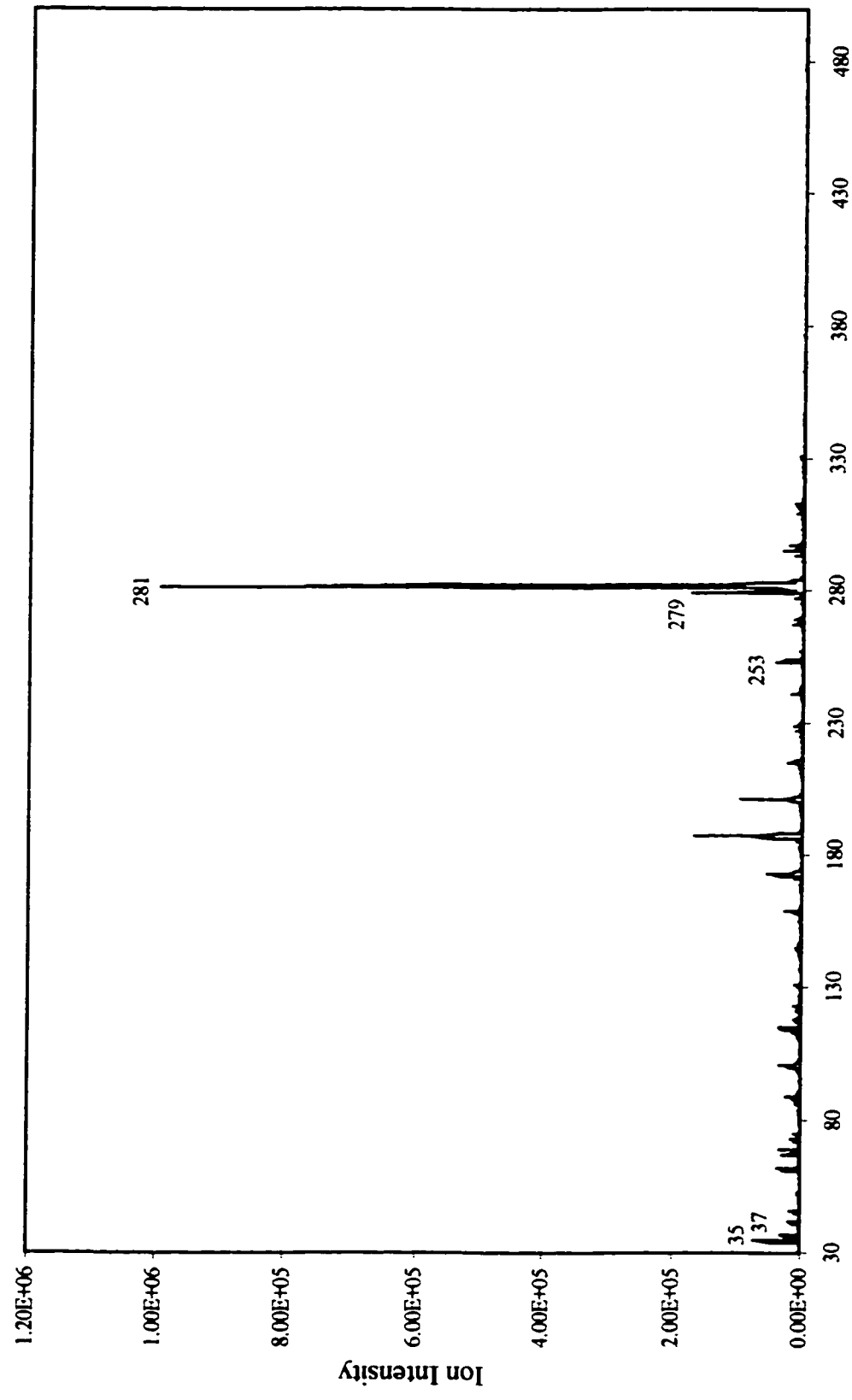


Fig. 5.25. APCI-, 15V, oleic acid

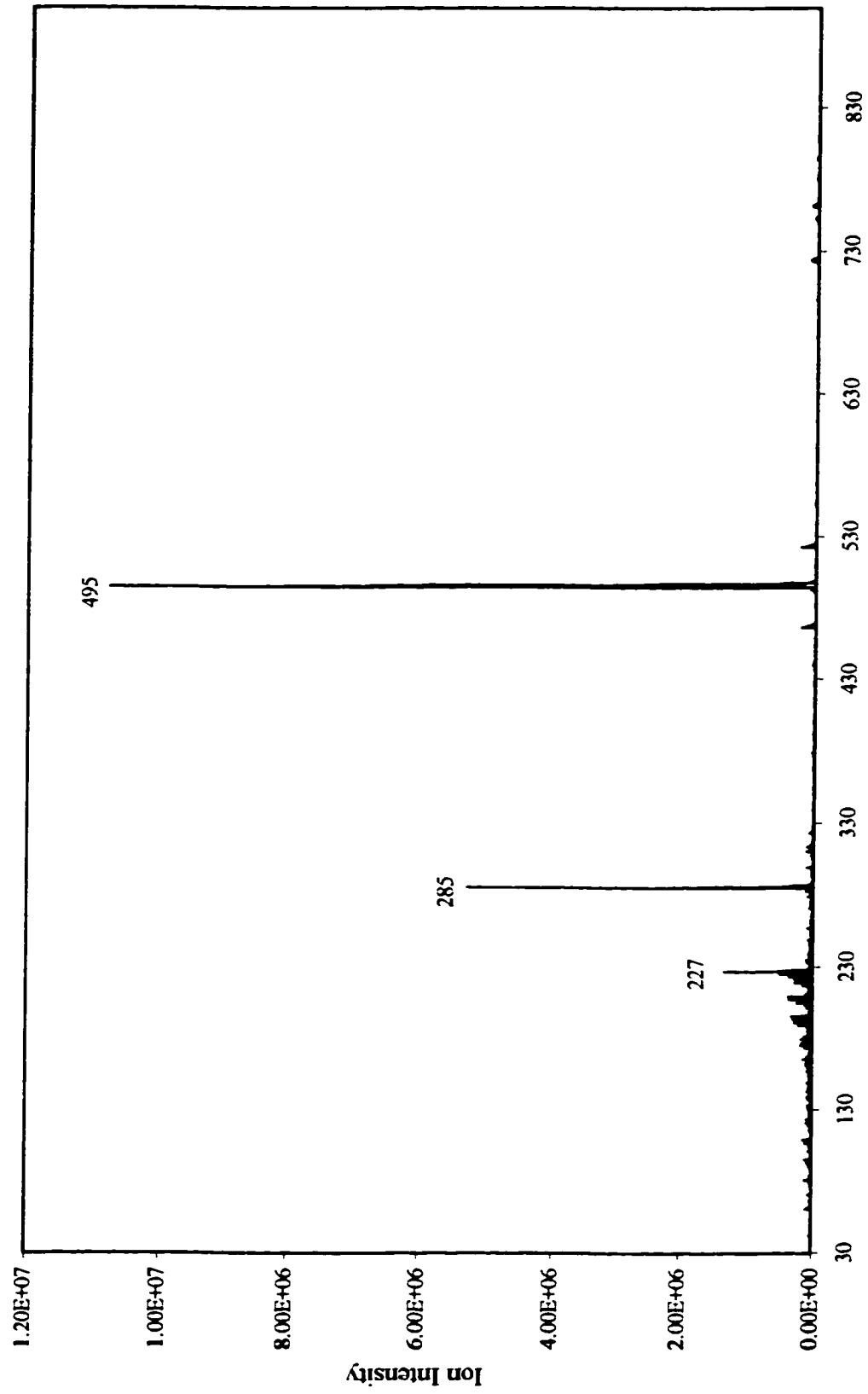


Fig. 5.26. APCI+, 15V, trimyristin

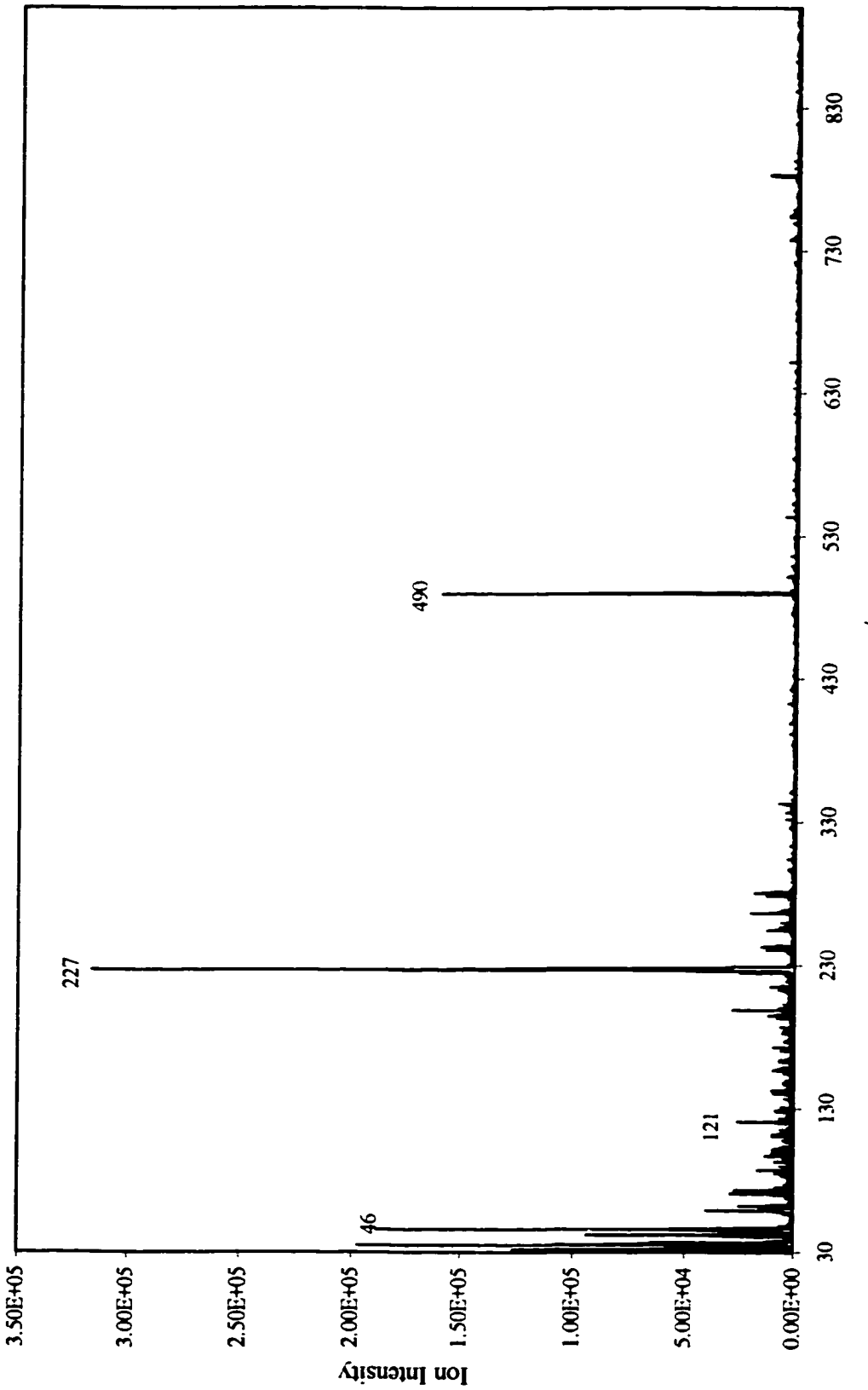


Fig 5.27. APCI-, 15V, trimyristin

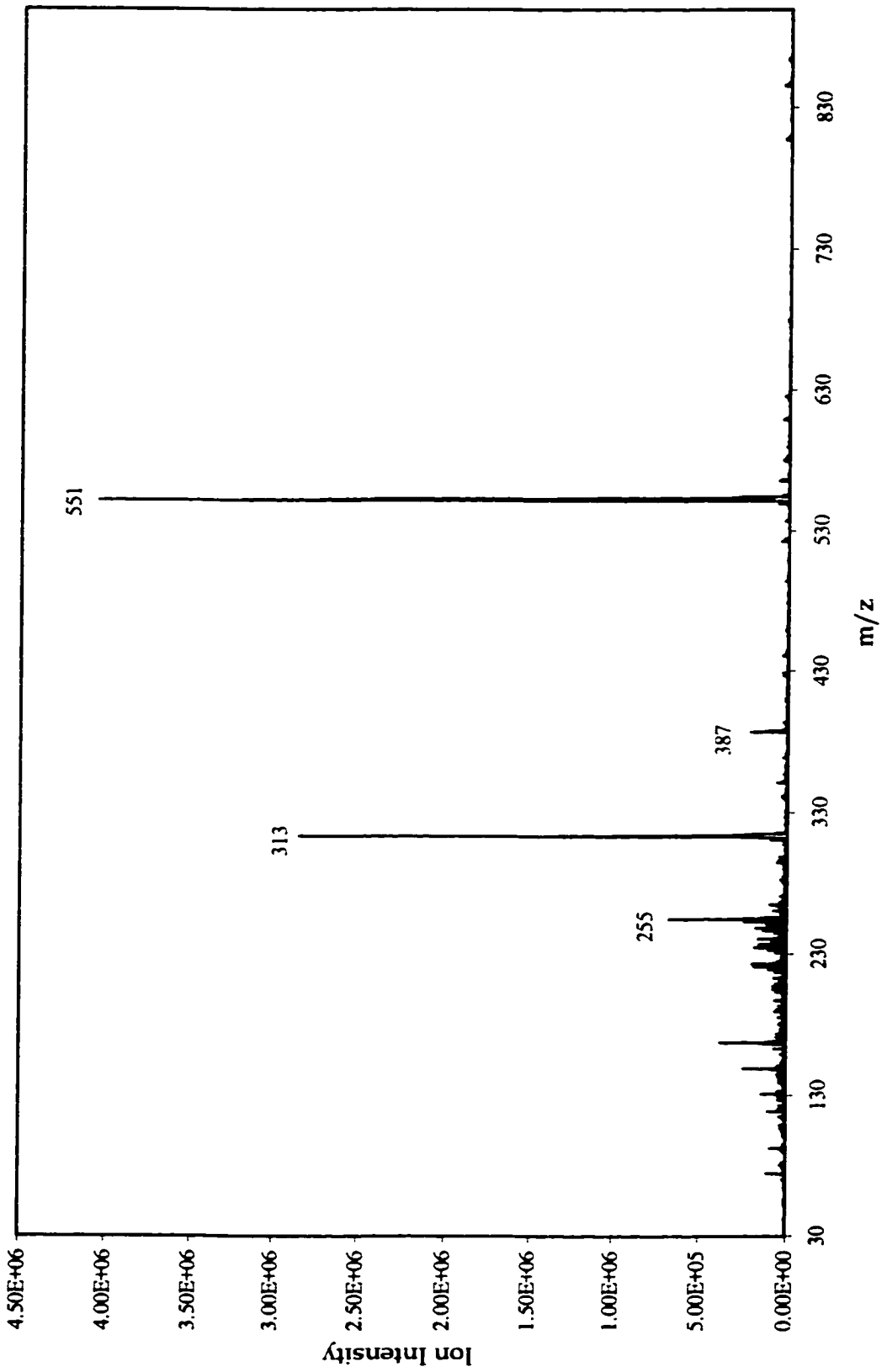


Fig. 5.28. APCI+, 15V, tripalmitin

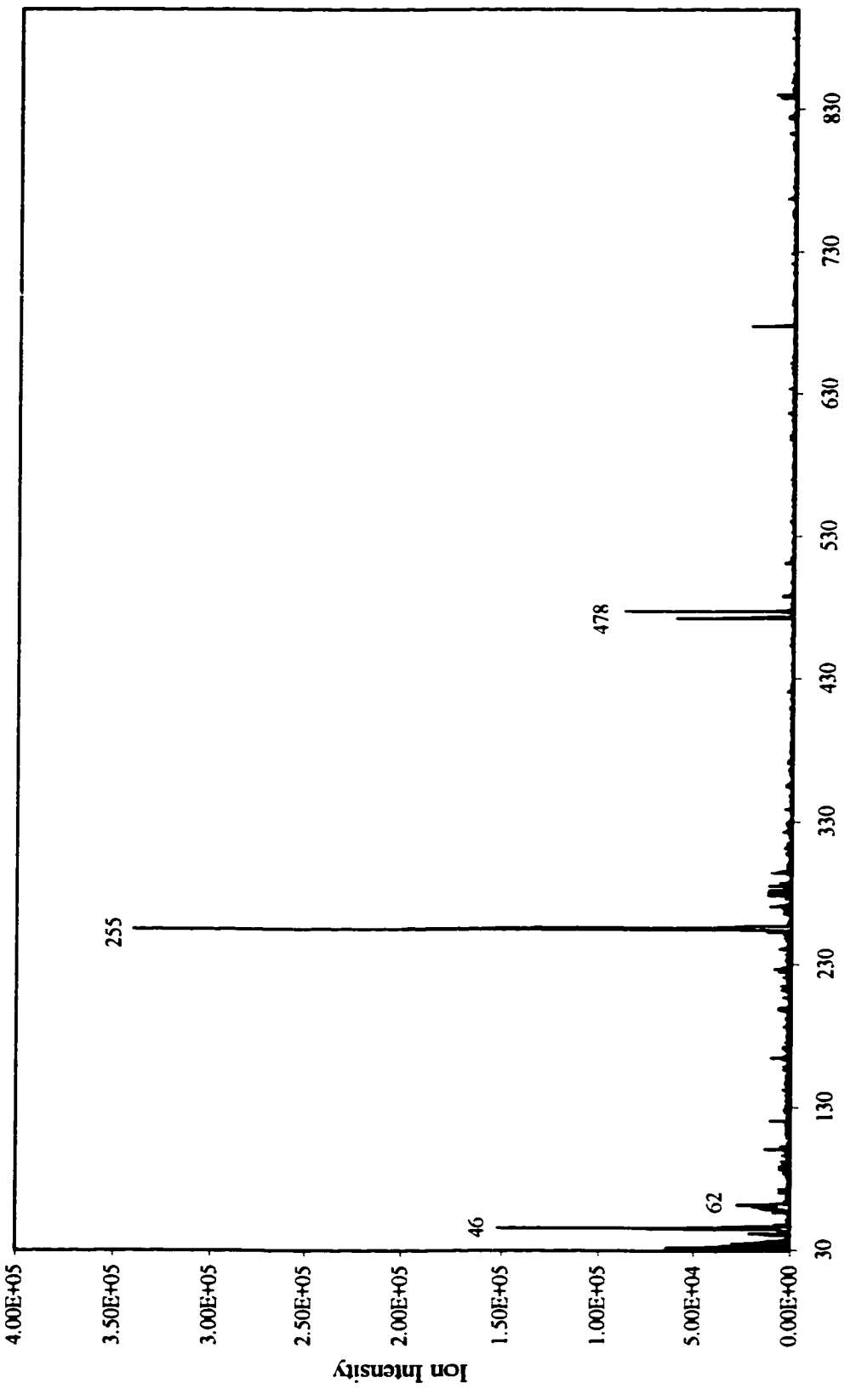


Fig. 5.29. APCI-, 15V, tripalmitin

The ion fragments proposed in Table 5.5 with two fatty acid substituents (*i.e.* m/z 523, 549 or 577) are only one possible combination of fatty acids that might comprise that ion. For example, if the ion has a mass suggesting that there are C-16 and C-18 fatty acids substituents, an ion with C-14 and C-20 fatty acid substituents would also give the same mass.

Table 5.5. Common Ions Observed in Positive Ion APCI Mass Spectrometry for Fractions of SEC135 and SEC140

m/z	Assignment
99	$[C_5H_7O_2]^+$
111	$[C_7H_{10}OH]^+$
285	$[CH_3(CH_2)_{12}CO_2\text{-}CH_2(CH)_2OH_2]^+$
311	$[CH_3(CH_2)_5CH{=}CH(CH_2)_7CO_2\text{-}CH_2(CH)_2OH_2]^+$
313	$[CH_3(CH_2)_{12}CO_2\text{-}CH_2(CH)_2OH_2]^+$
339	$[CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2\text{-}CH_2(CH)_2OH_2]^+$
367	$[CH_3(CH_2)_9CH{=}CH(CH_2)_7CO_2\text{-}CH_2(CH)_2OH_2]^+$
395	$[CH_3(CH_2)_{11}CH{=}CH(CH_2)_7CO_2\text{-}CH_2(CH)_2OH_2]^+$
411	$[CH_3(CH_2)_{11}CH{=}CH(CH_2)_7CO_2\text{-}CH_2(CH)_2O_2H_2]^+$
523	$[CH_2CHCH_2(\text{-}O_2C(CH_2)_{12}CH_3)(\text{-}O_2C(CH_2)_{14}CH_3]^+$
549	$[CH_2CHCH_2(\text{-}O_2C(CH_2)_7CH{=}CH(CH_2)_5\ CH_3)(\text{-}O_2C(CH_2)_{14}CH_3]^+$
577	$[CH_2CHCH_2(\text{-}O_2C(CH_2)_7CH{=}CH(CH_2)_5\ CH_3)(\text{-}O_2C(CH_2)_{16}CH_3]^+$

5.4 Conclusions

It can be concluded from the mass spectra of the SEC and Florisil fractions and from the TLC that the majority of the chlorine is in a mixture of acylglycerols and fatty acids. This fact combined with the results of the ^1H NMR suggest that much of the chlorinated components found in these fractions are fatty acids. The chlorinated fatty acids could be distributed throughout the various mono-, di-, and triacylglycerols in an extensive number of combinations.

6. CONCLUSIONS AND RECOMMENDATIONS

The study of EOX has many aspects and each of them lead to a better understanding of EOX, natural and man-made, in our environment. There is much further work that can be done to obtain more information about EOX in shrimp, in terms of their distribution and behavior in the environment as well as their structure.

6.1 Solvent Extraction and Extraction Methods

In Chapter 3, it was found through a QA program that the method developed was under good control and was reliable. Additionally, the washing procedure applied was effective for the removal of inorganic salts. Sodium sulfate was used effectively to remove water from the extract, and a rotary evaporator was most effective at reducing solvent volume. The best solvent system that was employed in this thesis was hexane-acetone, it had good extraction efficiency for lipid and EOX, as well as satisfactory precision and a low blank.

It is clear from the experiments in Chapter 3 that the various solvent systems gave different extraction efficiencies for EOX from different matrices. However, there is some question as to why there is a variation in the extraction efficiency, especially for EOCl. The answer may be related to the types of compounds extracted, and since most of the chlorine is found in the lipid fractions the compounds would likely be lipids. This could be explored further by identifying the lipids extracted using various solvent systems to those extracted using the standard $CHCl_3$ and methanol mixture. Hopkins *et al.* [246]

have described many of the methods for the separation and identification of lipid (as applied to *P borealis*) that might be employed to this end.

In Chapter 3 it was also found that the Polytron was superior to a shaking board for extraction of EOCl and EOI. As part of the experiments to determine the composition of lipids, alternative extraction methods, such as microwave-assisted extraction, might also be examined.

6.2 Spatial and Temporal Trends

In Chapter 4, it was found that a spatial trend existed for EOX in shrimp from the coast of Labrador. The general trend found that EOX in shrimp roe increased from North to South. It was also found that there was a high correlation between EOCl, EOBr and EOI in roe.

The spatial trends present for EOX in shrimp may be studied further by taking samples from a larger number of locations along the Labrador Coast and analyzing them further for trends in EOX. It would be advisable that all of these samples be taken at around the same time to eliminate the effects of seasonal fluctuations or temporal changes in distribution. It would also be useful to survey other areas in the range of the species, which includes but is not limited to Alaska and Greenland.

It would also be beneficial to analyze EOX from different locations for molecular size distribution to determine if the trends (if any are found) are related to any particular compound, at least in terms of size. If one is found, then the fraction containing this compound(s) could be characterized using NMR and MS.

Temporal trends may be examined as well, through monitoring of EOX in shrimp from one location over several years. Again it would be valuable to characterize the EOX to determine what types of compounds are changing or are static.

6.3 Characterization

It was found from the SEC (Chapter 5), that EOX is distributed over a wide range of molecular weights. This distribution was strongly correlated to lipid, where the highest amounts of EOX are found in the fractions with relatively large amounts of lipid. There was a strong relationship between the distribution of EOCl, EOBr and EOI in the fractions, and this relationship was most convincing in the samples from SFA6. A correlation for EOBr and EOI between shrimp roe from the two locations was also demonstrated. This may be due to the types of compounds that comprise EOX and MS and NMR could be used for verification.

The results in Chapter 5 exhibited clear differences in the size distribution of EOCl between shrimp muscle and roe, while EOI and EOBr did not show such marked variation. It would be of interest to investigate these differences for shrimp from a number of areas. The EOX should also be characterized to determine which compounds partition differently in the shrimp muscle and roe.

The results of the attempts at characterization in Chapter 5 indicate that some of the EOCl in shrimp are related to a chlorinated fatty acid. Part of the structure has been proposed to consist of a terminal chlorine with an n-2 double bond. Since no report of this particular fatty acid was found in the literature, chlorinated or otherwise, it is

especially important to do additional work to characterize the proposed chlorinated fatty acids, which would include 2D NMR experiments.

The first step in this process would be to form methyl esters of all the fatty acids present, including free fatty acids and those which may be part of phospholipids or acylglycerols. This could be accomplished with BF_3 and methanol or methanol-sulfuric acid. The methyl esters could be analyzed directly by GC-MS or alternatively they could be separated on a TLC plate and the individual spots on the plate submitted to NMR and MS. These would be the most practical ways to determine structure, since, given the small amounts of chlorinated compound found in the extracts, it would be difficult to separate enough of the pure chlorinated fatty acid from the unchlorinated compounds to use other methods, such as crystallography, for structure elucidation.

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