MEASUREMENT OF ARSENOBETAINE AND ARSENOCHOLINE IN FISH TISSUE BY FAST ATOM BOMBARDMENT MASS SPECTROMETRY

Michael L. Zimmerman
B.S., Ashland College, 1980

A thesis submitted to the faculty
of the Oregon Graduate Center
in partial fulfillment of the
requirements for the degree
Master of Science
in
Environmental Science

August, 1988

The thesis "Measurement of Arsenobetaine and Arsenocholine in Fish Tissue by Fast Atom Bombardment Mass Spectrometry" by Michael L. Zimmerman has been examined and approved by the following Examination Committee:

Douglas Barofsky
Professor
Thesis Research Advisor

James Penkow
Professor

Bill Pengelly
Associate Professor

Rick Johnson
Assistant Professor

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Measurement of Arsenobetaine and Arsenocholine in Fish Tissue by Fast Atom Bombardment Mass Spectrometry

Michael L. Zimmerman, M.S.
Oregon Graduate Center, 1988

Supervising Professor: Douglas Barofsky

A technique to measure arsenobetaine and arsenocholine in fish tissue by fast atom bombardment mass spectrometry was developed with particular attention to quantitative analysis. Experiments were performed which demonstrate analysis of the compounds desorbed directly from thin layer silica chromatography matrices, quantitative analysis of arsenobetaine in real fish samples, and accurate mass measurement of arsenobetaine in normal FAB/MS using peaks from the glycerol matrix as mass references. Improvements to the technique to quantitatively measure these important arsenic metabolites are suggested including optimization of the extraction/isolation procedures and use of isotopically labelled internal standards or surrogates for more accurate measurements.

INTRODUCTION

This thesis describes the development of a technique to measure arsenobetaine and arsenocholine in fish tissue by fast atom bombardment (FAB) mass spectrometry (MS). These arsenic metabolites are produced by algae and/or fish and, because of their zwitterion and water soluble character, are especially amenable to FAB/MS. The results of these experiments may be best summarized as follows:

a. Direct analysis of material desorbed from thin layer chromatography silica matrices was demonstrated;
b. Quantitative analysis of arsenobetaine in real samples was demonstrated; and
c. Accurate mass measurements of arsenobetaine using the normal FAB/MS glycerol matrix as mass reference was achieved with errors in the range of 4-10 ppm.

Arsenic is a ubiquitous metal occurring naturally in fossil fuels, mineral deposits, and some drinking water supplies. The production, use, and environmental impact of arsenic in its various forms has been the focus of a great deal of concern in recent years, owing to the relative toxicity of the different inorganic and organic arsenic compounds. The toxicity of arsenobetaine and arsenocholine is unknown, but the biochemical mechanisms that produce organoarsenicals are thought to remove or bind and detoxify the inorganic forms of arsenic.

Water soluble, biochemically active and toxic inorganic forms of arsenic are produced primarily by the refining of lead, nickel, and especially copper flue dust. It is interesting to note that no facility in the world is dedicated to arsenic production. Rather, arsenic trioxide, As_2O_3, is a byproduct of copper ore refining, and efficient production of arsenic trioxide therefore depends on efficient removal of the flue dust from the copper smelter stack. In this case, air pollution control is to the benefit of the refinery.

Approximately 70% of the 29,000 tons of arsenic trioxide produced in 1980 was used in agriculture to control weeds and as a wood preservative to inhibit fungal and bacterial growth. In both uses, arsenic is in the form of cacodylic acid. Other uses include specialty glass, electronics, and pharmaceuticals. Because of its conductive properties, arsenic and arsenic-gallium alloys are becoming more important in semiconductor industries.

The three biggest contributors of inorganic arsenic to the environment are agricultural application/runoff, inefficient stack scrubbing in ore refining operations, and fossil fuel burning. Although local environments may be severely perturbed by such inputs, further discussion of environmental and biochemical concerns should not be limited to 'polluted' environments. Arsenic exists naturally in seawater (3 ppb), freshwater (60 ppb), in algae and fish (40-6,000 ppb), and in man, who in some cases consumes between 29 and 169 μg per day (1). The metabolic production of organoarsenic compounds is thought to occur naturally and predominantly in algae of warm phosphate deficient seawater.

Excellent reviews of arsenic chemistry can be found in books by Brinkman and Bellama (2) and by Lederer and Fensterheim (3).

ARSENIC BIOCHEMISTRY and GEOCHEMISTRY

Understanding bioconcentration and conversion of arsenic in the food chain is essential to toxicology and risk assessment where arsenic pollution is considered. The inorganic speciation is important to vertebrate animals because the valence state determines toxicity, i.e., in vertebrate animals trivalent arsenite is much more toxic than pentavalent arsenate. These two oxidation states, +3 and +5, are the dominant forms of arsenic in aquatic systems. Wagemann (4) developed the Eh-pH diagram in Figure 1 for freshwater considering barium as the most likely element capable of complexing arsenic and holding the total dissolved levels relatively low. Under these conditions arsenic exists as solid pentavalent barium complex. Andreae (5) has shown the dominant seawater species to be pentavalent arsenate (H_3AsO_4), with some dependence on temperature and depth in the water column.

In the Tacoma Washington-Puget Sound area a copper smelter discharges large amounts of As_2O_5. Crecelius et. al. (6) have shown absorption or coprecipitation of arsenic in sediments to be the controlling mechanism when

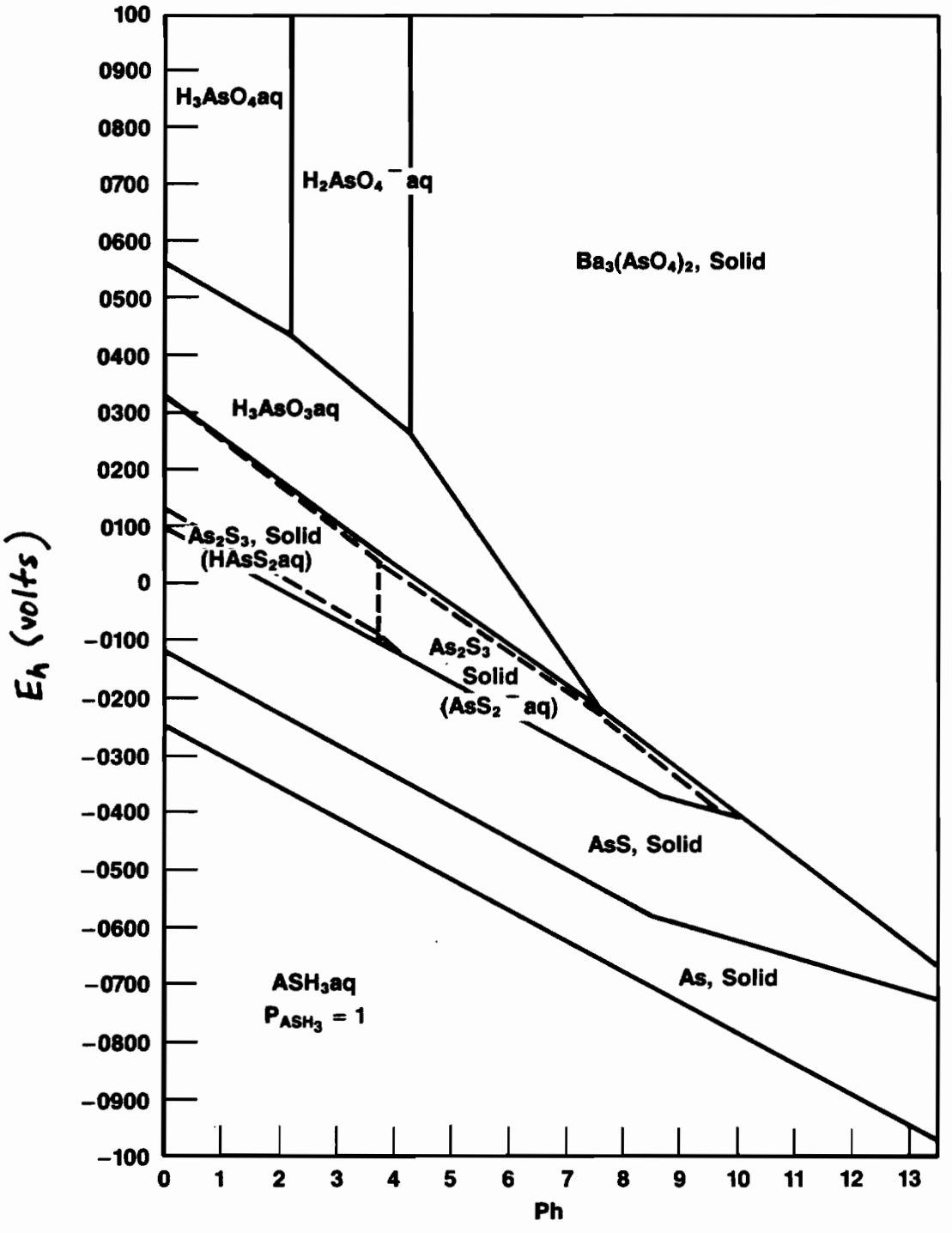


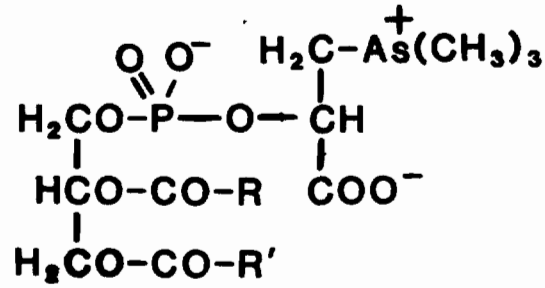
Figure 1. Eh-pH Diagram for Arsenic at 25°C and 1 atm. Pressure, Showing the Fields of Stability for the Most Important Arsenic Species in the Presence of 10^{-5} M of Total Arsenic, 10^{-3} M of Total Sulfur and 2.2×10^{-7} M of Total Barium. Dashed Lines Define Domains for Species Enclosed in Parentheses. From Wagemann (1978).

water concentrations drop from 1,200 ppb near the smelter to 4 ppb within one mile. Sediments near the smelter contain up 10,000 ppm As^{5+}.

The behavior of arsenic in plants and animals is often described analogous to its periodic table family member phosphorus. Both elements are most available in the pentavalent state, and it is no surprise that algae appear to pick up arsenate by using the phosphate transport system in the cell membranes (2). Also, the eventual substitution of arsenic for nitrogen in betaine and choline may be another example of family mimicry in the periodic table.

Bioconversion of arsenic from the inorganic state to various organic forms involves two general processes, namely, reduction from the pentavalent to trivalent state, and methylation. These general mechanisms are widely recognized as detoxifying processes used by living cells as a way of neutralizing the otherwise toxic effects of arsenic and to facilitate its excretion into the environment. Algae have been used as model organisms in arsenic studies because 1) they are easy to cultivate, 2) they represent the base of the food chain, and 3) they exhibit bioconcentration of arsenic, i.e., they maintain concentrations above the levels of the surrounding environment. At relatively low concentrations of arsenate the algae cell can reduce and methylate arsenic to replace nitrogen and either eliminate it as arsenobetaine or store it as a phospholipid. If the arsenate concentration becomes too high, the cell loses its ability to reduce, and the arsenate replaces phosphate in sugar metabolism, eventually stopping glycolysis and killing the cell. Figure 2 illustrates these mechanisms (3).

Arsenic metabolism is not, however, well defined. Virtually nothing is known of the reducing agent, and the methylating mechanism is unclear. In support of the reduction-methylation pathway Andreae and Klumpp (7) found algae able to produce at least 12 reduced inorganic and organoarsenic compounds, including methyl arsenate, dimethyl arsenate, and arsenite. Lunde (8,21) was the first to recognize high levels of arsenic containing lipids in algae and fish. At least one lipid was identified by Irgolic (9) and Cooney (10) as o-phosphatidyl trimethylarsonium lactate:



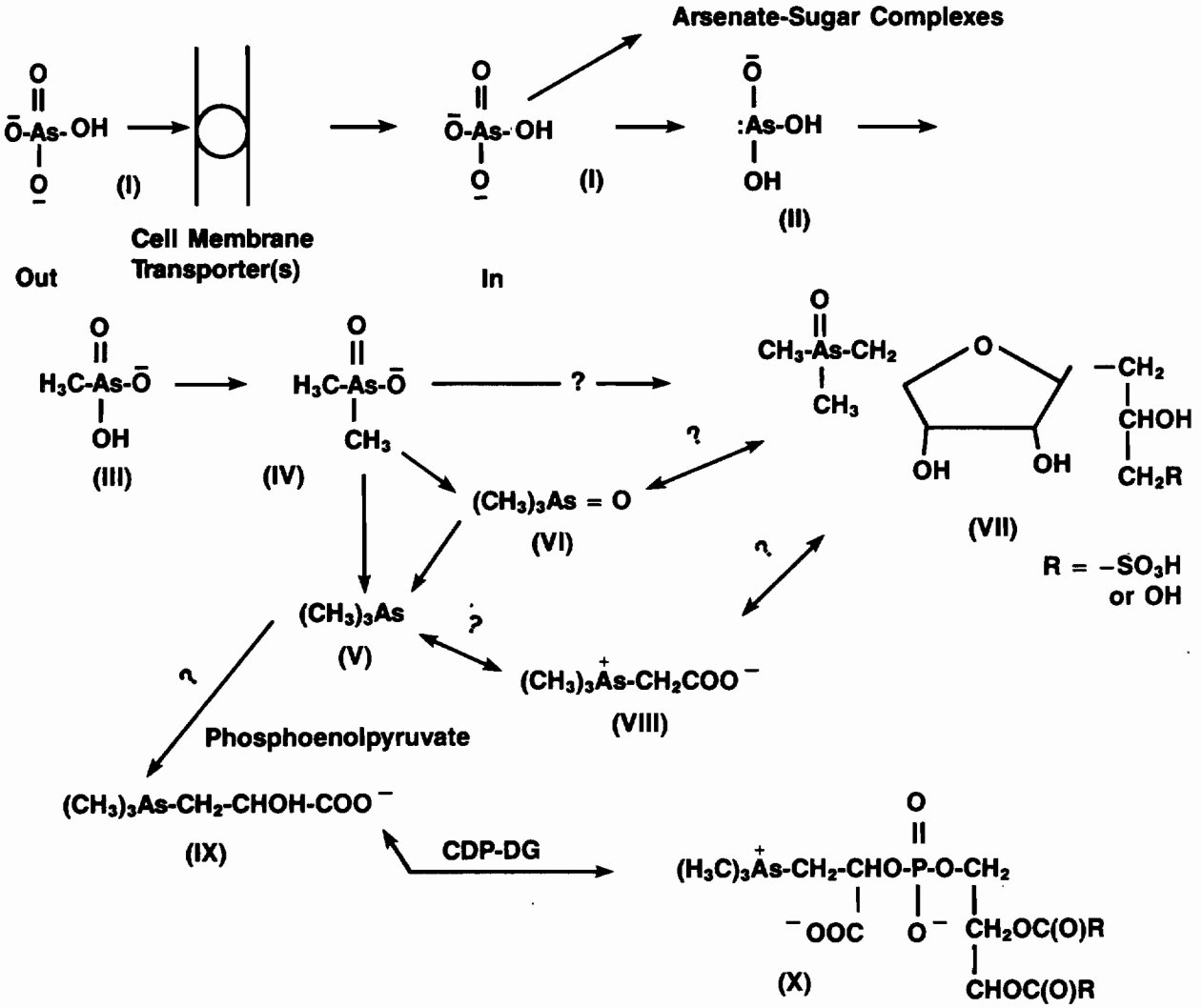
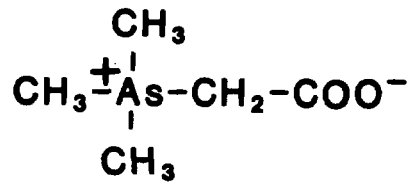


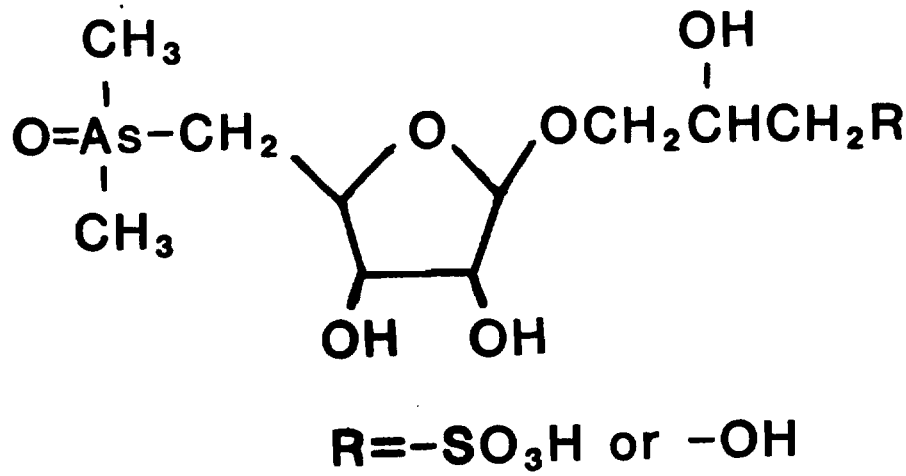
Figure 2. Biochemical Reactions of Arsenic in Algal Cells. (I) = Arsenate; (II) = Arsenite; (III) = Methyl Arsonate; (IV) = Dimethyl Arsinate; (V) = Trimethylarsine; (VI) = Trimethylarsine Oxide; (VII) = Sulfopropyl (Dimethylarsenoso) Deoxyribose or Dihydroxpropyl (Dimethylarsenoso)-Deoxyribose; (VIII) = Arsenobetaine; (IX) = Trimethylarsonium Lactate; (X) = O-Phosphatidyl-Trimethylarsonium Lactate; CDP-DG = Cytidine Diphosphate Diglyceride.

This led to the work of Edmonds and Francesconi (11) who first isolated arsenobetaine from the liver of the western rock lobster George:



Arsenobetaine has also been identified in the dusky shark (11) and school whiting (11).

Edmonds and Francesconi also identified an arsenosugar from the brown kelp (12), and suggest it as a precursor to arsenobetaine:



R = OH
2,3-dihydroxypropyl-5-
deoxy-5-(dimethylarsenoso)furanoside

R = SO3H
2-hydroxy-3-sulphopropyl-
5-deoxy-(dimethylarsenoso)furanoside

Evidence to support the more toxic phosphate replacement mechanism in sugar metabolism is much less conclusive (3). It is also unclear whether the organoarsenics found in fish are produced by the fish or are ingested. Reduction-methylation pathways in fish cells have been suggested that might

indicate de novo synthesis by the organism although no information is available concerning the chemical structure of the organoarsenic compound (13,14). Other studies indicate that the organoarsenics found in fish and crustacea are the result of accumulation through the food chain (15,16,17).

ANALYSIS OF ORGANOARSENIC COMPOUNDS

Virtually all the tools of analytical chemistry have been applied to the identification and recent quantification of organoarsenics. The difficulty with the organoarsenics is due at least in part to a lack of synthetic reference standards and the wide variety of chemical structures that have been described. Whereas inorganic speciation is a relatively simple matter of analysis by hydrid-generation atomic absorption spectroscopy, organoarsenics from natural matrices usually require careful isolation, matrix removal or clean-up, and structure specific analysis by crystallography, NMR, chromatography, mass spectrometry, or combinations of these techniques. The problem is further complicated if quantitative information is desired.

Cox (1925), (18), Chapman (1926), (19), Coulson (1935), (20), and Challenger (1945), (23), were the first to analyze and recognize organoarsenics in marine animals. Cox first observed elevated levels of arsenic in the urine of patients who consumed fish; Chapman showed that crustacea contained high levels of arsenic; Coulson identified 'shrimp arsenic'; and Challenger was concerned with poisoning and volatile arsines.

Lundes' work (1973), (21), provided the first data that quantitatively distinguished organoarsenic and inorganic arsenic in fish tissue. He used distillation to remove the inorganic fraction as volatile $AsCl_3$ and analyzed respective fractions by neutron activation and x-ray fluorescence. Over 90% of the total arsenic was found to be organic, but no specific compounds were identified.

The work of Andreae (1973), (22), although limited to seawater, is the first data identifying and quantifying methylated forms of arsenic in seawater. The method involves reduction to the arsine and volatilization followed by gas chromatography and detection with conventional FID or ECD detectors.

The first isolation and identification of arsenobetaine was performed by Edmonds and Francesconi (11) using X-ray crystallography techniques. Ion exchange and thin layer chromatography was also important in isolating the

compound. These workers were later responsible for isolating and identifying dimethylarsenoso furanosides from brown kelp employing NMR for structural elucidation. No quantitative analysis was attempted for either compound.

Chemical reactivities with enzymes, thin layer chromatography, and physical examination of o-phosphatidyl-trimethylarsonium lactate were keys to deducing the structure of the lipid isolated by Cooney, Mumma, and Benson (10).

The first use of mass spectrometry to confirm the structure of any organoarsenic was by Cheng and Focht (24), who published the EI (electron impact) spectra of trimethyl arsine. Norin and Christakopoulos (25) presented the first mass spectra of arsenobetaine derived from fish and later suggest the presence of arsenocholine in shrimp (26). Their work includes EI, FD (field desorption), and FAB (fast atom bombardment) mass spectrometry together with thin layer chromatography and novel pyrolysis gas chromatography.

High resolution FAB for organoarsenic analysis was first described by Luten and Riekwel-Booy (27), who also identified arsenobetaine in several fish species.

It should be noted that mass spectrometry has been used only to confirm the structure and presence of arsenobetaine and to suggest the presence of arsenocholine. Lipid and sugar metabolites have not been examined, and no quantitative analysis by mass spectrometry has been attempted. Quantitative analysis for arsenobetaine has only recently been attempted (28). The method of choice for quantification in this case, however, was HPLC with atomic absorption detection.

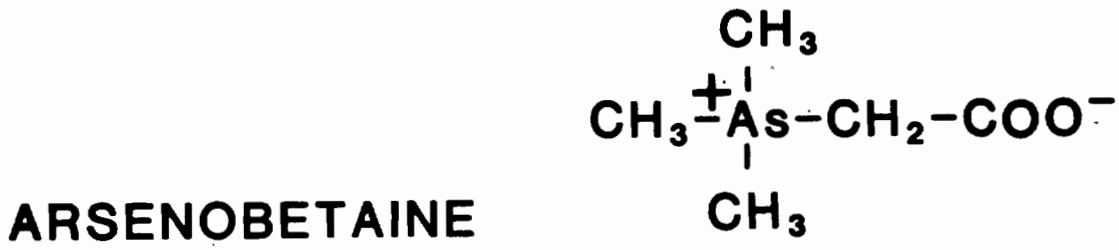
Since its introduction in 1980 (29), fast atom bombardment mass spectrometry has become a well used and valuable technique in studies of relatively large, labile compounds. The pseudo-molecular ion (M+H)+ and scarcity of fragmentation obtained using this relatively simple technique has encouraged many studies of peptides, organometallics, organic salts, and biochemicals that have not been mass analyzed before. Quantitative analysis, although not exploited to the extent of structural investigations, has also been successful (30,31).

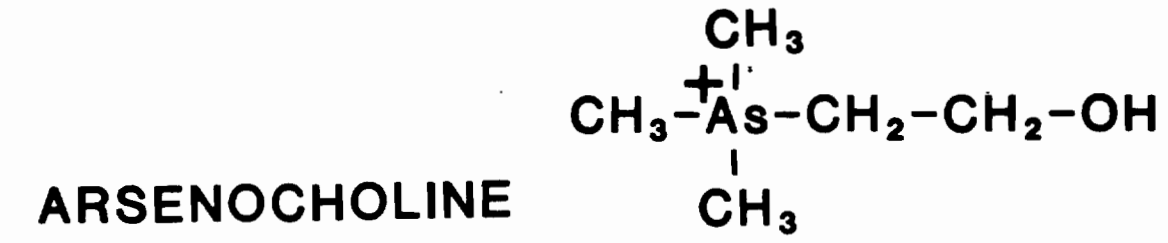
Difficulties with quantitative analysis for arsenobetaine in fish are not unlike the problems inherent in all environmental analyses where sensitivity is important, i.e., the compound of interest must be isolated efficiently from the matrix, and an appropriate internal standard or surrogate must be used to insure some measure of accuracy and precision.

DIRECT MASS SPECTROMETRIC ANALYSIS OF TLC PLATES

A. GENERAL METHODS

The initial FAB experiments with arsenobetaine and arsenocholine were intended to compare FAB and FD spectra previously reported (25,27) and to apply the FAB technique to the analysis of thin layer chromatograms. Real fish extracts chromatographed on electrophoretic plates were provided by Harold Norin of the National Institute of Environmental Medicine, Stockholm, Sweden. FAB/MS analysis directly from paper or two dimensional chromatograms where the compound is suspended on the matrix was suggested by Day, et.al.(32). The structures of the two organoarsenic compounds are shown below:





Synthetic samples of arsenobetaine and arsenocholine were supplied by M. O. Andreae of the Florida State Department of Oceanography.

All mass spectrometric data was obtained using a DuPont CEC-110 mass spectrometer fitted with an Ion Tech 11NF FAB gun. Analyses were performed at 6 and 8 kV accelerating voltage with the FAB gun operated at 6 kV using argon as the FAB collision gas. No accurate mass determinations were attempted. Data was collected with a UV oscillographic chart recorder.

Analyzing directly off the TLC plate was accomplished by cutting a probe size section of the plate (0.5 cm^2) and attaching it on the end of the FAB probe with vacuum stable silver paint (Figure 3). Although this brings the sample slightly closer to the FAB gun and mass spectrometer's optics, this difference in geometry does not significantly alter the instrument's performance. Also, the sample remains conductive, a requirement in FAB since the probe tip acts as the source repeller.

B. RESULTS

Regular FAB spectra of the synthetic compounds were first obtained by U. Geissmann who made several interesting observations (unpublished data). His experiments with arsenobetaine, using glycerol as the matrix, yielded a base peak at m/z=135 due to a loss of CO_2, and the characteristic (M+H)+ ion at m/z=179. Clusters were observed of the type (nM+H)+ where n=9 at m/z=1603 (1KV accelerating voltage). He also found that compared to published FD spectra, FAB results in a reduced decarboxylation of the protonated molecular ion. Published spectra show I135/I179 = 3.8. Spectra I obtained display I135/I179 = 1.8 - 2.5. Geissmann's FAB spectra of arsenocholine showed a base peak at m/z = 165, the (M+H)+ ion, and another at 147, the result of a loss of water.

Two interesting things happen when the arsenobetaine is analyzed from a silica gel-aluminum backed TLC plate. First, the ratio of I135/I179 becomes approximately 0.6, suggesting a significant difference in decarboxylation has occurred compared to normal liquid assisted FAB. And secondly, the glycerol background is reduced by a factor of 3 or 4. These differences in the spectra are likely a result of chemical or physical interaction between the silica, glycerol, and sample. An example of spectra of arsenobetaine is shown in Figure 4.

Silica gel is known to bind irreversibly to some polar molecules, especially when the silica is treated or activated with strong acid or base. Such treatment is used routinely in the preparation of waste water, oil, and solid samples where polar or oxidizable interferences are expected. Activated silica gel certainly participates in some ion exchange or redox in aqueous environments, and it is not unreasonable to suggest some ionic or redox effect in FAB-MS.

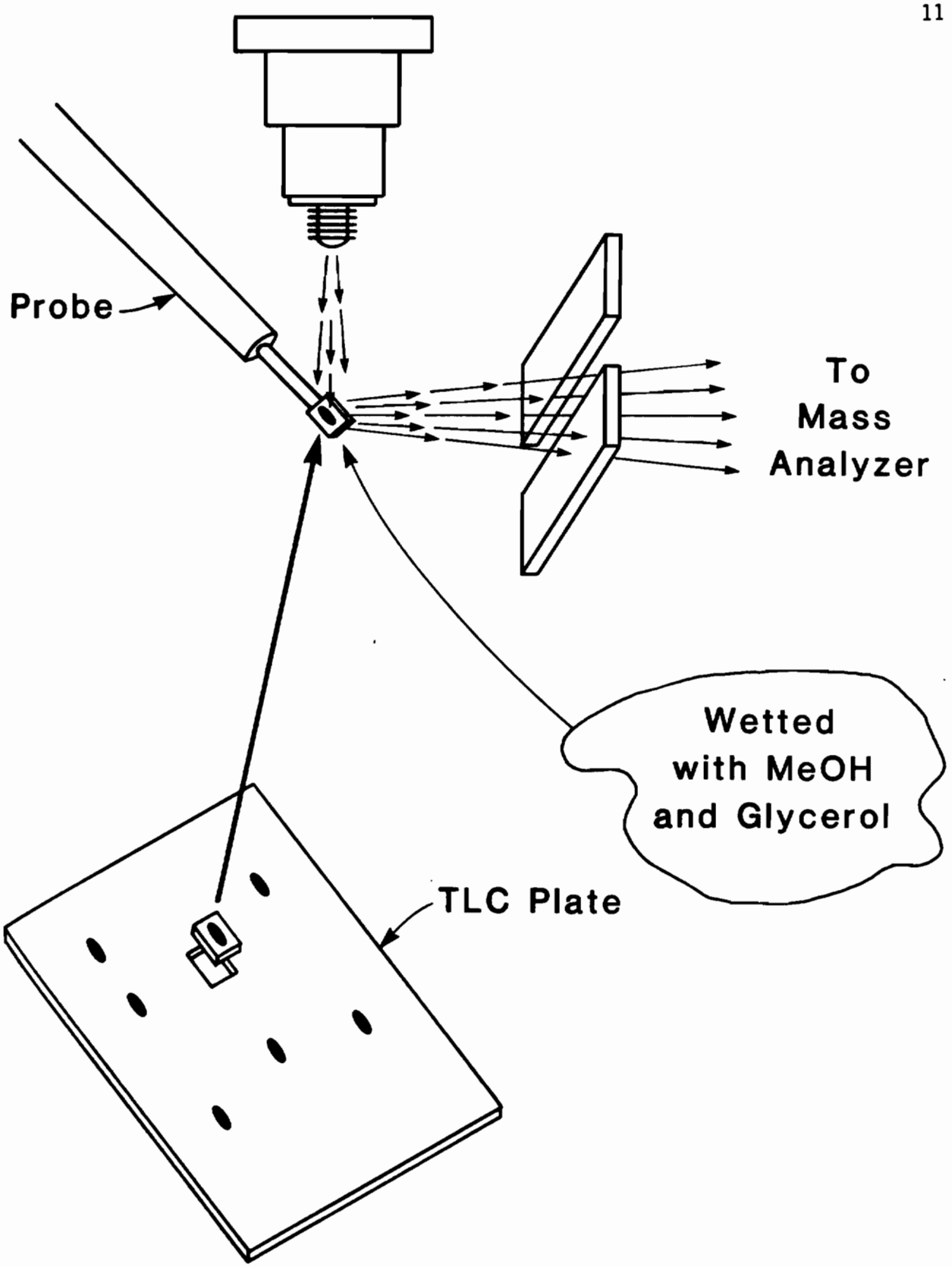


Figure 3. TLC-FAB Schematic Illustrating Scheme for Direct FAB Analysis of TLC Plates.

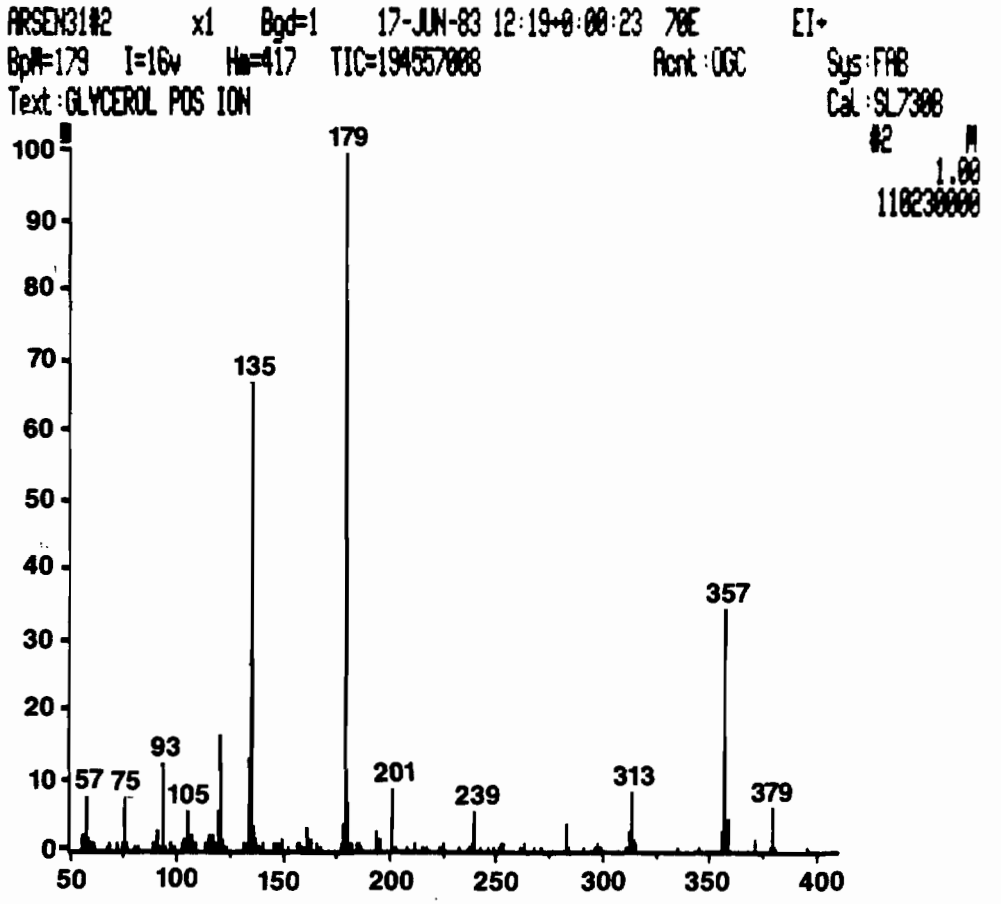


Figure 4. Arsenobetaine Spectrum in Glycerol, on Silica Plate.

The next experiments involved the analysis of the TLC or electrophoretic plates which had been prepared by Norin, Figures 5 and 6 (unpublished data). Synthetic arsenobetaine and arsenocholine had been subjected to the same chromatography as had the real fish extracts in a $CHCl_3$:MeOH:H_2O:NH_4OH (65:35:4:1) mobile phase. Spots were already indicated which were confirmed by observation under short wavelength UV light. Rf values, the ratio of the distance of sample migration to solvent migration, were estimated from Norin's plates as 0.53 for arsenobetaine and 0.63 for arsenocholine, comparable to the values obtained by Luten and Riekwel-Booy (27).

Spectra from fish extract spots taken from plates #2 and #3 are shown in Figures 7, 8, and 9. The spots of the synthetic compounds gave excellent spectra of arseno-betaine, m/z 179, and arsenocholine, m/z 165. The spots in the chromatogram of the fish extract did not, however, give any definitive evidence for either organoarsenic, although the peak at m/z 165 in the spectra of spot #13 might suggest arsenocholine. The peak at m/z 147, present in the spectra of synthetic arsenocholine as a result of a loss of water, is present in all these spectra from the TLC plates. The peak at m/z 151 is not present in non-silica spectra and cannot presently be rationalized. The strong peak at m/z 168 in the spectrum of the fish spot #13 also cannot be accounted for at present. A salt of silica or NH_4^+ may be possible.

The positive ion spectra of glycerol is tabulated in Figure 11 for reference.

Analysis of two other plates yielded similar results for synthetic spots but were entirely absent of evidence for the organoarsenics in spots from fish extracts. A concentrated fish extract in viscous liquid form, also provided by H. Norin, was analyzed and gave no indications of arsenobetaine or arsenocholine.

TLC, SPIKING, AND QUANTITATIVE STUDIES

A. GENERAL METHODS

Microgram amounts of N-betaine, N-choline, arsenobetaine, and arsenocholine were mixed with glycerol and analyzed by FAB-MS on the CEC-21-110 at 8 KV accelerating voltage using argon as the FAB collision gas. Peak heights at m/z 104 for N-choline, m/z 118 for N-betaine, m/z 165 for

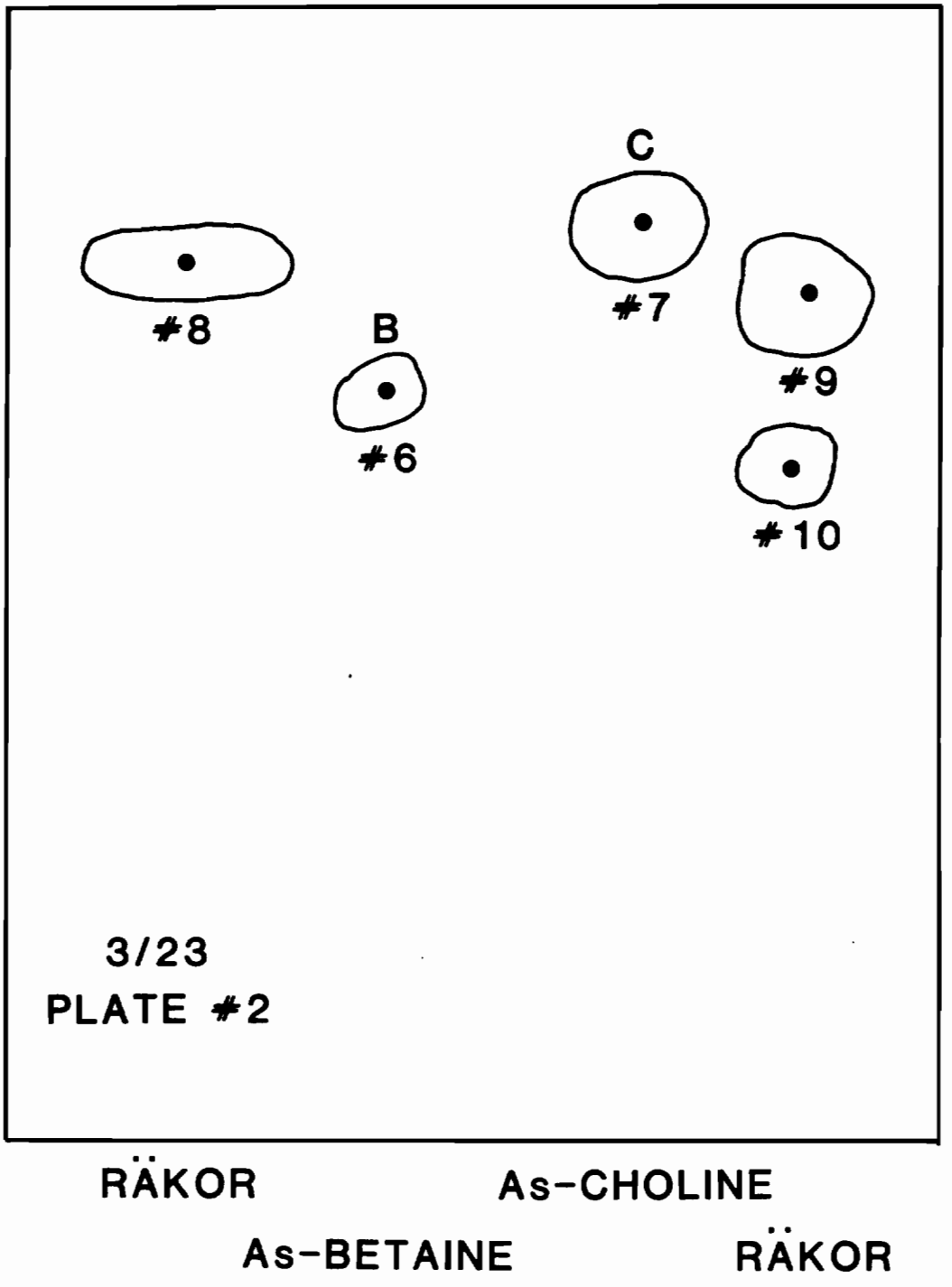


Figure 5. Thin-Layer Chromatogram of Organoarsenical Standards and Fish (RÄKOR) Extracts.

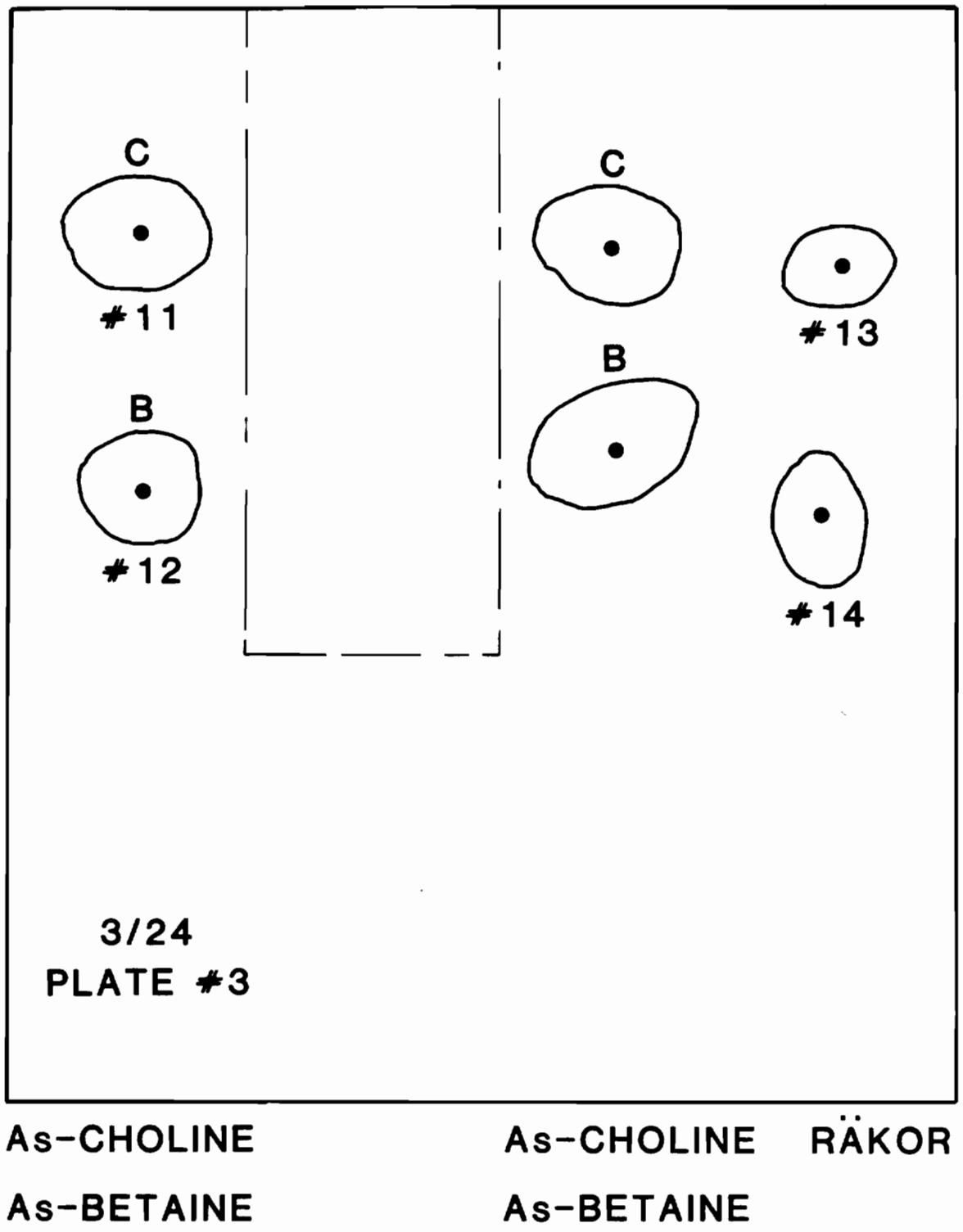


Figure 6. Thin-Layer Chromatogram of Organoarsenical Standards and Fish (RÄKOR) Extracts.

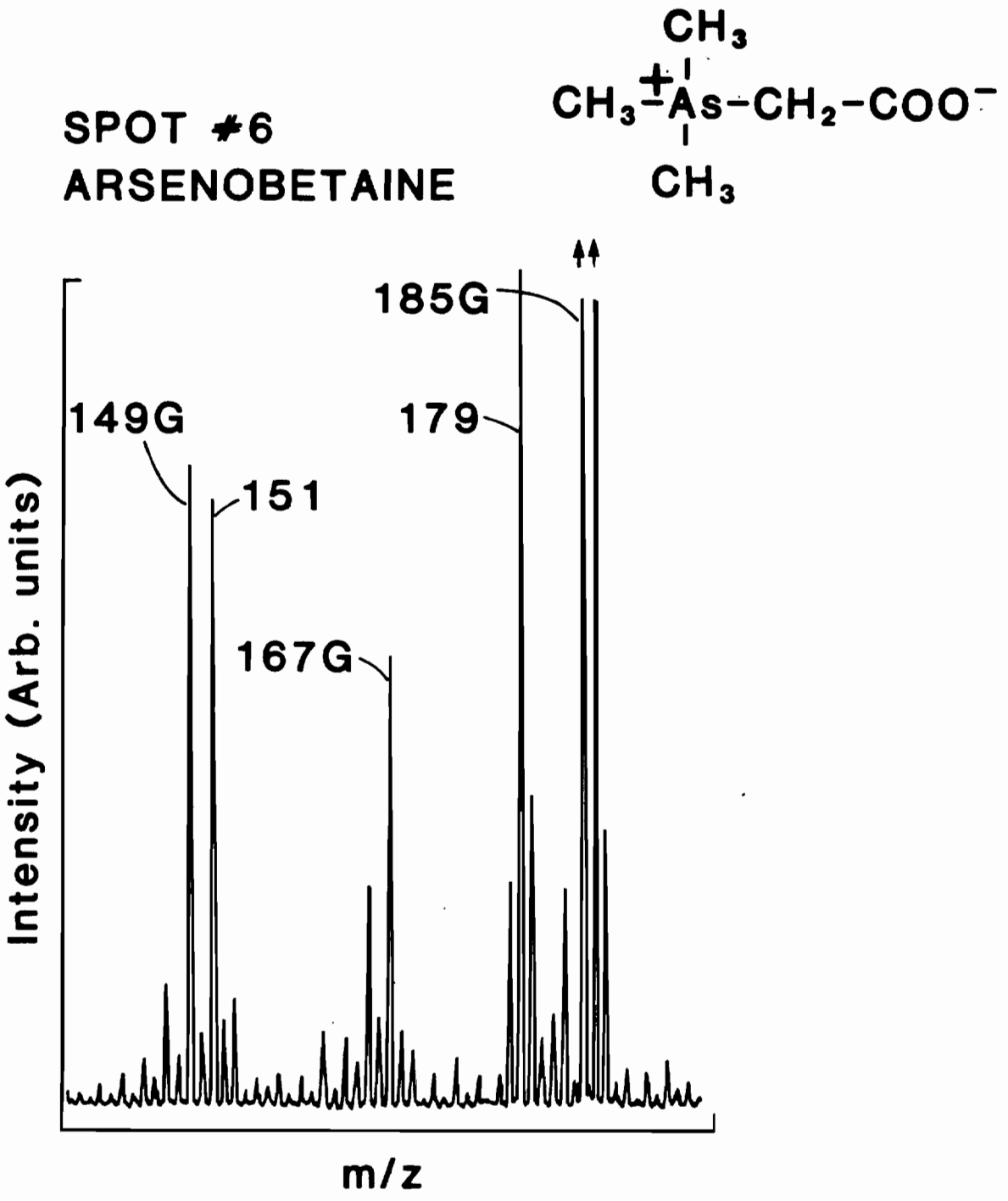


Figure 7. Direct FAB-MS of TLC Spot #6, Plate #2. Arsenobetaine Standard, m/z 179.

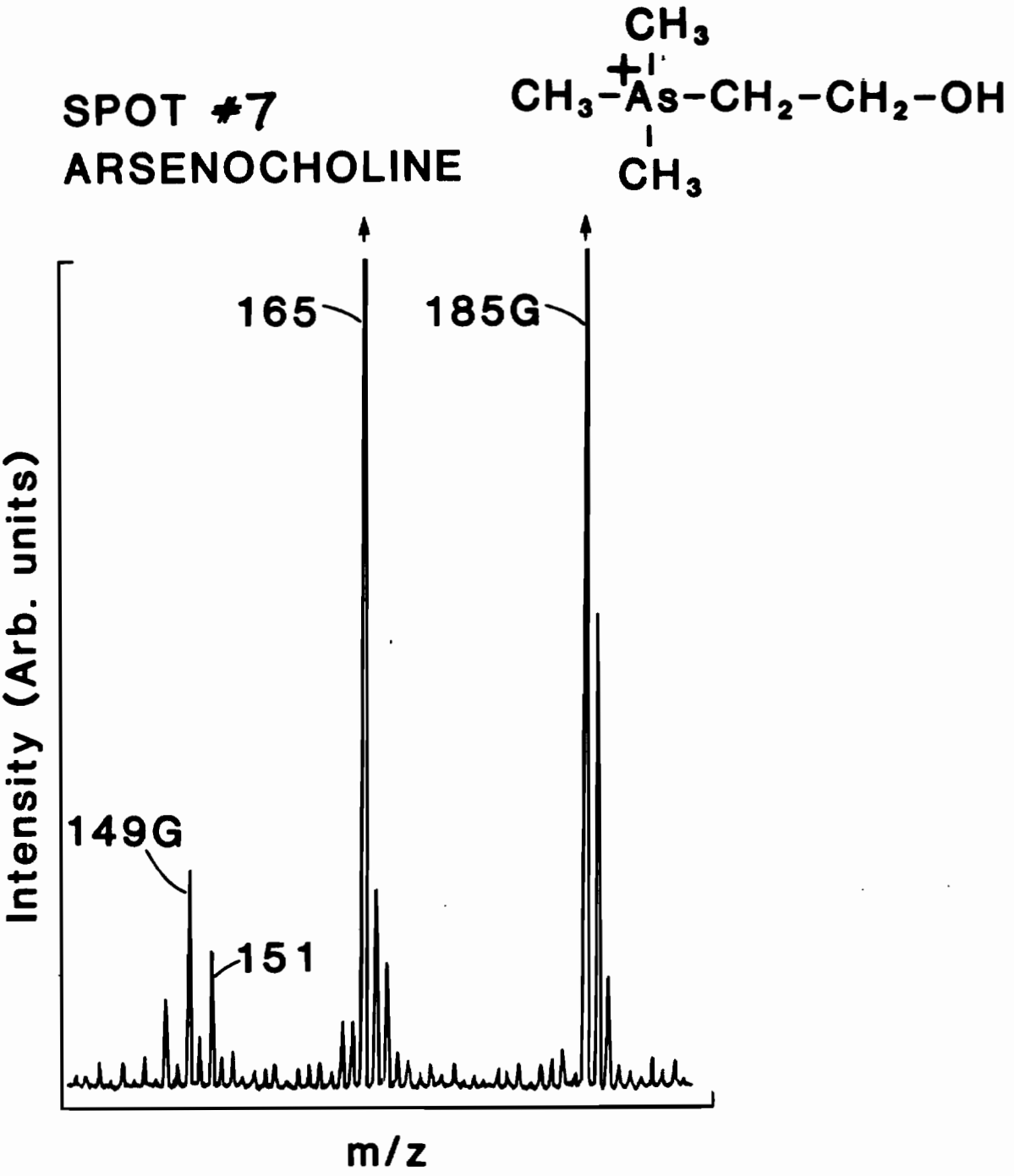


Figure 8. Direct FAB-MS of TLC Spot #7, Plate #2. Arsenocholine Standard, m/z 165.

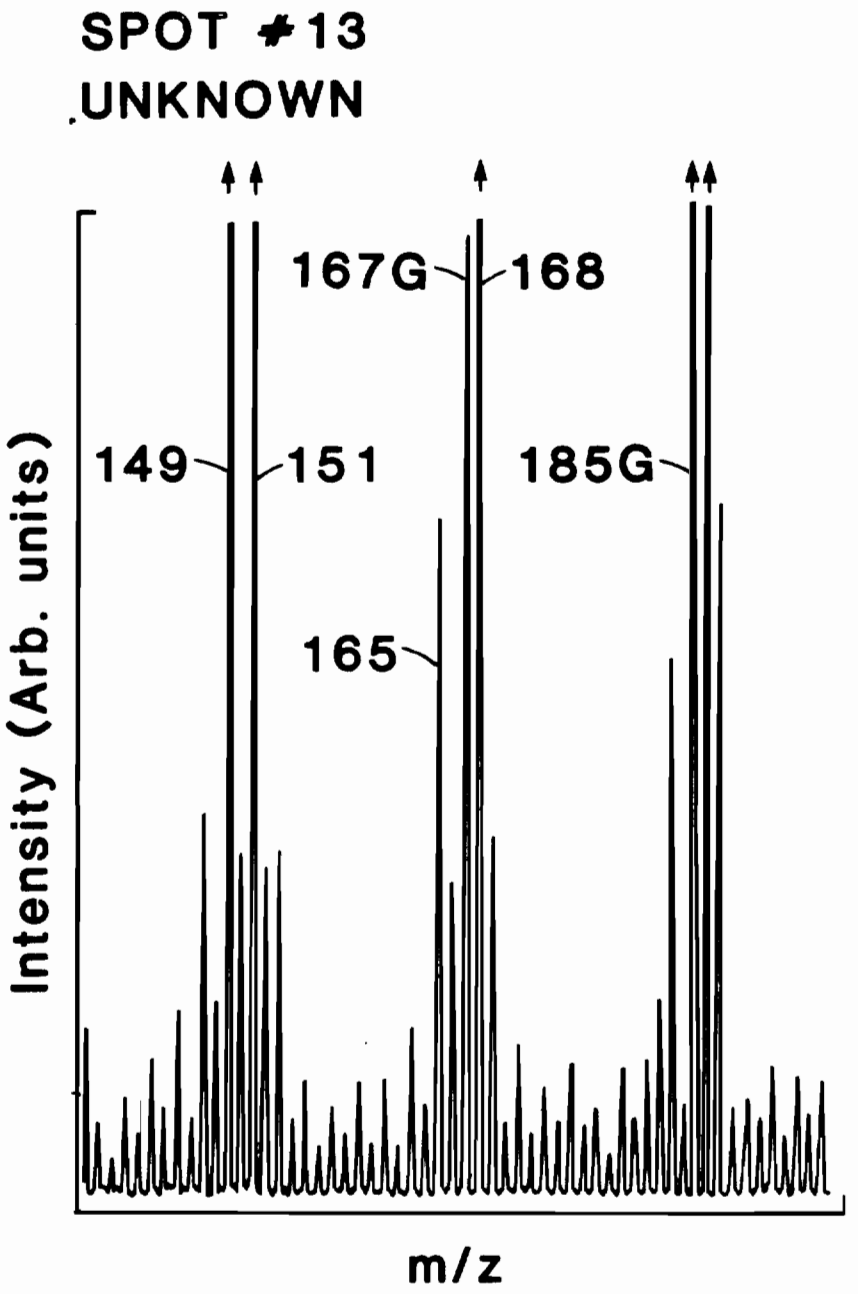


Figure 9. Direct FAB-MS of TLC Spot #13, Plate #3. Note m/z 165, 168.

arsenocholine, and m/z 179 for arsenobetaine were measured with a ruler from the oscillographic strip chart recording of the spectra. Silica TLC plates of 0.1 mm and 0.25 mm silica thickness were developed with high purity analytical grade solvents.

B. RESULTS

Experiments designed to duplicate the thin layer chromatograms of Norin and to assess the response of low levels of arsenobetaine when analyzed by FAB in the silica matrix were conducted by applying approximately 10 μg each of both the nitrogen and the arsenic compounds to TLC plates, developing the plates, and analyzing the developed spots either directly off the plates or by scraping off the plates and eluting with various solvent systems. Two mobile phases were used to develop the TLC plates: n-butanol, acetic acid, and water (60:15:25) and chloroform, methanol, water, and ammonium hydroxide (65:35:4:1). The first system of n-butanol gave no discernable betaine or choline spots under UV light. The chloroform system gave well resolved chromatograms with detectable spots at Rf 0.62 and 0.73 for the nitrogen compounds and 0.53, 0.63 for the arsenic compounds. The spots were cut out, painted to the FAB probe, wetted with 2-10 μl MeOH and 2-3 μl glycerol, and analyzed. Approximately 10 μg of each compound was applied to the TLC plate before development. The first results of FAB-MS analysis of the developed spots directly off the TLC plates are given below:

TABLE 1. FAB/MS ANALYSIS OF 0.5 cm^2 PORTION OF TLC SPOTS

Rf	Compound	m/z	Peak Height
0.62	N-betaine	118	>12 cm
0.73	N-choline	104	9.6 cm
0.53	Arsenobetaine	179	5.8 cm
0.63	Arsenocholine	165	1.0 cm

The same amounts of each compound show saturated peaks when analyzed in glycerol only. Assuming the difference in sensitivity is the result of dispersal of the compound in the silica matrix on the plate, the same TLC experiment was performed and the spots were analyzed indirectly by scraping them off the plates and extracting each with 100 μl MeOH. The MeOH was allowed to evaporate to approximately 10 μl and 2 μl of each concentrate was mixed with glycerol and analyzed. Considering a loss of 80% since only 20% of the concentrate is analyzed, results are give here;

TABLE 2. FAB/MS ANALYSIS OF METHANOL EXTRACTED TLC SPOTS

Rf	Compound	m/z	Peak Height
0.62	N-betaine	118	>12 cm
0.72	N-choline	104	>12 cm
0.51	Arsenobetaine	179	10 cm
0.60	Arsenocholine	165	8.5 cm

The difference in sensitivity between the TLC extract and the directly cut out spot analysis is clearly the result of dispersal of the compounds in an area of the silica gel too large for direct analysis. Only 0.5 cm^2 of a spot will fit on the FAB probe when cut out for direct analysis while the spots are over 1 cm^2 in area. Another consideration is that only 1 or 2 μl of an extract may be applied to the probe for analysis. For these reasons, we thought the best approach would be to mix the entire spot extract with glycerol, allow the MeOH to evaporate, and analyze the concentrate.

In the following experiments, TLC (chloroform system) spots were analyzed and compared to straight analyte in glycerol analysis. Two TLC silica plates of 0.25 mm and 0.1 mm silica thickness were developed with 0.3 μg of arsenobetaine. The resulting spots were detected under UV light, scraped and extracted with MeOH. The extract was allowed to concentrate to near dryness (<2 μl) the brought to 5 μl final volume. The entire 5 μl was added to 2 μl of glycerol, and the MeOH was evaporated under a heat lamp before applying the glycerol to the FAB probe. The results of FAB-MS analysis of arsenobetaine in

glycerol (0.3 μg in 2 μl glycerol), arsenobetaine (0.3 μg) developed on the 0.1 mm silica TLC plate, and arsenobetaine (0.3 μg) developed and extracted from the 0.25 mm silica plate are presented here:

TABLE 3. FAB/MS ANALYSIS OF ARSENOBETAINE IN GLYCEROL, 0.25 mm SILICA TLC PLATE, AND 0.1 mm SILICA PLATE

Rf	Compound	Sample	m/z	Peak Height
--	Arsenobetaine in Glycerol	#1	179	9.7 cm
		#2	179	10.3
		#3	179	9.8
		#4	179	10.0
0.51	Arsenobetaine	#1	179	2.0 cm
0.51	TLC Spot	#2	179	2.6
0.51	(0.25 mm silica)	#3	179	2.2
0.53		#4	179	2.2
0.51	Arsenobetaine	#1	179	6.5 cm
0.50	TLC Spot	#2	179	5.0
0.51	(0.1 mm silica)	#3	179	5.5
0.52		#4	179	4.2

In these experiments, a clear and significant difference can be seen between normal liquid assisted FAB-MS (no silica matrix) and TLC silica extraction analysis. The TLC-chloroform system is a very reproducible chromatographic system, even if it does not lend itself to particularly sensitive FAB-MS analysis.

As a demonstration of linear response over a range of analyte concentrations, data is presented later in which peak areas, rather than heights, were measured on the VG 70E instrument (Figure 10).

For the experiments intended to assess the recovery of N-betaine and N-choline from real fish, an extraction and cleanup method described by several authors (25,26,27) was used. Briefly, an appropriate amount of fish tissue was homogenized in a tissue blender and subjected to a lipid extraction with chloroform, methanol, and water, according to the method of Bligh and Dyer (33), see APPENDIX A. The fish used for these experiments were ocean sole purchased from a supermarket. The lipid extraction results in a large volume (500 ml) of $CHCL_3/MeOH/H_2O$, depending on the initial mass of fish tissue

extracted. The method relies on the proper proportions of $CHCL_3$, MeOH, and water, to effect a phase separation. The $CHCL_3$ and tissue residue separate to the bottom of the extraction apparatus while the MeOH and water, which contains the water soluble compounds of interest, is in the layer above. Each of four 10 g fish homogenates were spiked with the following levels of nitrogen compounds to allow detectable concentrations in the final 5-10 ml concentrates:

TABLE 4. LEVELS OF NITROGEN COMPOUND SPIKES

Fish #1	15.9 mg N-betaine
Fish #2	15.4 mg N-betaine
Fish #3	18.8 mg N-betaine
	12.6 mg N-choline
Fish #4	15.4 mg N-betaine
	14.9 mg N-choline
Fish #5	0 (blank)

After allowing the MeOH phase to separate from the $CHCl_3$ phase, the MeOH/water was easily recovered and filtered through Whatman filter paper. The MeOH was evaporated at 40°C and the remaining water was concentrated at 80°C to a final volume of 10 ml.

The aqueous concentrates were acidified to pH 3 and passed through a cation exchange column of Amberlite IR-120, 20-50 mesh, H^+ form. The ion exchange column contained 2 grams of resin packed in a disposable pipette. The entire concentrate was passed through the column and the water eluate was discarded. The columns were then eluted with 5 ml of 5% NH_4OH and 2 ml of water to elute the compounds of interest. The 7 ml eluate was collected and neutralized with 30% HCL.

Assuming complete recovery of the nitrogen compounds, it was expected that the 7 ml extracts from the spiked fish homogenates would yield detectable amounts of N-betaine and N-choline when 1 to 5 μl aliquots were analyzed by FAB/MS. Approximately 2 $\mu g/\mu l$ should be present. Results of the analyses are given below:

TABLE 5. FAB/MS ANALYSIS OF FISH CONCENTRATES SPIKED WITH NITROGEN COMPOUNDS

FISH SPIKE	N-Choline m/z 104 Peak Height	N-Betaine m/z 118 Peak Height
#1	0.2, 0.3	6.6, 8.2
#2	0.5, 0.5	4.3, 7.0
#3	4.1, 4.5	8.5, 7.8
#4	5.0, 6.0	10.0, 8.8
#5 (blank)	0.2, 0.4	0.6, 1.0

Efforts to further concentrate the spiked extracts to volumes amenable to TLC were only partly successful. Extracts #2, 4, and 5 were overheated and lost. Extracts #1 and #3 were concentrated to approximately 100 μl and developed in the TLC system of $CHCl_3$:MeOH:H_2O:NH_4OH on 0.25 mm silica gel plates over 35 minutes. The developed plates yielded spots at Rf 0.54 and 0.61. A 2 μg N-betaine standard was developed on the same plate and yielded a spot at Rf 0.56. These Rf values are low compared to previous data. Nevertheless, FAB analysis of the scraped, extracted, and concentrated TLC extract gave the following peak intensities:

TABLE 6. FAB/MS ANALYSIS OF TLC SPOTS FROM NITROGEN COMPOUND SPIKED FISH CONCENTRATES

FISH SPIKE	N-Choline m/z 104 Peak Height	N-Betaine m/z 118 Peak Height
#1	0.5, 0.2	>12, >12
#3	10, 9.0	>12, >12
2 μg N-betaine	0.2	>12

These FAB analyses were performed on the CEC 21-110 at 6 KV accelerating voltage using Xenon as the FAB collision gas.

Results to this point indicate that some measurable extraction is possible, at least with the nitrogen compounds. Weaknesses in the method include the relatively poor sensitivity of FAB/MS, estimated as 10 to 100 ng, and the difficult task of concentrating an aqueous extract to an appropriate volume where detection of concentrated compounds is possible.

NITROGEN ANALOGUES AS INTERNALS STANDARDS

A. GENERAL METHODS

Without resources for synthesizing labelled organoarsenics or for determining the natural background levels of betaine and choline in animal tissue, it was decided to use the nitrogen containing analogues as internal standards. Calibration curves were generated by analyzing mixtures of N-betaine and arsenobetaine and varying only the amount of arsenobetaine. One curve for arsenocholine resulted from similar analysis of N-choline and arsenocholine.

During the installation of the VG 7070E mass spectrometer (the instrument used in this part of the study) I made a fishing trip to Commencement Bay, Tacoma, Washington, with the intention of catching some arsenic ladden fish from the area of the Asarco copper refinery. I caught a dogfish and two small mouth ocean bass from the Bay and collected a large brown kelp growing near the refinery. I also received two shellfish extracts from the Washington Department of Environmental Quality (DEQ) that they had analyzed for total arsenic and had found to contain 150 μg/kg and 350 μg/kg, respectively. The two ocean bass from Commencement Bay were extracted and analyzed in duplicate along with the shellfish from the Washington DEQ.

In addition to the fish and shellfish from Commencement Bay, ocean fish fillets were acquired from a supermarket. Three of these supermarket fillets were homogenized and spiked with 1.3 mg of arsenobetaine; one other homogenate of the supermarket fillet was extracted and analyzed without the arsenobetaine spike. The spiking level, 13 mg/kg (ppm), was chosen to represent an amount of arsenobetaine that might be expected in fish tissue (21).

All of the samples were extracted by the Bligh-Dyer procedure, and the extracts were passed through an ion exchange column. Both procedures were

described in the preceding section. Following extraction and ion exchange, all the samples, except spike #2 and fish #2, were mass analyzed twice.

In an attempt to remove the native N-betaine, the DEQ extracts were passed through a second ion exchange column. Spike #3 was treated in the same way. The 1 ml extracts were passed onto IR-120 H^+ columns and eluted with 5 ml of NH_4OH and 2 ml of water. The 7 ml eluates were concentrated to 1.0 ml and mass analyzed.

All the Washington samples, Commencement Bay and DEQ, were developed on individual silica TLC plates in $CHCl_3$:MeOH:NH_4OH:H_2O. Resulting spots on all the plates were measured at Rf 0.8 and 0.5 which might indicate separation of betaine analogs. Standards of N-betaine and arsenobetaine were developed under identical conditions and resulted in spots at Rf 0.75 and 0.65. The lower spots thought to contain the arsenic compounds were scraped from the plates and extracted with 100 μl MeOH. The MeOH extract was evaporated to a final volume of approximately 10 μl.

B. RESULTS

The similar FAB-MS behavior of N-betaine and arsenobetaine in glycerol is shown in Figure 12 where the molecular ion intensities are plotted against time. This similarity is important for internal standard considerations.

The calibration data is given in the following tables and shown in Figure 10. The calculated ratios are based on nanomoles of compound and peak areas averaged over at least 20 scans of the mass spectrometer. Data was plotted as: Area m/z 179 /Area m/z 118 versus nanomoles arsenobetaine/nanomoles N-betaine, respectively. Such plots should have slopes of 1 and intercepts of zero.

The first calibration was performed with 6.5 μg (55 nmol) of N-betaine. At least two replicates were analyzed at each level of arsenobetaine:

TABLE 7. FAB/MS CALIBRATION OF ARSENOBETAINE AND N-BETAINE (55 nmol N-BETAINE)

Amount Arsenobetaine	Amt As/Amt N	Area 179/Area 118
3.3 nmol	0.06	0.035, 0.064
6.5	0.12	0.14, 0.14
11	0.20	0.25, 0.16
16	0.29	0.36, 0.29
33	0.60	0.69, 0.62

The second calibration was performed with 130 ng (1.1 nmol) of N-betaine. Replicates were analyzed at each level of arsenobetaine except the lowest and highest:

TABLE 8. FAB/MS CALIBRATION OF ARSENOBETAINE AND N-BETAINE (1.1 nmol N-BETAINE)

Amount Arsenobetaine	Amt As/Amt N	Area 179/Area 118
0.33 nmol	0.30	0.62
0.81	0.74	0.90, 0.65, 0.70
1.6	1.45	1.3, 1.4, 1.7
8.1	7.4	5.9, 9.1
16	15	10

The third calibration was performed with 650 ng (5.5 nmol) of N-betaine. Replication was performed at only three levels of arsenobetaine:

TABLE 9. FAB/MS CALIBRATION OF ARSENOBETAINE AND N-BETAINE (5.5 nmol N-BETAINE)

Amount Arsenobetaine	Amt As/Amt N	Area 179/Area 118
0.33 nmol	0.06	0.09
0.65	0.12	0.26
0.81	0.15	0.24, 0.19

TABLE 9. FAB/MS CALIBRATION OF ARSENOBETAINE AND N-BETAINE (5.5 nmol N-BETAINE) (Continued)

Amount Arsenobetaine	Amt As/Amt N	Area 179/Area 118
1.2	0.22	0.39, 0.35
1.6	0.29	0.55, 0.47
3.3	0.60	0.86
8.1	1.5	1.8
16.3	3.0	3.1

All three calibrations result in roughly linear plots with correlation coefficients greater than 0.9 (Figure 10A, B, C, and D); regression statistics are also presented in Figure 10. Sensitivity, as defined by the area of a peak 2.5X the area of the average background glycerol peak at the same m/z, is approximately 0.3 nmols (0.05 μg).

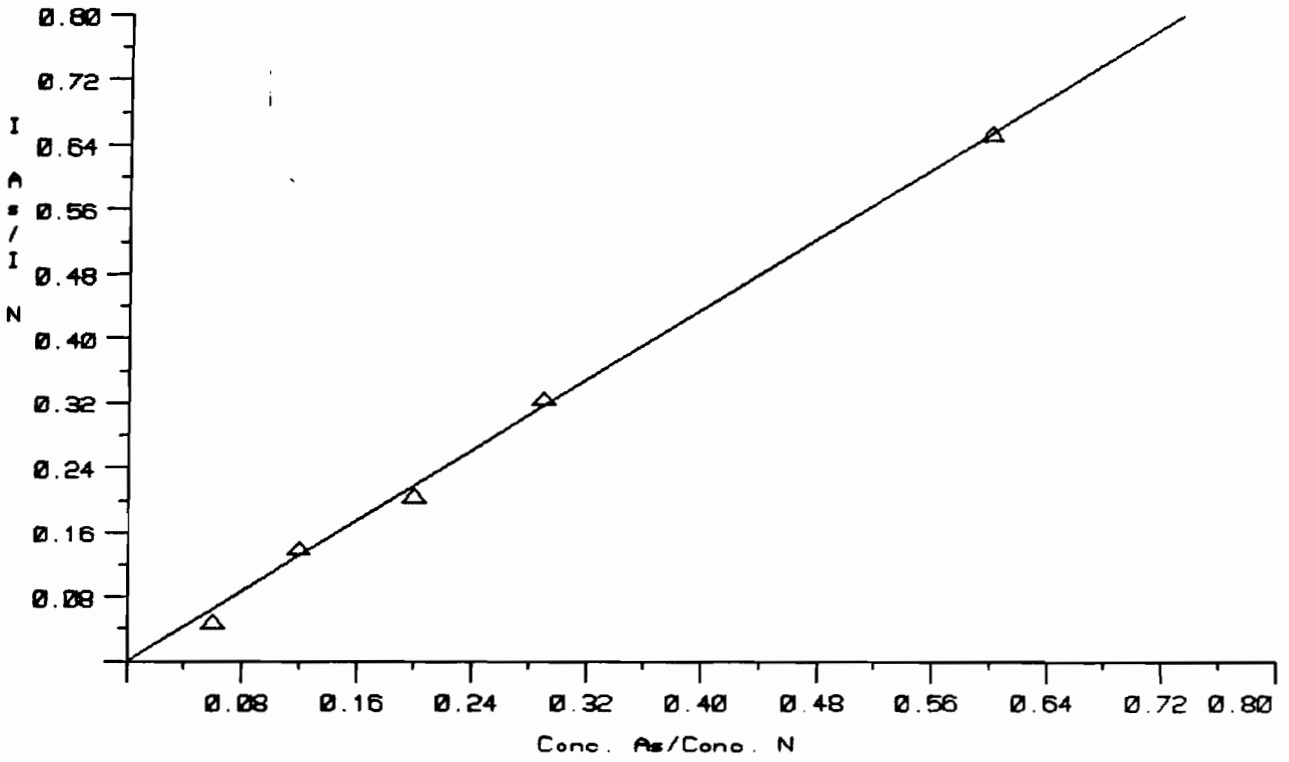
The arsenocholine data resulted from the analysis of 360 ng (3.5 nmol) of N-choline:

TABLE 10. FAB/MS CALIBRATION OF ARSENOCHOLINE AND N-CHOLINE (3.5 nmol N-CHOLINE)

Amount Arsenocholine	Amt As/Amt N	Area 165/Area 104
1.7 nmol	0.5	1.1
2.7	0.8	1.2
5.1	1.5	1.6
15	8.8	4.6

Ideally, quantification includes spiking the sample before extraction with a known amount of isotopically labelled internal standard which can be used to quantify the analyte. A surrogate compound of similar structure and behavior might also be used. In this work, however, the internal standard was spiked into the sample immediately prior to analysis. This approach prohibits accounting for losses which may have occurred in the extraction and clean-up of the sample.

A
(from Table 7)



Arsenobetaine 1

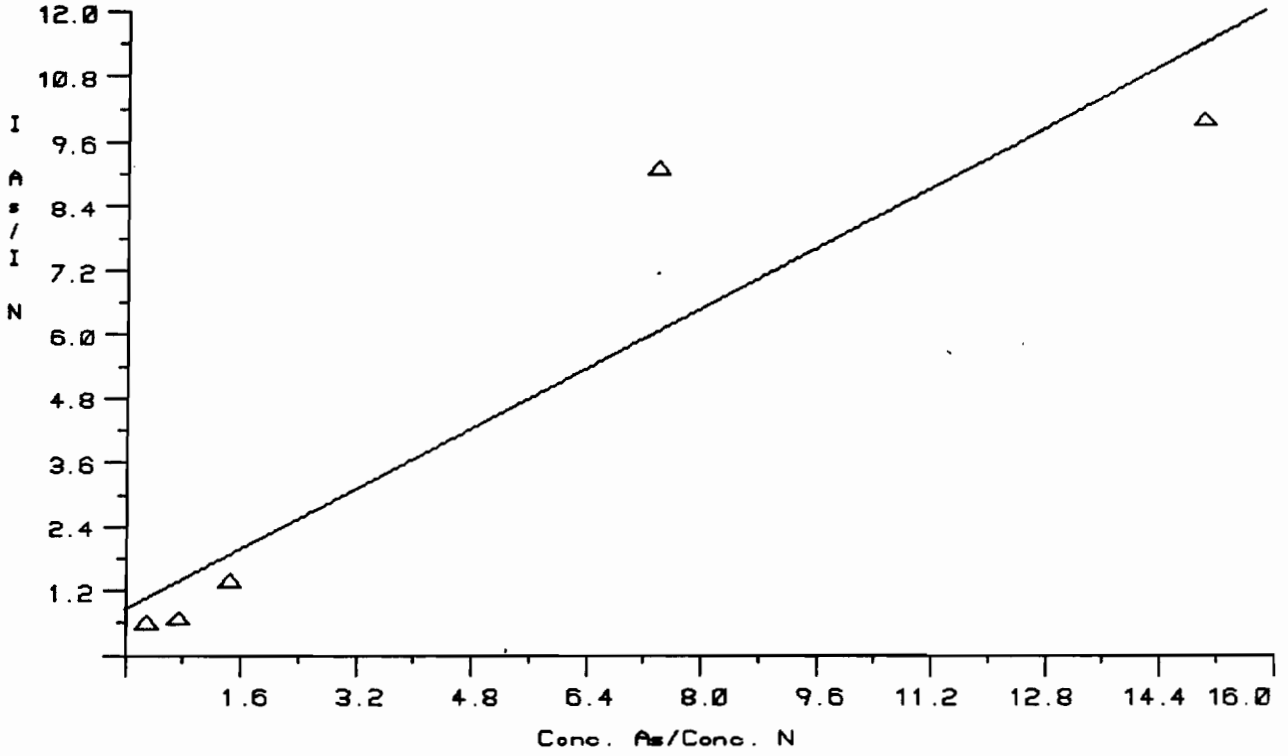
X-Values	Y-Values	Average
0.060	0.035, 0.064	0.050
0.120	0.14, 0.14	0.14
0.200	0.25, 0.16	0.205
0.290	0.36, 0.29	0.325
0.600	0.69, 0.62	0.655

A SLOPE OF 1.10793 GIVES AN INTERCEPT OF -0.00651
A SLOPE OF 0.90257 GIVES AN INTERCEPT OF 0.00588

THE DATA HAS A CORRELATION OF COEFFICIENT OF 0.99884

Figure 10. Arsenobetaine Calibration

B
(from Table 8)



Arsenobetaine 2

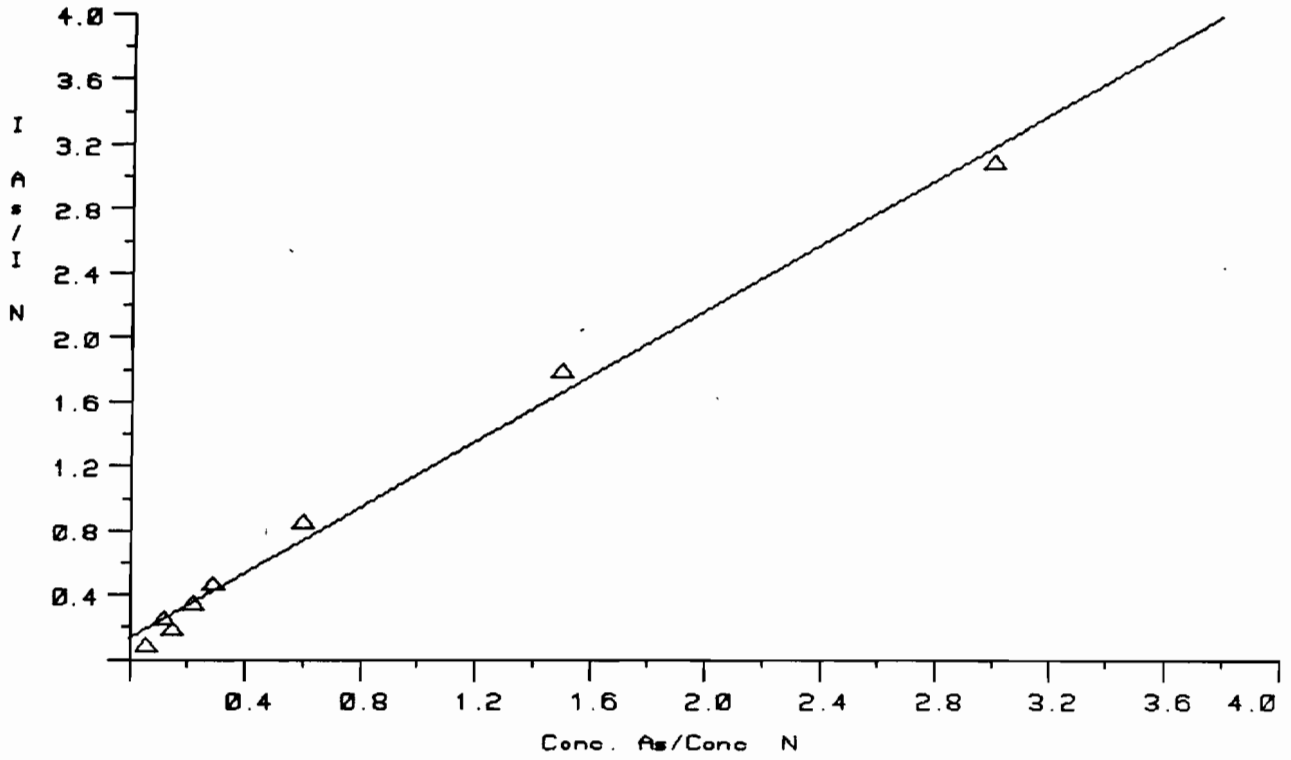
X-Values	Y-Values
0.300	0.62
0.740	0.70
1.45	1.40
7.40	9.10
15.0	10.0

A SLOPE OF 0.70146 GIVES AN INTERCEPT OF 0.89269
A SLOPE OF 1.42559 GIVES AN INTERCEPT OF -1.27261

THE DATA HAS A CORRELATION OF COEFFICIENT OF 0.93166

Figure 10. (Continued)

C
(from Table 9)



Arsenobetaine 3

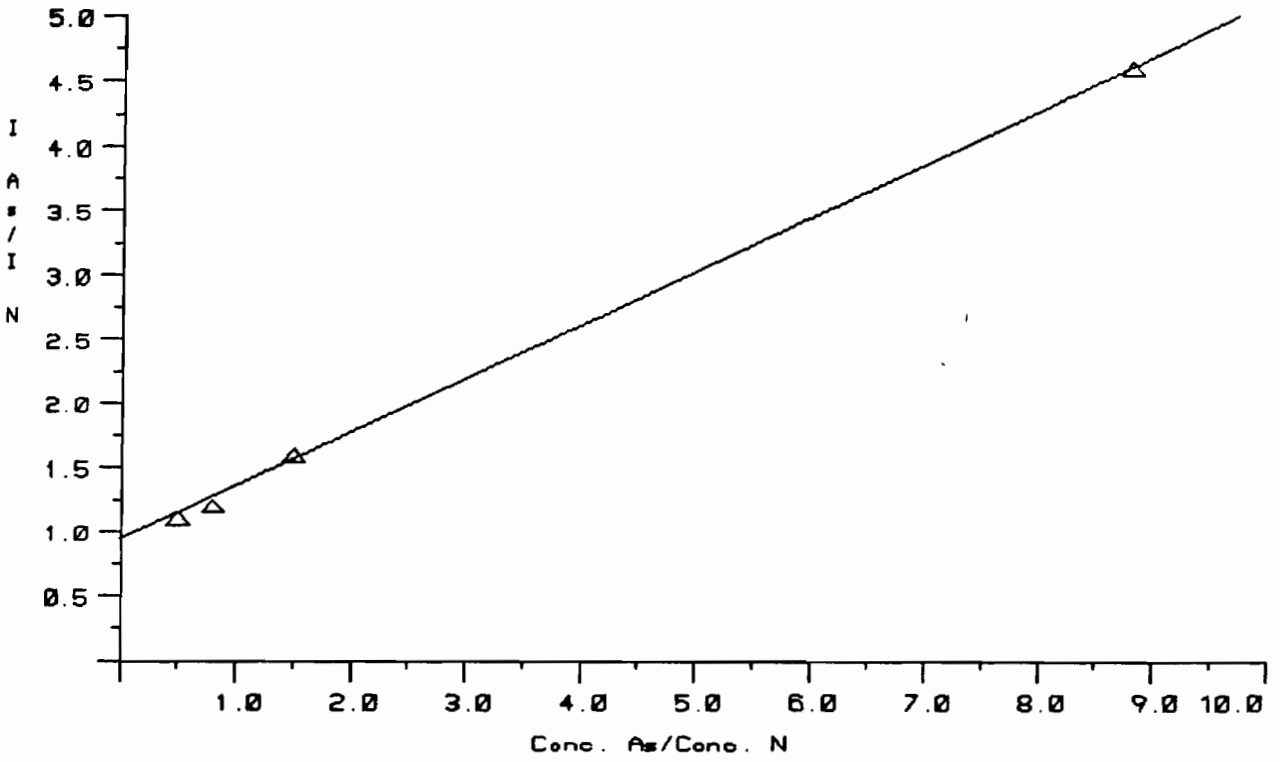
X-Values	Y-Values
0.060	0.09
0.120	0.26
0.150	0.19
0.220	0.35
0.290	0.47
0.600	0.86
1.50	1.8
3.00	3.1

A SLOPE OF 1.01834 GIVES AN INTERCEPT OF 0.13387
A SLOPE OF 0.98198 GIVES AN INTERCEPT OF -0.13146

THE DATA HAS A CORRELATION OF COEFFICIENT OF 0.99593

Figure 10. (Continued)

D
(from Table 10)



Arsenocholine 1

X-Values	Y-Values
0.50	1.1
0.80	1.2
1.50	1.6
8.80	4.6

A SLOPE OF 0.42053 GIVES AN INTERCEPT OF 0.90544
A SLOPE OF 2.37791 GIVES AN INTERCEPT OF -2.15306

THE DATA HAS A CORRELATION OF COEFFICIENT OF 0.99963

Figure 10. (Continued)

The data below summarizes the extraction and concentration of the fish and shellfish samples:

TABLE 11. EXTRACTION OF COMMENCEMENT BAY AND SUPERMARKET FISH AND SHELLFISH

Sample	Initial Wt.	Final Volume
Fish 1 - S1	22.8 g	1.0 ml
Fish 1 duplicate	18.7 g	1.0 ml
Fish 2 - S2	60 g	2.0 ml
Fish 2 duplicate	74 g	1.0 ml
11MNL (DEQ)	18.5 g	0.8 ml
16502 (DEQ)	33.2 g	0.8 ml
Supermarket Fish Spike #1 (1.3 mg As-betaine)	100 g	1.0 ml
Supermarket Fish Spike #2 (1.3 mg As-betaine)	100 g	1.0 ml
Supermarket Fish Spike #3 (1.3 mg As-betaine)	100 g	1.0 ml
Supermarket Fish (No Spike)	100 g	1.0 ml

The data in the following table summarizes the FAB/MS analysis of all the samples without TLC:

TABLE 12. FAB/MS ANALYSIS OF COMMENCEMENT BAY AND SUPERMARKET FISH

Sample	Vol Anal	Amt N-betaine (internal Standard)	Area m/z 179 (As-betaine)	Area m/z 118 (N-betaine)
Glycerol	1.0 µl	0	60	210
Fish Blank	1.0 µl	5.5 nmol	84	1987
Spike #1	1.0 µl	5.5 nmol	4779	3597
Spike #1	1.0 µl	5.5 nmol	3204	2516
Spike #2	1.0 µl	5.5 nmol	2969	2077
Spike #3	1.0 µl	1.1 nmol	2249	678
Spike #3	1.0 µl	1.1 nmol	1555	799
Fish #1	2.0 µl	1.1 nmol	359	1901
Fish #1	2.0 µl	0	1171	2079
Fish #1 D.	2.0 µl	1.1 nmol	504	2596
Fish #1 D.	2.0 µl	0	692	1584
16502	1.0 µl	1.1 nmol	713	8850

TABLE 12. FAB/MS ANALYSIS OF COMMENCEMENT BAY AND SUPERMARKET FISH (Continued)

Sample	Vol Anal	Amt N-betaine (internal Standard)	Area m/z 179 (As-betaine)	Area m/z 118 (N-betaine)
16502	1.0 µl	0	807	9148
11MNL	1.0 µl	1.1 nmol	321	5899
11MNL	2.0 µl	0	518	3660
Fish #2	2.0 µl	1.1 nmol	197	1515
Fish #2 D.	2.0 µl	1.1 nmol	187	1503

The Commencement Bay fish and DEQ samples all show a background at m/z 118 that might be due to N-betaine; this is especially evident in the shellfish extracts. Fish #2 did not show a m/z 179 peak above the normal glycerol background, and therefore no calculations were made for this sample.

In developing an expression for computing the level of arsenobetaine in the sample extracts, the linear calibrations make it reasonable to assume a response factor for arsenobetaine equal to 1.0. The formula for calculation of arsenobetaine concentration is derived in APPENDIX B.

TABLE 13. CALCULATED AMOUNT OF ARSENOBETAINE IN COMMENCEMENT BAY AND SUPERMARKET SPIKED FISH SAMPLES

Sample	Concentration (µg/g)	
Spike #1	13.1 µg/g	(100% recovery)
	12.5 µg/g	(96% recovery)
Spike #2	14.1 µg/g	(108% recovery)
	13.6 µg/g	(105% recovery)
Spike #3	6.5 µg/g	(50% recovery)
	3.8 µg/g	(29% recovery)
Fish 1 Duplicate	31 µg/g	
DEQ 11MNL	8.0 µg/g	

Within reasonable error limits, the recoveries of arsenobetaine from Spike samples #1 and #2 are 100%; the recovery from Spike sample #3 is anonymously low. The background N-betaine in the spike extracts is low and does not affect

the recovery calculations when 5.5 nmol of internal standard is used. Calculations when 1.1 nmol of N-betaine is used (spike #3) do not yield good recoveries. This suggests that sample analysis may be better using the higher level of internal standard, which results in a signal at m/z 118 that swamps the background signal.

The data from fish #1 and the DEQ shellfish 16502 show larger 118 peaks without the addition of 1.1 nmol N-betaine than with; thus no estimate of their arsenobetaine content was possible from this data. The reason for the large background at m/z 118 in these two samples and the loss in intensity with addition of N-betaine is not understood.

Analysis of spike #3 and the DEQ extracts after subjecting them to a second ion exchange treatment gave the following results (Table 14):

TABLE 14. FAB/MS ANALYSIS OF SPIKE #3 AND DEQ SHELLFISH AFTER SECOND ION EXCHANGE

Sample	Amount N-betaine	Area 179	Area 118	CONC (μg/g)	Percent Recovery
Spike #3	1.1 nmol	607	804		
Spike #3	1.1 nmol	1214	453	5.3	40
Spike #3	1.1 nmol	2082	1031	4.0	31
Spike #3	1.1 nmol	1660	664	4.9	37
11MNL	1.1 nmol	163	1736		
11MNL	1.1 nmol	550	3287		
16502	0	339	1497		
16502	1.1 nmol	690	1832		
Glycerol	0	646	285		

The recovery from spike #3 is still poor but consistent with the preceding determination. The 179 areas for the DEQ extracts are not significantly above the glycerol background and do not permit meaningful quantification.

FAB/MS analysis of the samples recovered from the chloroform developed TLC plates yielded the following data (Table 15):

TABLE 15. FAB/MS OF COMMENCEMENT BAY FISH EXTRACTS FROM TLC SPOTS

Sample	Amount N-betaine	Area 179	Area 118
Arsenobetaine (9 μg)	1.1 nmol	22874	553
Arsenobetaine (0.18 μg)	1.1 nmol	494	502
Silica TLC blank	0	229	151
Fish #1 5 μl	0	993	1541
Fish #1 5 μl	1.1 nmol	1046	1950
Fish #2 5 μl	0	156	5779
11MNL 5 μl	0	792	6682
11MNL 5 μl	1.1 nmol	676	6993
16502 5 μl	0	937	3641
16502 5 μl	1.1 nmol	1019	4427

Calculations of the arsenobetaine level in the fish samples yielded the following results (Table 16):

TABLE 16. CALCULATED AMOUNT OF ARSENOBETAINE IN COMMENCEMENT BAY FISH AND SHELLFISH FROM TLC ANALYSIS

Sample	Calculated Arsenobetaine	% Recovery
9.0 μg STD	8.1 μg	90
0.18 μg STD	0.19 μg	106
Fish #1	43 μg/g	
Fish #2	None detected	
11MNL	50 μg/g	
16502	15 μg/g	

These TLC extracts yielded results not unlike the results of the analysis of the extracts before TLC. Fish #1 was found to contain 43 and 31 μg/g while 11MNL results were 8.0 and 50 μg/g, respectively.

REF Listing of Reference file GLYCEROL

Mass	Height(%)	Mass	Height(%)	Mass	Height(%)
1800.89999	0.01	1700.82999	0.01	1565.81299	0.01
1473.76599	0.01	1381.71799	0.01	1289.67099	0.01
1197.62299	0.14	1105.57599	0.05	1013.52800	0.08
921.48119	0.16	829.43390	0.23	737.38649	0.60
645.33919	0.96	553.29189	2.29	461.24449	4.87
369.19719	14.34	277.14980	57.89	185.10249	100.00
167.09193	10.00	149.08138	5.00	93.05515	100.00
75.04459	100.00				

RETURN=continue CTRL/A=abort 'H=hardcopy

THE JOHNS HOPKINS UNIVERSITY
NATIONAL SCIENCE FOUNDATION REGIONAL INSTRUMENTATION FACILITY
MIDDLE ATLANTIC MASS SPECTROMETRY LABORATORY
DEPARTMENT OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
725 NORTH WOLFE STREET • BALTIMORE, MARYLAND 21205
(301) 955-3022

MAJOR AND MINOR GLYCEROL IONS IN POSITIVE ION FAB SPECTRA

	$-H_2O$	$-2H_2O$	$-3H_2O$	$-2CH_2OH$	+Na	?	-92
93							
185	167	149	131	123	115	101	93
277	259	241	223	215	207	193	185
369	351	333	315	307	299	285	277
461	443	425	407	399	391	377	369
553	535	517	499	491	483	469	461
645							
737							
829							

Figure 11. Edited Reference File (VG 11-250 Data System); Johns Hopkins Reference.

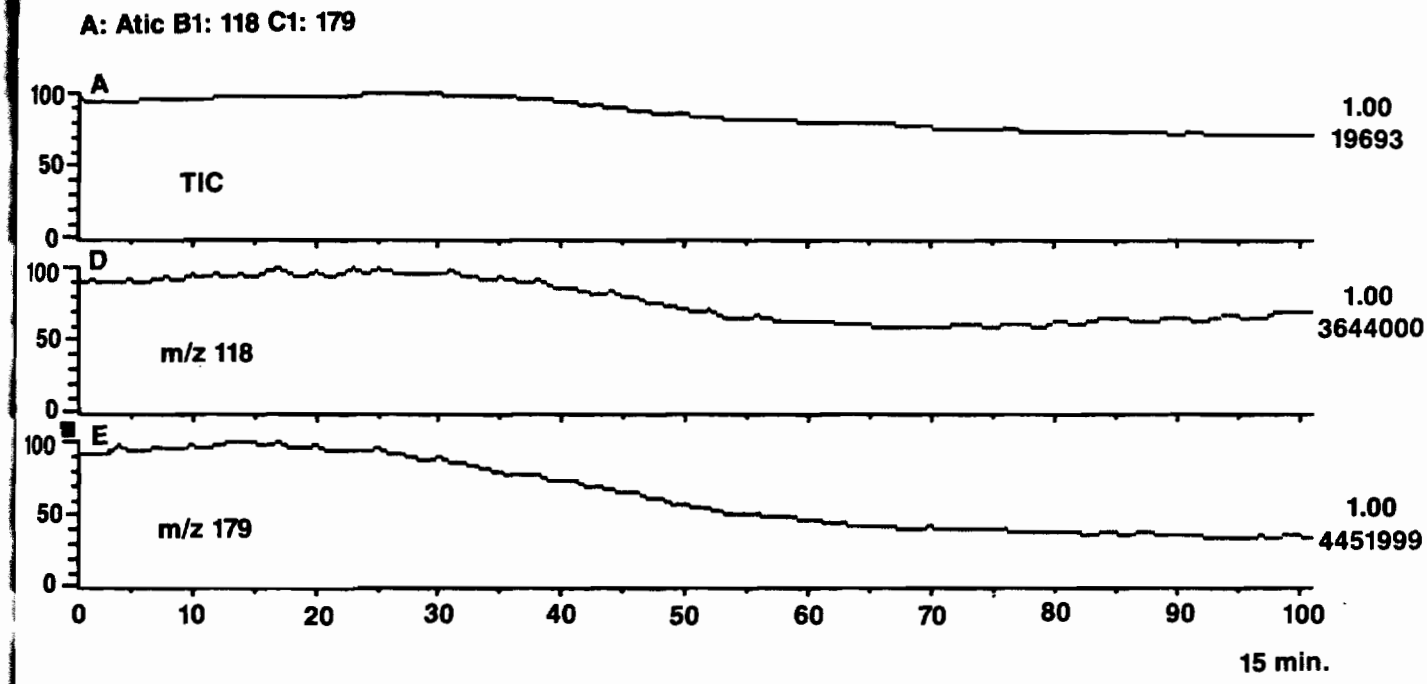


Figure 12. N-Betaine and Arsenobetaine Molecular Ion Intensity Over 15 Minutes of FAB-MS Analysis.

MEDIUM RESOLUTION FAB-MS

In order to confirm the presence of arsenobetaine in real samples, accurate mass determinations were performed. Quantitative calibration was also established to determine whether better quantification might be possible at higher mass resolution.

A. GENERAL METHODS

Part of the data presented in this section were acquired in the full scanning mode of the VG7070E-HF mass spectrometer, scanning from m/z 300 to m/z 70 at 2 sec/decade using an exponential down scan law. Mass calibration was performed using an edited glycerol reference file (Figure 11). The mass spectrometer was tuned to a resolution of approximately 5,000 (10% valley definition); with attention to minimizing any change in sensitivity.

The remaining data presented here were acquired by an alternate method of accurate mass determination. The instrument was operated by scanning the accelerating voltage and electric sector (KVE scan) while keeping the magnetic field fixed. By collecting continuum, time-based data and using glycerol reference peaks at m/z 185.1025 and 167.0919, accurate mass determinations were possible. This technique is a method of software peak matching called 'simple accurate mass' or 'SAM'.

Eight samples of edible fish and shrimp known to have been caught in Southern California waters were purchased from a fish market in Los Angeles and extracted according to the lipid method of Bligh-Dyer. The methanol phase was filtered 3X and concentrated to 100 μl. This concentration required several days of evaporation using Kuderna-Danish apparatus and a light stream of nitrogen. No ion exchange or TLC was performed. The extracts were first analyzed at baseline resolution (1,200) by Dr. Douglas Barofsky and Lorne Isabelle on the VG 7070E-HF at OGC.

Subsequently, the extracts from fish samples #1, #2, and #6 were analyzed by medium resolution (R=5,000) FAB/MS using the VG 7070E at Battelle Memorial Institute in Columbus, Ohio. Both full scan and SAM data were acquired. A slightly different approach for quantification using standard addition of arsenobetaine was attempted.

B. RESULTS

Measured amounts of arsenobetaine and N-betaine (1.1 nmol) analyzed at medium resolution (R=5,000) yielded the following data:

TABLE 17. FAB/MS CALIBRATION OF ARSENOBETAINE AND N-BETAINE (1.1 nmol) AT R = 5,000

Amount Arsenobetaine	Amt As/Amt N	Area 179/Area 118
24.7 nmol	22.3	26.9
12.4	11.3	12.3
4.94	4.49	3.91
2.47	2.23	2.08
1.24	1.11	0.74

The masses measured were 179.013 (±0.005) and 118.092 (±0.004). Glycerol shows two peaks at each nominal mass, 179.095, 179.2372 and 118.059, 118.178. The exact masses of the $(M+H)^+$ ions of arsenobetaine and N-betaine are 179.0059 and 118.0868, respectively.

Full scan medium resolution FAB analysis of the TLC extracts described in the preceding section resulted in the intensity data and calculated concentrations tabulated below.

TABLE 18. MEDIUM RESOLUTION FAB/MS ANALYSIS OF COMMENCEMENT BAY TLC EXTRACTS

Sample	Amt N-betaine	Area 179.006/Area 118.07	Conc
Spike #3 5 µl	1.1 nmol	6621 / 4493	
Spike #3 5 µl	0	4630 / 3353	159 µg (12%)
Fish #1 5 µl	1.1 nmol	803 / 1671	
Fish #1 5 µl	0	760 / 1202	3.4 µg/g
11MNL 5 µl	1.1 nmol	226 / 7871	
11MNL 5 µl	0	309 / 5161	0.24 µg/g
16502 5 µl	1.1 nmol	512 / 4753	
16502 5 µl	0	520 / 3928	0.75 µg/g
Glycerol	0	230 / 108	

The Washington DEQ value for total arsenic in shellfish 11 MNL is 0.15 μg/g. They also report 0.35 μg/g total As in shellfish 16502. The m/z 179 areas in these samples are near the background level of m/z 179 in glycerol and the native N-betaine levels are high. These two effects introduce uncertainty into the accuracy of the calculated concentrations.

Some of the data acquired using the SAM method is presented below:

TABLE 19. ACCURATE MASS MEASUREMENTS OF COMMENCEMENT BAY TLC EXTRACTS

Sample	Mass measured	ppm difference from exact mass
Spike #3	179.0040	10.6
Fish #1	179.0039	11.1
11MNL	179.0051	4.5
16502	179.0038	11.7
12 μg Arsenobetaine	179.0062	1.7
" "	179.0053	3.4

No intensity data was available using this acquisition system.

The Southern California fish samples are identified here:

TABLE 20. SOUTHERN CALIFORNIA MARKET FISH EXTRACTIONS

Sample	Amount Extracted	Final Volume
#1 Scrod	105 g	100 μl
#2 Scrod	92 g	100 μl
#3 Sole	100 g	100 μl
#4 Sole	110 g	100 μl
#5 Red Snapper	90 g	100 μl
#6 Red Snapper	98 g	100 μl
#7 Shrimp	55 g	100 μl
#8 Shrimp	72 g	100 μl

Barofsky and Isabelle's analyses revealed that scrod samples #1 and #2 definitely show evidence for arsenobetaine. The mass peaks at m/z 135 and 179 were strong and easily reproducible. No evidence for arsenocholine was observed.

Sole samples #3 and #4 and Red Snapper #5 gave no evidence for either arsenobetaine or arsenocholine.

Red Snapper #6 gave ambiguous evidence for arsenobetaine and none for arsenocholine. The occurrence of m/z 135 is strong and continuous but that of m/z 179 is sporadic and not well correlated.

The shrimp samples yielded no evidence for either organoarsenic.

Based on Barofsky and Isabelle's preliminary survey of the samples, quantitative analyses of #1, #2, and #6 was attempted. These data are presented below:

TABLE 21. FAB/MS ANALYSIS OF SOUTHERN CALIFORNIA FISH EXTRACTS WITH STANDARD ADDITION OF ARSENOBETAINE

Sample	Amt As added	Area 179	Area 135	d179	d135
Scrod #1	0	45979	-		
Scrod #1	0.3 μg	88331	-	42352	-
Scrod #2	0	5693	8923		
Scrod #2	0.3 μg	15242	20729	9549	11806
Red Snapper #6	0	5859	7841		
Red Snapper #6	0.3 μg	11441	18862	5582	11021
Arsenobetaine	0.3 μg	10989	-		
Arsenobetaine	0.3 μg	7750	11858		
"	"	8118	12339		
"	"	7502	11103		
Glycerol	0	420	970		

These values, like all previous data, represent the average peak areas calculated for at least 20 scans. 'Magic bullet', a matrix of dithiothreitol and dithioerythritol (5:1), was used for Scrod #1 and the first arsenobetaine standard. This matrix unfortunately gives a strong m/z 135 peak and therefore was not the best choice for analysis of these compounds. In analogy to the case when standard additions of N-betaine are used, calculations are based on the difference between areas at m/z 179, i.e, the difference between the areas with and without the addition of 0.3 μg arsenobetaine. The arsenobetaine standards gave very reproducible values for the areas at m/z 179 and 135 but were not used for the calculations. In each case, 2 μl of each 100 μl extract was analyzed.

The arsenobetaine content of the extracts were determined to be:

TABLE 22. CALCULATED CONCENTRATIONS OF ARSENOBETAINE IN SOUTHERN CALIFORNIA FISH

Scrod #1	0.14 μg/g
Scrod #2	0.25 μg/g
Red Snapper #6	0.14 μg/g

Simple accurate mass determinations gave the following:

TABLE 23. ACCURATE MASS MEASUREMENTS OF SOUTHERN CALIFORNIA FISH EXTRACTS

Sample	Accurate Mass Measured	ppm Difference from Exact Mass
Scrod #1	179.0038	11.7
Scrod #2	179.0050	5.03
Red Snapper #6	179.0041	10.0
Arsenobetaine Std.	179.0045	7.82

Examples of the accurate mass spectra are shown in Figure 13.

CONCLUSIONS

FAB-MS provides a semi-quantitative method for the determination of arsenobetaine in fish tissue. The values found for arsenobetaine agree rather well with the values reported by Lawrence, et.al. (28). It would be best to use ^{13}C or As labelled internal standards to achieve better FAB quantitative results. Medium resolution FAB provides excellent calibration and accurate mass data which confirms the presence of the quaternary organoarsenic. Sensitivity and sample prep limitations might be overcome with MS-MS analysis.

The data from the fish of Commencement Bay and Southern California do not differ significantly. Both show low ppm or high ppb levels of arsenic and organoarsenics. This may lend support to the hypothesis that organoarsenic

production is most dependent on the phosphate:arsenic ratio rather than only arsenic concentration since Southern California and other warmer waters are known to be relatively low in phosphate.

The use of a glycerol matrix for accurate mass FAB/MS measurements proved to be very useful and convenient. Other methods, which suggest the use of other mass markers, do not result in significantly more accurate mass measurement (28).

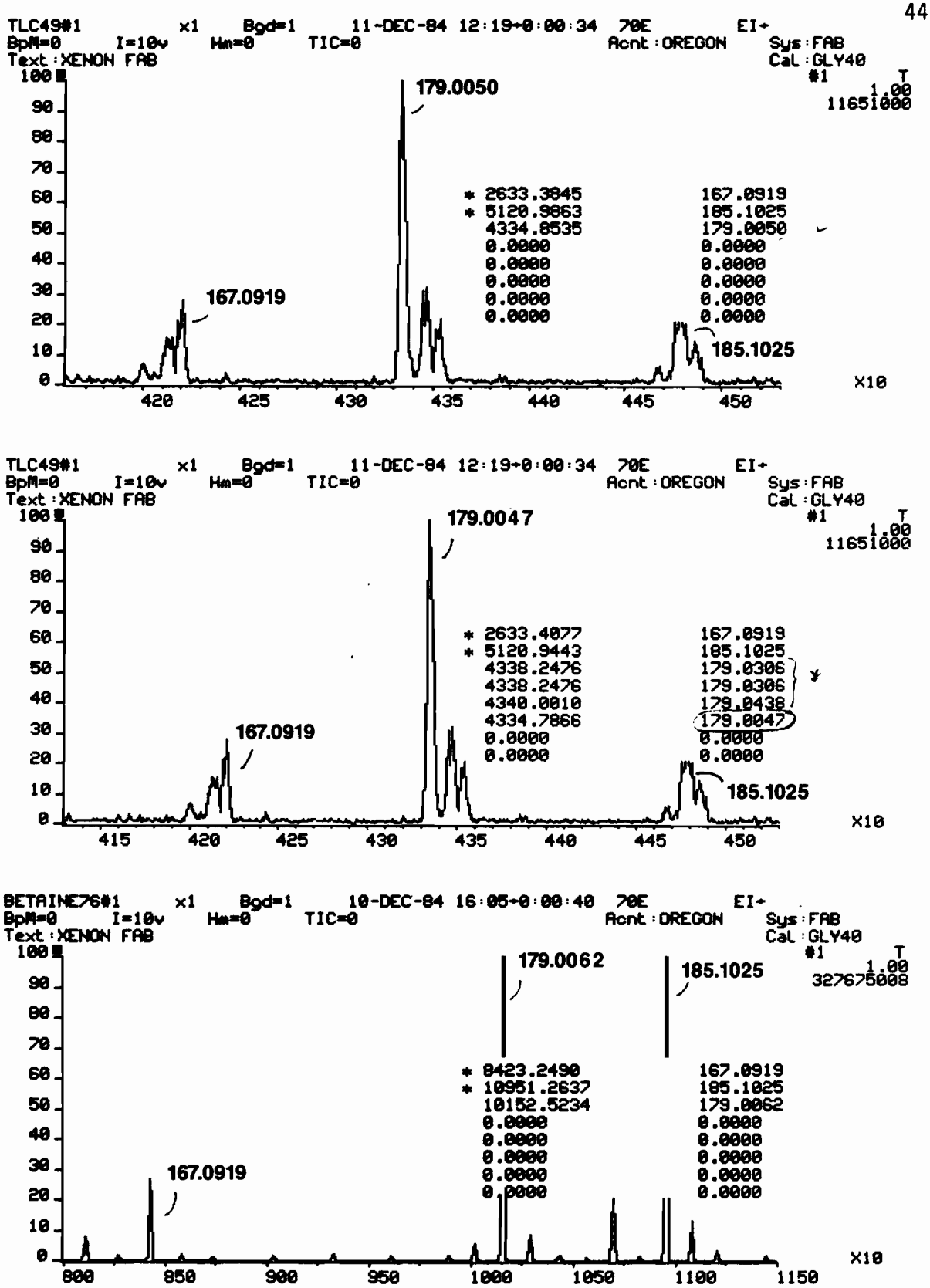


Figure 13. Simple Accurate Mass Spectra.

REFERENCES

1) Shunichi, Hariguchi, et. al., An attempt at comparative daily intake of several metals from foods in thirty countries in world, Osaka City Med.J., 24, 237 (1978) in Chemical Abstracts 92: 16530h, (1980).

2) Brinkman, F. E., and Bellama, J. M., Book, Organometals and Organometalloids, ACS Symp Ser No. 82, 1978.

3) Zingaro, R. A. in Book, Arsenic, Ch. 24, p 327-47, Proceedings of the Arsenic Symposium, edited by W. H. Lederer and R. J. Fensterheim, Van Nostrand Reinhold Environmental Series #33, 1983.

4) Wagemann, R., Some theoretical aspects of stability and solubility of inorganic arsenic in the freshwater environmen. Water Resources, **12**, 139 (1978).

5) Andreae, M. O., Distribution and speciation of arsenic in natural waters and some marine algae. Deep Sea Research, **25**, 391 (1978).

6) Crecelius, E. A., et. al., Geochemistries of arsenic, antimony, mercury, and related elements in sediments of Puget Sound. Environ. Sci. Technol., **9**, 325 (1975).

7) Andreae, M. O. and Klumpp, O., Biosynthesis and release of organoarsenic compounds by marine algae. Environ. Sci. Technol., **13**, 738 (1979).

8) Lunde, G., The analysis of arsenic in the lipid phase of marine limnetic algae. Acta Chem. Scand., **26**, 2642 (1972).

9) Irgolic, K. J., et. al., Characterization of arsenic compounds formed by 'Daphnia Magna' and 'Tetraselmis Chui' from inorganic arsenate. Environ. Health Persp., **19**, 61 (1977).

10) Cooney, R. V., et. al., Arsonium phospholipid in algae. Proc. Natl. Acad. Sci. USA, **75**, 4262 (1978).

11) Edmonds, J. S., Francesconi, K. A., et. al., Isolation, crystallization, crystal structure and synthesis of arsenobetaine, the arsenical constituent of the western rock lobster George. Tetrahedron Lett., **18**, 1543 (1977).

12) Edmonds, J. S., and Francesconi, K. A., Arseno-sugars from brown kelp as intermediates in cycling of arsenic in a marine ecosystem. Nature, **289**, 602 (1981).

13) Oladimeh, A. A., et. al., Metabolism of inorganic arsenic to organoarsenicals in rainbow trout. Ecotoxicology Environ. Safety, **13**, 394 (1978).

REFERENCES CONTINUED

14) Penrose, W. R., et. al., Implications of inorganic/organic interconversions on fluxes of arsenic in marine food webs. Environ. Health Persp., **19**, 53 (1977).

15) Sorensen, E. M. B., et. al., Arsenic accumulation, tissue distribution and cytotoxicity in teleosts following indirect aqueous exposure. Bull. Environ. Contam. Toxicl., **21**, 162 (1979).

16) Cooney, R. V., and Benson, A. A., Arsenic metabolism in Homarus Americanus. Chemosphere, **9**, 335 (1980).

17) Wrench, J., and Addison, J., Marine Pollution Bull., **10**, 18 (1979).

18) Cox, H. E., Analyst (London), **50**, 3 (1925).

19) Chapman, A. C., Analyst (London), **51**, 548 (1926).

20) Coulson, E. J., et. al., J. Nutr., **10**, 255 (1935).

21) Lunde, G., Separation and analysis of organic-bound and inorganic arsenic in marine organisms. J. Agriculture and Food Science, **24**, 1021 (1973).

22) Andreae, M. O., Determination of arsenic species in natural waters. Anal. Chem., **49**, 820 (1977).

23) Challenger, F., Biological methylation. Chem Rev., **36**, 315 (1945).

24) Cheng, C. N. and Focht, D. D., Applied and Environ. Microbiology, **38**, 494 (1979).

25) Norin, H. and Christakopoulos, A., Evidence for the presence of arsenobetaine and another organoarsenical in shrimps. Chemosphere, **11**, 287 (1982).

26) Norin, H., Ryhage, R., Christakopolous, A., and Sandstrom, M., New evidence for the presence of arsenocholine in shrimps by use of pyrolysis gas chromatography-atomic absorption spectrometry/mass spectrometry. Chemosphere, **12**, 299 (1983).

27) Luten, J. B. and Riekwel-Booy, G., Identification of arsenobetaine in sole, Lemon Sole, Flounder, Dab, Crab and Shrimps by Field Desorption and Fast Atom Bombardment Mass Spectrometry. Chemosphere, **12**, 131 (1983).

28) Lawrence, J. F., Michalik, P., Tam, G., Conacher, H. B. S., Identification of arsenobetaine and arsenocholine in Canadian fish and shellfish by high performance liquid chromatograph with atomic absorption detection and confirmation by fast atom bombardment mass spectrometry. J. Agric. Food Chem., **34**, 315 (1986).

REFERENCES CONTINUED

29) Barber, M., Bordoli, R. D., Sedgwick, R. D., and Tyler, A. N., J. Chem. Spc. Chem. Commun., **7**, 325 (1981).

30) Caprioli, R. M., Fast atom bombardment mass spectrometry for determination of dissociation constants of weak acids in solution. Anal. Chem., **55**, 2387 (1983), and references therein.

31) Riley, T. L., et.al., Quantitative determination of paint additives with fast atom bombardment mass spectrometry. Anal. Chem., **56**, 2145 (1984), and references therein.

32) Day, R. J., Unger, S. E., and Cooks, R. G., Anal. Chem., **52**, 557A (1980).

33) Bligh, E. G., and Dyer, D. J., Lipid extraction, Can. J. Biochem. Physiol., **37**, 911 (1959).

APPENDIX A

BLIGH-DYER LIPID EXTRACTION AND ISOLATION OF ORGANOARSENICS

GENERAL SAMPLE PREPARATION STEPS:

Bligh-Dyer extraction of water soluble organoarsenics

Concentration - FAB/MS analysis

Ion Exchange - FAB/MS analysis

Thin Layer Chromatography - FAB/MS analysis

BLIGH-DYER EXTRACTION SUMMARY:

The wet tissue is homogenized with a mixture of chloroform and methanol in such proportions that a miscible system is formed with the water in the tissue. Dilution with chloroform and water separates the homogenate into two layers, the chloroform layer containing all the lipids and the methanol/water layer containing all the non-lipids.

BLIGH-DYER PROCEDURE:

The following procedure applies to tissues that contain 80+/-1% water and about 1% lipid. It is imperative that the volumes of chloroform, methanol, and water, before and after dilution, be kept in the proportions 1:2:0.8 and 2:2:1.8, respectively.

1) Each 100 gram sample is homogenized in a blender or other more rigorous system for 2 to 3 minutes with a mixture of 100 ml chloroform and 200 ml methanol.
2) 100 ml of chloroform is added to the mixture and the blending is repeated for another 2 to 3 minutes.
3) 100 ml of water is added and the mixture is blended again for 2 to 3 minutes.
4) The entire mixture is filtered through Whatman #1 paper on a Buchner funnel with slight suction. The filtrate is collected and allowed to separate.
5) The upper layer of methanol/water is collected for further isolation and concentration of the compounds of interest.

Appropriate adjustments can be made to the volumes of solvent when the tissue does not contain 80% water. No such adjustments, however, were found to be necessary with the fish samples that were extracted in these experiments.

ISOLATION AND CONCENTRATION FOLLOWING EXTRACTION:

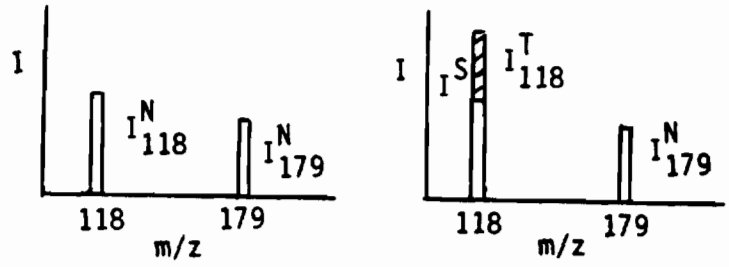
The methanol/water extracts which contain the organoarsenics are concentrated for FAB/MS analysis using Kuderna-Danish glassware over a water bath at 40-80°C followed by gentle heat and a light stream of nitrogen, or acidified to pH 3 with HCl and passed through a cation or mixed ion exchange column (Amberlite IRA-400, 20-50 mesh, OH form, Amberlite IRC-50, H form) to isolate the organoarsenics of interest. Elution from the ion exchange columns was performed with 2% NH_4OH. The elutes were collected and concentrated for FAB/MS analysis, or thin layer chromatography on aluminum backed silica plates followed by FAB/MS analysis, as described in the text.

APPENDIX B

DERIVATION OF FORMULA FOR CALCULATION OF ARSENOBETAINE CONCENTRATION WITH N-BETAINE AS INTERNAL STANDARD

Area 179/Area 118 X Amount N-betaine/Amount arsenobetaine = 1.0.

For the following:



I (N) 118 = intensity (area) of peak at m/z 118 in sample
I (S) 118 = increase in intensity of peak at m/z 118 due to spike
I (T) 118 = total intensity (area) of peak at m/z 118

Assuming:

I (N) 118 = amount of N-betaine in sample = [native] (nmol)
I (T) 118 = amount of N-betaine in spiked sample
I (S) 118 = amount of N-betaine used to spike = [spike] (nmol)

Therefore:

$$I(T) / I(N) = I(N) + I(S) / I(N) = [native] + [spike] / [native]$$
$$= 1 + [[spike] / [[native]]$$

And: $[native] = [spike] (I(T)/I(N) - 1)exp-1 = [spike] I(N) / [I(T)-I(N)]$

Rewriting the expression for Rf = 1 yields:
Amount of Arsenobetaine (nmol) = [179] = [native] I(N) 179 / I (N) 118 =
[I (N) 179 / I (T) 118 - I (N) 118] X [spike]

In order to estimate [179], it is necessary to measure the background N-betaine, I (N) 118. If I (N) 118 = 0, then:
[179] = [I (N) 179 / I (T) 118] X [spike]

The formula for calculating the concentration of arsenobetaine in the samples is given by:

[conc] = [[179] X 179(MW)] X VC / (VA X M)
where: VC = Volume of Concentrate (μl)
VA = Volume Analyzed (μl)
M = Mass Extracted (grams)

BIOGRAPHICAL NOTE

The author was born the proud son of Paul and Joann Zimmerman on November 21, 1957, in Columbus, Ohio. He attended grammar school and high school in Clyde, Ohio, where he graduated in 1976. He then entered Ashland College in Ashland, Ohio, and received his Bachelor of Science degree in June, 1980.

In August, 1980, through December, 1982, the author worked for O. H. Materials Co. in Findlay, Ohio.

The author then began study at the Oregon Graduate Center in January, 1982. The requirements for the degree Master of Science were completed in August, 1988.

The author left the Graduate Center to work for Montgomery Laboratories in Pasadena, California. He is currently employed by Battelle Memorial Institute in Columbus, Ohio. He is married to the former Linda Fedorovich and they have two children, Kristen, age 8, and Sarah, age 2.