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# Water-soluble arsenic residues from several arsenolipids occurring in the tissues of the starspotted shark *Musterus manazo*†

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**Alkali-labile and alkali-stable arsenolipid fractions were prepared from 12 tissues of the starspotted shark *Musterus manazo* and analyzed by high-performance liquid chromatography–inductively coupled mass spectrometry. At least six arsenolipids were found in the shark. Two major alkali-labile arsenolipids (a dimethylated arsenic-containing lipid and an arsenocholine-containing lipid) were shown in ordinary muscle, dark muscle, heart, bone, skin and stomach, whereas a single major arsenolipid, the dimethylated arsenic-containing lipid, was shown in the intestine, liver, kidney, spleen and brain. Besides these lipids, four other minor alkali-labile arsenolipids were present. On the other hand, as for the alkali-stable arsenolipids, a dimethylated arsenic-containing lipid and an arsenocholine-containing lipid were also found in dark muscle, skin, stomach and intestine, whereas only dimethylated arsenic-containing lipid was found in the liver. Copyright © 2001 John Wiley & Sons, Ltd.**

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## INTRODUCTION

The occurrence of both lipid-soluble and water-soluble arsenic compounds in marine organisms was reported for the first time by Lunde in 1968.[1] Water-soluble arsenic compounds were shown to have many structures and to be widely distributed in the marine environment; since the isolation and identification of arsenobetaine in the tail muscle of western rock lobster,[2] many kinds of arsenicals have been identified in various animals and algae and reviewed by various authors.[3–7] On the other hand, relatively little is known about lipid-soluble arsenic compounds, mostly because of the limited amounts of these compounds and the difficulty of isolating them. However, the investigation of the structure of the lipid-soluble arsenic compounds is important for the elucidation of arsenic circulation in marine ecosystems.

Recently, we investigated arsenolipids in a demersal shark, *Musterus manazo*, in which almost all the water-soluble arsenical is accumulated as arsenobetaine,[8,9] and reported that its tissues can be classified into three types:[10] (1) tissues mainly containing alkali-labile arsenic compounds; (2) those mainly containing alkali-stable compounds; (3) those containing both types of arsenic compound. Furthermore, our data suggested the presence of an arsenocholine-containing lipid in the muscle and a dimethylated arsenic-containing lipid in the liver.

In this study, with mild alkaline hydrolysis, the alkali-labile and alkali-stable arsenolipid fractions were prepared from 12 tissues of *M. manazo* and analyzed by high-performance liquid chromatography–inductively coupled mass spectrometry (HPLC–ICP MS). Some fractions were further

analyzed after severe acid- or alkali-hydrolysis. The results obtained from these experiments suggest the presence of several kinds of arsenolipid in the shark. This is in agreement with the expected diversity of arsenolipids in marine ecosystems.

## MATERIALS AND METHODS

### Authentic arsenic compounds

Arsenobetaine (AB), arsenocholine (AC), trimethylarsine oxide (TMAO), and tetramethylarsonium iodide (TMAI) were purchased from Trichemical Co.; dimethylarsinic acid (DMAA) was from Nakarai Chemical Co.; methanarsonic acid (MMA) was from Ventron Co.; disodium arsenate was from Wako Pure Chemical Co.; arsenic trioxide was from Mallinckrodt Co.

### Tissues

The tissues used [ordinary muscle (130 g), dark muscle (191.5 g), stomach (104.5 g), heart (6.9 g), gall bladder (2.6 g), intestine (60.0 g), skin (211.5 g), spleen (15.0 g), brain (8.5 g), liver (290.0 g), kidney (26.0 g) and bone (114.5 g)] from five fresh starspotted sharks (average weight 1300 g) were obtained fresh from a market.

### Extraction of lipid-soluble arsenic compounds

Each sample was minced with a knife and extracted twice with ten volumes of chloroform/methanol (2:1). Water was then added to reach a water/chloroform–methanol ratio of 1:4. After shaking for 2 min, the mixture was allowed to stand overnight.[11] The arsenic compounds that separated into the chloroform (lower) layer were referred to as lipid-soluble arsenic compounds.

### Preparation of polar lipid fractions

The lipid-soluble arsenic compound fraction was fractionated into polar and neutral lipid fractions as follows: after drying with a vacuum evaporator, the dried lipid-soluble arsenic compound fraction was dissolved in ten times its weight of chloroform and mixed with five times its weight of silicic acid (Mallincrokdt, 100 mesh) in a beaker. After filtration with No. 2 filter paper on a No. 3 glass-filter, the silicic acid on the paper was washed six times with five times its weight of chloroform. The chloroform filtrates were gathered and concentrated as the neutral lipid-soluble arsenic compound fraction. The silicic acid on the paper was then washed four times with methanol ten times the weight of the dried lipid-soluble arsenic compound fraction.[10] The methanol filtrates were gathered and concentrated as the polar lipid-soluble arsenic compound fraction.

### Partial hydrolysis of lipid-soluble arsenic compounds

According to Dawson's method,[12] the polar lipid-soluble arsenic compound fraction extracted from each tissue was subjected to mild alkaline hydrolysis: the lipids in the fraction of each tissue were incubated in 0.027 mol $dm^{-3}$ sodium hydroxide for 20 min at 37 °C to prepare alkali-labile and alkali-stable fractions. 5 $cm^3$ of water (alkali-labile fraction) or chloroform (alkali-stable fraction) was added to each fraction after it had been dried.

For severe hydrolysis of the water-soluble arsenic residues derived from alkali-labile arsenolipids, an alkali-labile fraction was separated by HPLC using a Nucleosil 100 SA column under the same conditions as described below, and each fraction containing arsenic was collected six times and dried. To each residue were added 0.25 $cm^3$ of water and 0.25 $cm^3$ of conc. HCl and this was heated in boiling water for 1 h. After the hydrolysate was neutralized with 3 M sodium hydroxide, water was added to a volume of 2 $cm^3$ (alkali-labile/HCl fraction). For severe hydrolysis of the alkali-stable arsenolipids in the alkali-stable fraction the mixture was further hydrolyzed with saturated barium hydroxide under reflux for 5 h [alkali-stable/$Ba(OH)_2$ fraction].[13]

These fractions were stored at −30 °C until used.

### Arsenic determination

Arsenic was determined by hydride generation–quartz furnace atomic absorption spectrometry after the tissue samples were digested with a mixture of nitric, sulfuric and perchloric acids as described previously.[14] For the lipid-soluble fraction, an aliquot of each fraction was saponified (100 °C, 10 min) with 12.5 volumes of 2.4 mol $dm^{-3}$ ethanolic potassium hydroxide before the digestion.

### HPLC–ICP MS

A Tosoh CCP 8000-series chromatograph (Tosoh

Co.) was used for the chromatographic separation. AB, TMAO, AC and TMAI were separated from each other at a flow rate of 1.0 $cm^3 min^{-1}$ on a Nucleosil 100 SA cation-exchange column (250 mm × 4.6 mm i.d., Wako Pure Chemical Co.) with a 0.1 mol $dm^{-3}$ pyridine–formic acid buffer (pH 3.1). On the other hand, arsenite, arsenate, MMA and DMAA were separated from each other at a flow rate of 1.5 $cm^3 min^{-1}$ on a Nucleosil 100 SB anion-exchange column (250 mm × 4.6 mm i.d., Wako Pure Chemical Co.) with a 0.02 mol $dm^{-3}$ phosphate buffer (pH 6.8). The outlet of the column was connected to a concentric type A nebulizer. A HP 4500 (Yokogawa Analytical, Tokyo, Japan) inductively coupled plasma mass spectrometer served as an arsenic-specific detector. Twenty $mm^3$ of each sample was injected onto the column directly, except for the alkali-labile/HCl fraction which was neutralized before the injection.

The ion intensities at *m/z* 77 ($^{40}Ar^{37}Cl$, $^{77}Se$) and *m/z* 82 ($^{82}Se$) were monitored to detect possible interferences on *m/z* 75.

## Thin layer chromatography

Thin layer chromatography was performed on a cellulose thin layer (Avicel SF, thickness: 0.1 mm, Funakoshi Yakuhin Co., Ltd). In order to confirm the position of the fractionated arsenic compound, the cellulose thin layer was removed at 5 mm intervals. Each of the samples removed was added to a portion of 20% ethanol, mixed with a vortex mixer for 20 s and analyzed by graphite furnace atomic absorption spectrometry. Dragendorff reagent[15] was used to authenticate AC (Trichemical Co. Ltd).

# RESULTS

## Total arsenic in each tissue

The total arsenic concentration ($\mu g\ g^{-1}$ polar lipid) in each fraction has already been reported in a previous paper (ordinary muscle: alkali-labile fraction 8.4, alkali-stable fraction 0.3; dark muscle: 3.3, 3.5; stomach: not detected, 3.1; heart: not detected, 0.8; gall bladder: 9.5, 57.2; intestine: 3.8, 6.0; skin: 5.6, 6.7; spleen: 4.6, 4.7; brain: 5.5, not detected; liver: 22.4, 209.7; kidney: 30.1, 2.0; bone: 2.1, 2.2).[10]

## HPLC–ICP MS chromatograms of alkali-labile fractions

Each alkali-labile fraction from 12 tissues was analyzed by HPLC–ICP MS (Fig. 1). On the whole, two major peaks (peaks 1 and 2) and at least two minor ones (peaks 3 and 4) were found. The occurrence of these four compounds was clearly demonstrated in ordinary muscle, dark muscle, heart, bone, skin and stomach. On the other hand, a single major peak (peak 1) was found in the remaining organs (intestine, liver, kidney, spleen and brain), except for the gall bladder. Especially in the kidney, spleen and brain, almost all of the arsenic in this fraction was detected as peak 1. In other words, soft tissues had one major alkali-labile arsenolipid, whereas the muscles (white and dark muscles) and mellow tissues (stomach, skin and intestine) had two.

The gall bladder, in that it had a few more peaks near peak 1 and 2, showed a considerably different chromatogram from other tissues.

## HPLC–ICP MS chromatograms of the derivatives from peaks 1, 2 and 4

Each of the three compounds that appeared as peak 1, peak 2 and peak 4 in the alkali-labile fraction prepared from the ordinary muscle was separately subjected to severe acid-hydrolysis (alkali-labile/HCl fraction). The derived water-soluble arsenic compound in the alkali-labile/HCl fraction from each peak was analyzed by HPLC–ICP MS. Figure 2 shows the chromatograms on Nucleosil 100 SA. AC was derived from the peak 2 compound, whereas peak 4 was resistant to the hydrolysis. The retention time of the compound derived from the peak 1 compound agreed with that of DMAA. However, because the retention time of DMAA overlaps with MMA in this column, this compound was further confirmed as DMAA by chromatography on Nucleosil 100 SB (data not shown).

## Confirmation of the derivatives from peaks 1 and 2

The alkali-labile/HCl fraction from the peak 1 or peak 2 compounds was subjected to thin layer chromatography with authentic arsenic compounds. The $R_f$ value of the arsenic compound in the former agreed with that of DMAA and that in the latter agreed with that of arsenocholine (Table 1).

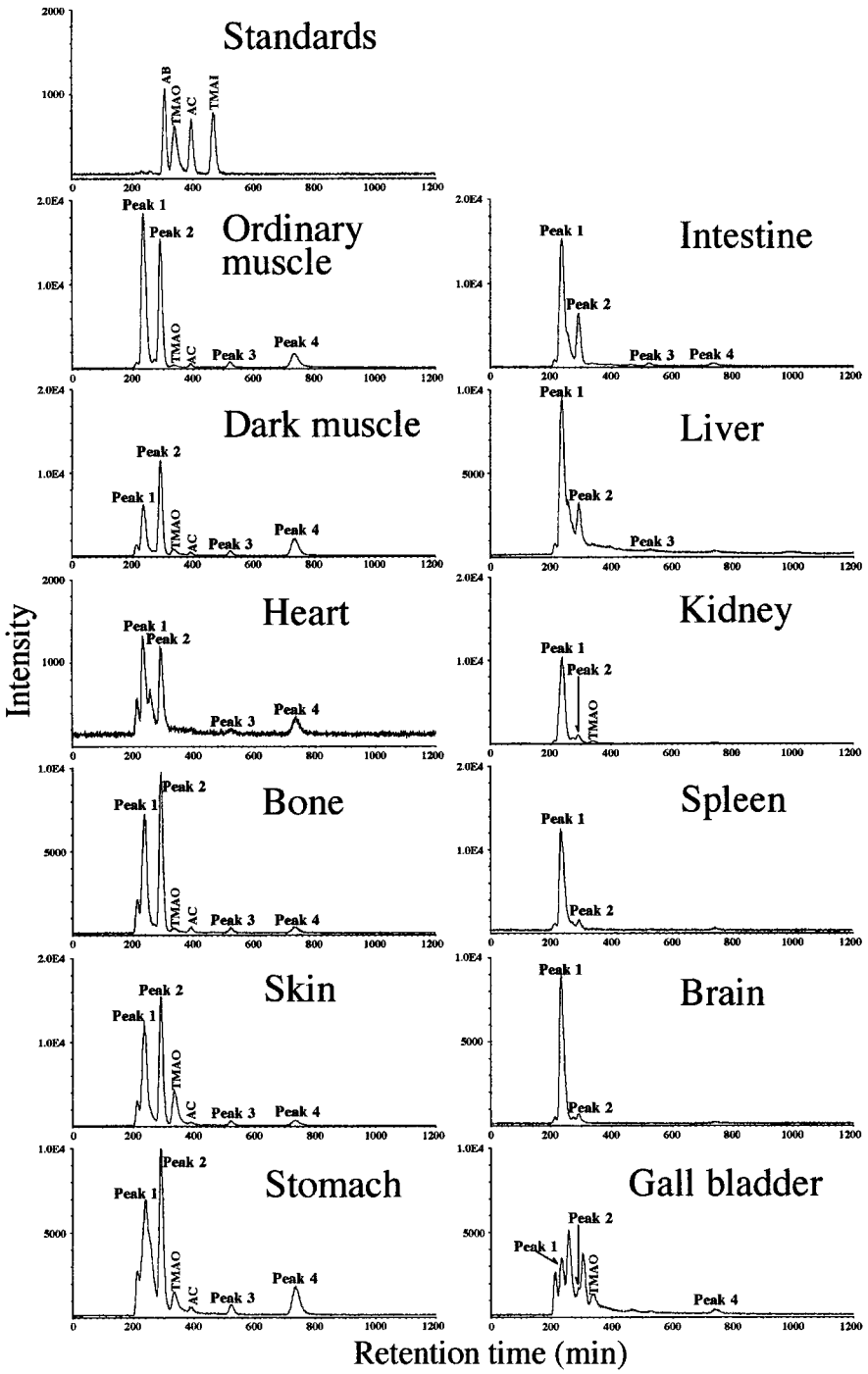


**Figure 1** HPLC–ICP MS qualitative chromatograms (Nucleosil 100 SA) of authentic arsenicals (AB: arsenobetaine, TMAO: trimethylarsine oxide, AC: arsenocholine, TMAO: tetramethylarsonium ion) and the alkali-labile fractions prepared from 12 tissues of the starspotted shark *M. manazo*. Experimental conditions were as described in the text.

 

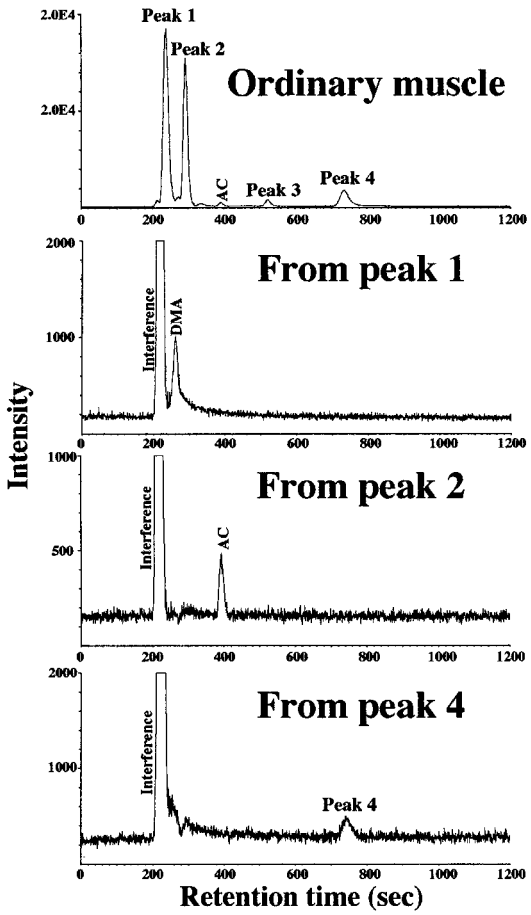


**Figure 2** HPLC–ICP MS qualitative chromatograms (Nucleosil 100 SA) of the water-soluble arsenic residues obtained with severe acid hydrolysis of the peak 1 compound, peak 2 compound or peak 4 compound prepared from the ordinary muscle of the starspotted shark *M. manazo* (Fig. 1). For abbreviations see Fig. 1. Experimental conditions were as described in the text.

## HPLC–ICP MS chromatograms of water-soluble residues derived from alkali-stable fractions

Each water-soluble arsenic residue in the alkali-stable/$Ba(OH)_2$ fraction was analyzed by HPLC–ICP MS. Although all 12 hydrolysates were analyzed by HPLC–ICP MS, those of low arsenic content did not show a clear separation of each derived arsenical. Figure 3 shows only the results for the tissues that showed a clear separation.

A few arsenic compounds were detected in the hydrolysates (Fig. 3). The major arsenicals were shown to be DMAA and AC: these two compounds were detected in all of the hydrolysates except for the liver hydrolysate, in which only DMAA was detected. The compound referred to as DMAA in the figure was confirmed as DMAA by the chromatography on Nucleosil 100 SB (data not shown).

## Confirmation of the water-soluble residues derived from alkali-stable fractions

Arsenicals in the alkali-stable/$Ba(OH)_2$ fraction from the stomach were separated by HPLC and the fractions containing the peaks referred to as DMAA or AC were collected and analyzed by thin layer chromatography. The $R_f$ value of the arsenic compound referred to as DMAA agreed with that of DMAA and that referred to as AC agreed with that of AC (Table 1).

# DISCUSSION

## Alkali-labile arsenolipids

The presence of at least four alkali-labile arsenolipids is suggested in this study. They are discussed below.

### Peak 1

The DMAA-containing residue (peak 1) was clearly demonstrated as a major peak in all of the tissues of *M. manazo* investigated: an alkali-labile DMAA-containing arsenolipid is thought to occur ubiquitously in *M. manazo*.

The structure of the lipid is expected to be analogous to that of phosphatidylcholine for the following two reasons: (1) a water-soluble arsenic residue (peak 1) was derived with a mild alkaline hydrolysis and (2) DMAA was derived from the water-soluble arsenic residue with a severe hydrolysis with HCl. Arsenosugars, which are mainly dimethylated arsenic compounds, have been confirmed in various algae as water-soluble arsenicals.[3–7] However, at the present stage, it is rather hard to expect in shark, an animal of the highest trophic level, the presence of arsenosugar-containing glycerophospholipid analogous to that reported in the brown algae *Undaria pinnatifida*.[16]

### Peak 2

An AC-containing arsenic residue was shown to account for another major peak (peak 2) in some

**Table 1** $R^f$ values in thin layer chromatography of the water-soluble arsenic residues derived with severe hydrolysis

| Sample | $R_f$ value in solvent system[a] | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| Alkali-labile/HCl fraction | | | | | |
| from peak 1 | 0.86 | 0.80 | 0.68 | 0.27 | 0.77 |
| from peak 2 | 0.81 | 0.92 | 0.69 | 0.63 | 0.80 |
| Collected fraction | | | | | |
| containing first As peak[b] | 0.87 | 0.81 | 0.68 | 0.30 | 0.79 |
| containing second As peak[c] | 0.81 | 0.91 | 0.68 | 0.62 | 0.82 |
| DMAA | 0.86 | 0.81 | 0.69 | 0.28 | 0.79 |
| AC | 0.81 | 0.91 | 0.69 | 0.62 | 0.81 |

[a] Solvent systems: (1) ethyl acetate/acetic acid/water (3:2:1); (2) chloroform/methanol/28% aq. ammonia (2:2:1); (3) 1-butanol/acetone/formic acid/water (10:10:25); (4) 1-butanol/acetone/28% aq. ammonia/water (10:10:2:5); (5) 1-butanol/acetic acid/water (4:2:1).
[b] The peak referred to as DMAA in Fig. 2.
[c] The peak referred to as AC in Fig. 2.

tissues, i.e. muscles (ordinary muscle and dark muscle), heart, bone, skin and stomach. Thus, these tissues have two major arsenolipids, those containing DMAA containing and those containing AC. The AC-containing lipid, however, was only a minor compound in the spleen, brain and kidney.

As for ordinary muscle from *M. manazo*, we already expected the occurrence of phosphatidylarsenocholine as an alkali-labile arsenolipid because: (1) a water-soluble arsenic residue is derived with a mild alkaline hydrolysis; (2) AC was derived from the water-soluble arsenic residue with a severe hydrolysis with HCl; (3) phosphatidylarsenocholine was actually reported to be present in the muscle of yelloweye mullet following oral administration of arsenocholine.[17] Whether or not the AC-containing lipid is phosphatidylarsenocholine, it was confirmed to be present not only in ordinary muscle but also in a considerable number of other tissues in this study.

### Peak 4

Although relatively smaller than peak 1 and peak 2, peak 4 was also found mainly in the tissues in which the two major peaks were shown (Fig. 1). The arsenolipid from which the peak 4 compound was derived cannot be a glycerophospholipid, because this water-soluble residue was resistant to severe hydrolysis with HCl (Fig. 2). At present, all that is known about this compound is that it is an alkali-labile arsenolipid.

### The peaks overlapping with peak 1

In some tissues, such as heart, two additional peaks that appeared before and after peak 1 that considerably overlapped each other were found. We are now attempting to separate these two compounds and peak 1 using other HPLC conditions.

### TMAO and DMAA

In some tissues TMAO and/or AC were found. These water-soluble arsenic compounds are considered to be derived from arsenolipids with mild alkaline hydrolysis; for example, it is possible to degrade peak 2 further to produce a small amount of AC with mild alkaline hydrolysis.

The occurrence of TMAO suggested the occurrence of alkali-labile trimethylated arsenolipids in the tissues such as skin and stomach; further experiments will be needed to prove this.

## Alkali-stable arsenolipids

DMAA and AC were found in the alkali-stable/$Ba(OH)_2$ fraction in all tissues (dark muscle, skin, stomach and intestine) except for liver, in which only DMAA was detected.

The arsenolipid from which DMAA or AC was derived could not have been a glycerophospholipid, such as phosphatidylcholine, because glycerophospholipids are sensitive to mild alkaline hydrolysis. We previously reported the finding of an alkali-

 

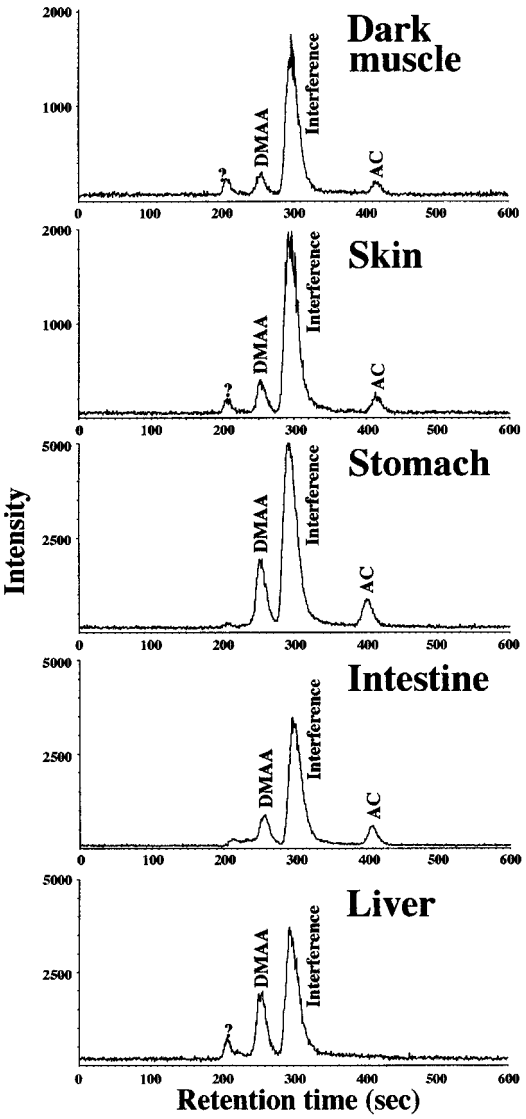


**Figure 3** HPLC–ICP MS qualitative chromatograms (Nucleosil 100 SA) of the water-soluble arsenic residues obtained with severe alkaline hydrolysis of the alkali-stable arsenic fractions prepared from five tissues of the starspotted shark *M. manazo*. For abbreviations see Fig. 1. Experimental conditions were as described in the text.

stable arsenolipid in liver that was also resistant to acid hydrolysis (3.3 mol $dm^{-3}$ trichloroacetic acid) and suggested that it was arsenosphingomyelin. DMAA- or AC-containing alkali-stable arsenolipids found in some tissues in this study were also suggested to be arsenosphingomyelin: these lipids, for example, may have a structure like sphingomyelins, in which the choline moiety is replaced with arsenocholine or a DMAA-containing water-soluble residue.

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