78/003

An Investigation of the Trace Elements Present in Bronze Whaler Sharks and Rock Lobsters

Final Report

Introduction

It can be argued that realistic health standards for trace elements in seafoods should be based on a detailed knowledge of the chemical structure and biological activity of the particular compound of the trace metal involved. Although there is no question that it is proper to be cautious in setting health standards in the absence of such information, further research may reveal that the original standard was unduly stringent.

From the work described below, this seems to be the case as far as the arsenic compound (arsenobetaine) present in the flesh of the bronze whaler shark and in the western rock lobster, is concerned.

On the other hand, we have failed to identify the compounds of selenium present in the flesh of the bronze whaler shark which may have a protective effect against poisoning by the mercury compounds which also occur in this species. In addition, with the exception of arsenobetaine, we have been unable to identify particular compounds of other trace elements, such as cadmium, which exceed the accepted limit, in the hepatopancreas of the western rock lobster. As a result, we cannot provide grounds for relaxing the health standard for mercury in the case of the bronze whaler shark; nor can we recommend that the hepatopancreas be incorporated in products derived from the offal of rock lobsters and intended for consumption by either humans or domestic stock.

The research carried out on these two topics will be summarized separately.

Compounds of Mercury and Selenium in the Bronze Whaler Shark

A comprehensive report on mercury in fish and fish products has appeared and the difficulties being faced by the Australian fishing industry over the presence of mercury compounds in seafood have been discussed.[2] The situation is particularly serious as far as the shark fishing is concerned, for the flesh of commercial species frequently contains mercury levels in excess of the accepted limit of 0.5 mg/kg. However, there is a considerable weight of opinion that unidentified naturally-occurring selenium compounds have a protective effect against mercury poisoning[1] and we sought to isolate the selenium compound(s) from the bronze whaler shark so that the biological activity of the pure substance(s) could be studied.

Unfortunately, we have failed in this undertaking. The flesh of the bronze whaler sharks we examined contained only 0.79-1.4 ppm Se but at first we were encouraged to find that a considerable proportion (32-79%, depending on the animal) could be extracted with methanol. This finding suggested

that the selenium compound extracted was a relatively simple molecule. However, attempts to fractionate the methanolic extract using methods similar to those which were successful in our work on the isolation of arsenobetaine[3] proved fruitless. In all cases the selenium was distributed between a number of fractions and the considerable losses of selenium which were observed indicated that the compound(s) was also unstable.

We have not abandoned work on this problem but the low levels of selenium compounds present in the flesh coupled with the apparent instability of these substances continue to hamper progress.

Trace Element Concentrations in the Western Rock Lobster

The average concentrations of trace elements present in the fresh tail muscle and the fresh hepatopancreas of western rock lobsters caught in Cockburn Sound, Western Australia, are set out in the Table.

(INSERT TABLE HERE)

It is clear that, with the exception of arsenic,[3] the levels of trace metals in the tail muscle are within the acceptable limits and are comparable with those of edible teleost species caught in Cockburn Sound.[4] On the other hand, much higher levels of Cd, Cu, Fe, Zn and, surprisingly, Ag, are accumulated by the hepatopancreas and the level of cadmium exceeded the acceptable limit in all of the animals examined. The wide range of levels of copper might be due to the presence of varying amounts of blood, which contains haemocyanin, in the samples but the same explanation cannot be used to rationalize other variations. The presence of significant levels of silver in the hepatopancreas is intriguing; little appears to be known concerning the rôle of silver in biological systems.

From these results it seems that the hepatopancreas acts as an efficient barrier to the accumulation of trace metals in the tail muscle and this finding will probably be welcomed by the major part of the fishery which is concerned with the production of tail muscle. However, for the same reason, it seems necessary to proceed cautiously with attempts to produce additional edible products from the offal of the western rock lobster. Admittedly, there is no information available concerning the toxicity of the particular cadmium compounds present in the hepatopancreas nor is it known if these would be absorbed by the mammalian digestive system. Nevertheless, until these questions are resolved, it seems that it would be prudent to remove the hepatopancreas from the offal before the latter is used for the preparation of edible products.

The Arsenic Compounds Present in the Hepatopancreas of the Western Rock Lobster

A bulk sample of the hepatopancreas was found to contain ca. 39 ppm As, the great majority (95%) of which was present as a water-soluble compound. By means of solvent extraction followed by extensive chromatography this substance was concentrated and finally isolated as the reineckate. This salt proved to be identical with the reineckate of synthetic arsenobetaine.[3] During this work, no other water-soluble arsenic compound was detected in the hepatopancreas.

Considerable efforts were also made to isolate the minor water-soluble arsenic compounds present in the hepatopancreas. It was thought that these might be derived from the diet of the rock lobster or might be intermediates in the pathway to arsenobetaine.

One of the main difficulties encountered in this work was that the lipid soluble arsenic compounds appeared to be unstable and losses of 70-80% were common during attempts to fractionate the extract by chromatography on such standard adsorbents as silicic acid and alumina. Other workers[5] have experienced similar difficulties in their attempts to isolate lipid-soluble arsenic compounds from cultures of marine algae. Because of this difficulty, it was necessary to use even milder methods of fractionation. Countercurrent distribution showed that at least four arsenic compounds were present in the lipid-soluble fraction. Further extensive fractionation of the most polar of these by chromatography on Sephadex LH20, DEAE Sephadex and Cellulose, as well as high performance liquid chromatography, failed to yield a pure compound. Eventually 25 mg of a viscous oil which contained 32,000 ppm As was obtained from 97 kg of hepatopancreas, but this product was still a mixture. Attempts to isolate a lipid-soluble arsenic compound are continuing.

A Preliminary Toxicological Evaluation of Arsenobetaine

Arsenobetaine has now been isolated from the tail muscle[3] of the western rock lobster as well as from the hepatopancreas and from the flesh of the bronze whaler shark.[3] Recently arsenobetaine has been identified in the flesh of another shark Prionace glaucus[6] and it seems that this substance will prove to be widely distributed in marine organisms.

It had been known for many years that the arsenic compound present in seafood was rapidly excreted by the human kidney; arsenobetaine has been isolated from human urine obtained after eating the tail muscle of the western rock lobster.[3] There does not appear to be any evidence that the presence of arsenobetaine in seafood is a health hazard and a preliminary toxicological examination of synthetic arsenobetaine has now reinforced this view.

Intraperitoneal injection of mice with a massive dose (500 mg/kg) of arsenobetaine did not result in any mortality and no symptoms of poisoning were observed. Mice treated with arsenobetaine at 360 mg/kg rapidly eliminated the compound in their excreta and no evidence of any metabolic alteration could be obtained by thin layer chromatography.

When tested in the Ames Salmonella typhimurium system for chemical mutagens,[7] both in the presence and absence of liver microsomal oxidase fraction, arsenobetaine gave consistently negative results.

From these experiments, there is again no evidence that arsenobetaine is either acutely toxic or is likely to have any long term effect.

Conclusion

So far, attempts to isolate the selenium compounds from the flesh of the bronze whaler shark have failed and, although some progress has been made, efforts to isolate and identify the lipid-soluble arsenic compounds present in the hepatopancreas of the western rock lobster have not yet been successful. On the other hand, arsenobetaine has been isolated from the hepatopancreas.

A preliminary toxicological examination of arsenobetaine has failed to reveal any biological activity, and there is no evidence that the presence of high levels of arsenobetaine in the flesh of the bronze whaler shark and in the western rock lobster represents any toxic hazard whatsoever.

Analyses of the tail muscle of the western rock lobster have shown that the levels of trace elements are well within accepted limits. On the other hand, the hepatopancreas contains a significant level of cadmium which exceeds the current health standard. For this reason, it is believed that the hepatopancreas should be removed before the offal is processed to yield edible products for either humans or domestic stock.

References

1. Report on Mercury in Fish and Fish Products. Australian Government Publishing Service, Canberra 1979.

2. Australian Fisheries, October 1980, 2.

3. J.R. Cannon, J.S. Edmonds, K.A. Francesconi, C.L. Raston, J.B. Saunders, B.W. Skelton and A.H. White, Aust. J. Chem., 1981, 84, 787.

4. D. Plaskett and I.C. Potter, Aust. J. Mar. Freshwater Res., 1979, 30, 607.

5. K.J. Irgolic, E.A. Woolson, R.A. Stockton, R.D. Newman, N.R. Bottino, R.A. Zingaro, P.C. Kearney, R.A. Pyles, S. Maeda, W.J. McShane and E.R. Cox, Environ. Health Perspectives, 1977, 19, 61.

6. S. Kurosawa, K. Yasuda, M. Taguchi, S. Yamazaki, S. Toda, M. Morita, T. Uehiro and K. Fuwa, Agric. Biol. Chem., 1980, 44, 1993.

7. B.N. Ames, J. McCann and E. Yamasaki, Mutation Res., 1975, 31, 347.

TABLE. Concentration of trace metal (µg/g)

	As	Ag	Cd	Cu	Cr,Co,Ni	Fe	Hg	Se	Zn
Fresh tail muscle									
mean ± S.D.	33.3 ± 10.4	*	*	5.2 ± 0.84	*	*	0.033 ± 0.005	0.29 ± 0.02	18.74 ± 0.60
range	27.1 - 51.4			3.8 - 5.9			0.026 - 0.040	0.25 - 0.31	17.5 - 19.9
Fresh hepato-pancreas									
mean ± S.D.	39.3 ± 15.98	9.1 ± 4.06	6.3 ± 1.88	76.9 ± 58.84	*	43.7 ± 17.03	≦ 0.02	≦ 0.02	67.8 ± 20.38
range	17.3 - 74.0	4.2 - 21.2	3.5 - 9.9	10.2 - 270.6		19.8 - 84.4			35.8 - 107.9

* not detected

FRACTIONATION OF LIPID SOLUBLE ARSENIC COMPONENTS FROM THE HEPATOPANCREAS OF THE WESTERN ROCK LOBSTER

Report by R.W. Dunlop

May 1981

RESULTS

Extraction of Hepatopancreas : The hepatopancreas of the western rock Lobster *Panulirus cygnus* George was collected from the Fisherman's Cooperative processing plant, Fremantle. From preliminary analysis,[1] there was an estimated 3.9 g of total arsenic in 100 kg of hepatopancreas. Chloroform and chloroform/methanol extraction of the blended pancreas gave respectively 34 mg and 104 mg of arsenic, at a concentration of 7.5 and 12.5 ppm; a yield of 0.0002% from the pancreas.

Fractionation of lipid soluble arsenic : The chloroform(A) and chloroform/methanol(B) extracts were each partitioned between hexane and methanol. Thirty percent of arsenic from the chloroform extract remained in the hexane fraction and subsequent partitioning (on an analytical scale) demonstrated that this arsenic cannot be extracted into methanol.

The methanol fraction of extract B was dissolved in chloroform and a precipitate formed with acetone. The precipitate contained 42% of the arsenic, but was subsequently lost. The chloroform solubles and the methanol fraction of extract A were each partitioned between hexane and methanol by counter current distribution to each yield four arsenic containing fractions (see figure 1).

The most polar fraction was chromatographed on Sephadex LH20 in methanol and then chloroform/methanol (40:60) yielding two arsenic containing fractions. DEAE Sephadex chromatography of the most retained arsenic containing fractions, precipitation from methanol of non-arsenic components followed by HPLC on a μ Bondapack C-18 reverse phase column and DEAE Sephadex chromatography (see figure 2) gave a pale yellow viscous oil containing 22,000 ppm arsenic.

Cellulose thin layer chromatography gave a pale viscous syrup containing 32,000 ppm arsenic. (I)

The second most polar fraction from counter current distribution of extract A and the least retained fraction of Sephadex LH20 chromatograms were fractionated by cadmium chloride precipitation. DEAE Sephadex chromatography of this oil (see figure 2) gave a dark yellow oil containing 2,100 ppm arsenic (II). The fractions I and II contain different arsenic species as defined by their distinct elution volumes on DEAE Sephadex.

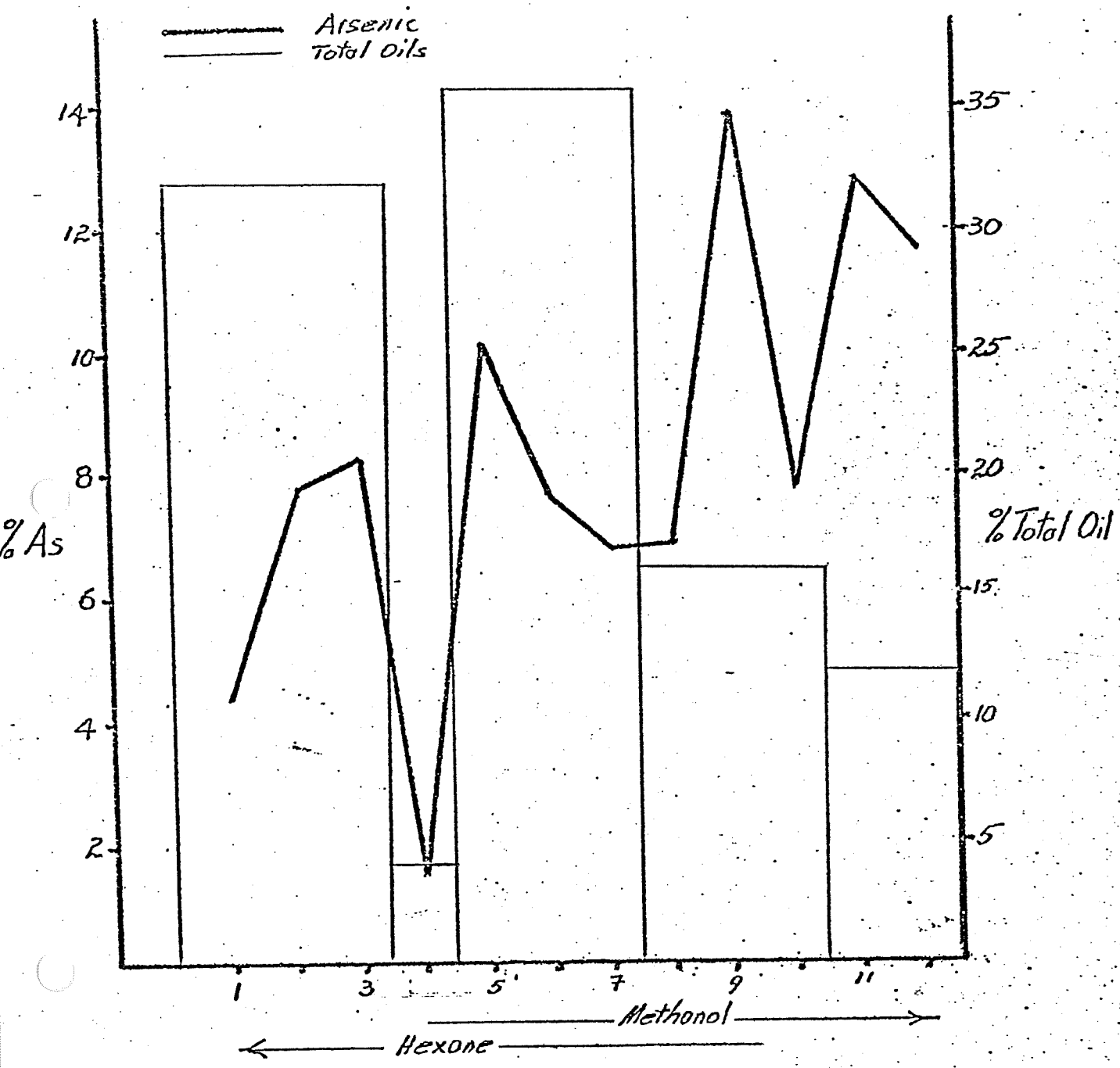


Figure 1. Counter Current Distribution

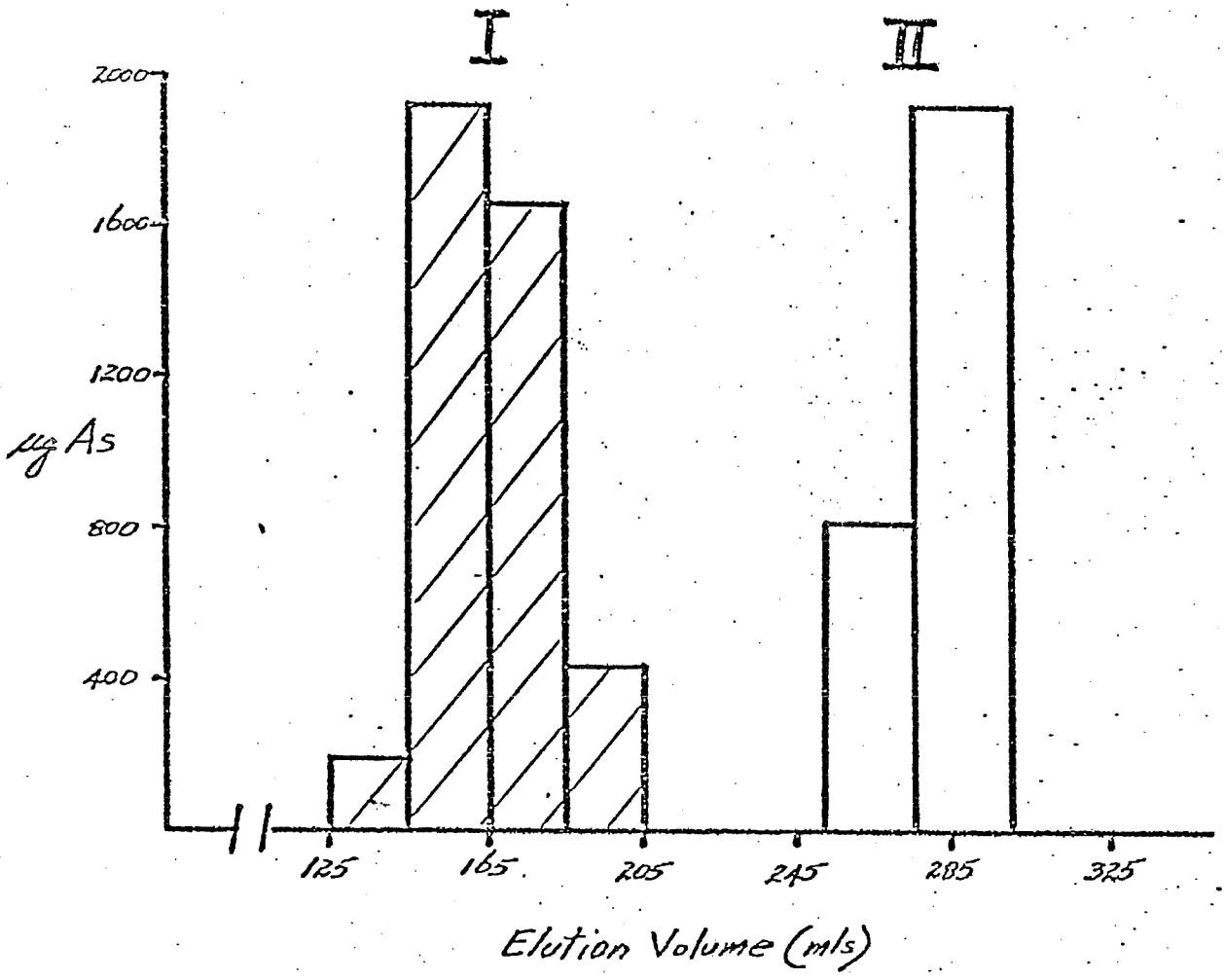


Figure 2. Arsenic Elution from DEAE Sephadex

DISCUSSION

Arsenic levels in marine organisms are significantly higher than in those of terresterial origin.[3] Moreover, it has been shown that the arsenic present in different marine organisms, including seaweeds, is in the form of water soluble and lipid soluble organo-arsenic compounds.[4-6] Three water soluble compounds have been isolated from marine sources. Arsenobetaine (1) was isolated from the western rock lobster Panulirus cygnus[7] and subsequently from the dusky shark Carcharhinus obscurus,[8] the school whiting Sillago bassensis[9] and another shark Prionace glaucus.[10] The other water soluble arsenic compounds are 2-hydroxy-3-sulphopropyl-5-deoxy-5-(dimethylarsenoso) furanoside(2) and 2, 3-dihydroxypropyl-5-deoxy-5-(dimethylarsenoso) furanoside(3) isolated locally from the brown kelp Ecklonia radiata.[11]

Biochemical approaches to structural elucidation of these marine organo arsenic compounds havebeen attempted by two groups. Benson[12, 13] examined the uptake of ^{74}As arsenate by a marine algae and Irgolic[14] performed similar radiolabelled experiments on marine algae and Daphnia species. Both groups reported the incorporation of ^{74}As into lipid soluble compounds which, on chromatographic and hydrolysis experiments, they assigned as phospholipids; Benson's as a phosphatidyl arsenolactate (4) and Irgolic's as a phosphatidyl choline(5).

Lipid soluble organo-arsenic components were detected in the hepatopancreas of the western rock lobster. Initially, the oil could be separated by centrifugation of the pancreas but with intermittant success; solvent extraction was finally adopted. The first indication that more than one arsenic component was present, was shown in the hexane/methanol partition of the chloroform extract.

Counter current distribution between hexane/methanol gave a range of polarity (see figure 1). The two most polar fractions gave the highest concentration of arsenic and these were chosen to study further.

Of the chromatographic supports available for lipid soluble extracts, silica gel and alumina are of choice because of their capacity as absorbents. Unfortunately, recovery of polar arsenic fractions was very poor (ca. 30%) and they were not further considered. This phenomenon was observed by Irgolic[14] who reported "an appreciable amount of radioactivity remained at the top of the [silica] column".

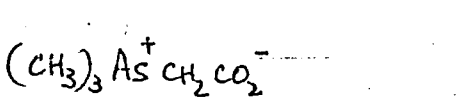
Acetone precipitation, while apparently successful on an analytical scale, gave poor fractionation and was of little value.

The first indication that initial fractionation by counter current distribution was real, came with Sephadex chromatography. Two distinct arsenic containing fractions were resolved, the most polar fraction being contained in higher elution volumes. This is very clear in figure 2. No chemical information can be obtained from this result except for the general one of polarity. Liquid liquid partition will be the major separation phenomenon ahead of exclusion chromatography.

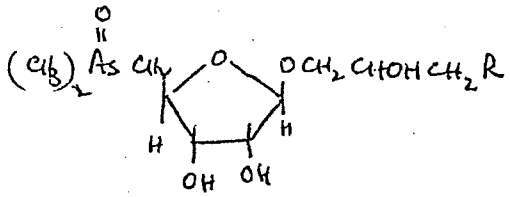
Cadmium chloride forms an insoluble complex with an ethanolic solution of the polar fractions. This technique is an excellent fractionation procedure for the crude bulk oil because of the high arsenic recovery (78%). However, the technique of cadmium removal on an alumina column,[15] developed for lecithin purification, is unsuitable for the arsenic components. EDTA extraction proved to be simpler with no loss of arsenic.

The isolation of a pure arsenic compound has not been achieved. The most concentrated fraction (I) appears to be a mixture of four components on HPLC, only one of which appears to be separable under the conditions attempted. Speculation as to the nature of this compound has not been fruitful. It is now water soluble. Very likely, it is a hydrolysis product obtained soon after extraction from the pancreas. In general, the oil from the pancreas, on KOH hydrolysis, yields only ca 35% water soluble arsenic, and possibly the arsenic in (I) is equivalent or similar to this hydrolysis product. The mass spectrum of (I) is inconclusive. An 80 MHz FT ^{1}H nmr spectrum[16] in D_2O has no signals below δ4. The major signals are at δ2 and δ3.2 with a broad methyl doublet at ca δ2.6 coupled to a multiplet at ca δ4. The broadening (ca 2Hz) remains unexplained but it could be postulated that P-H coupling is involved.

The fraction II is still a multi-component mixture. The major ^{1}H nmr signals indicate unsaturated methylene chains and the overall spectrum is not unlike that of lecithin.

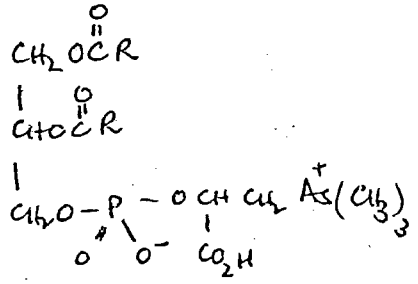


(I)

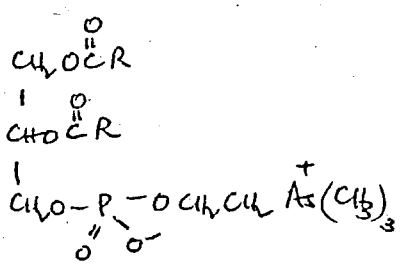


(2) $R = SO_3H$

(3) $R = OH$



(4)



(5)

References

1. An analytical survey of 21 western rock lobsters gave a mean concentration of 39 μgAsg^{-1} of hepatopancreas (see J.B. Saunders report).

2. A water soluble extract was also obtained and contained some 2-3gAs.

3. G.Lunde, Acta Chem. Scand., 1973 , 27, 1586.

4. G. Lunde, Acta Chem. Scand., 1972 , 26, 2642.

5. G. Lunde, J. Sci. Fd. Agric., 1975, 26, 1257

6. E. Sadolin, Biochem Z., 1928, 201, 323.

7. J.S. Edmonds, K.A. Francesconi, J.R. Cannon, C.L. Raston, B.W. Skelton and A.H. White, Tetrahedron Letters, 1977, 1543.

8. J.R. Cannon, J.S. Edmonds, K.A. Francesconi and J.B. Langsford, Management and Control of Heavy Metals in the Environment, Int. Conf. London 283 (CEP Consultants, Edinburgh 1979).

9. J.S. Edmonds and K.A. Francesconi, to be published in Mar. Pollut. Bull.

10. S. Kurosawa, K. Yasuda, M. Taguchi, S. Yamazaki, S. Toda, M. Morita, J. Uehiro and K. Fuwa, Agric. Biol. Chem., 1980, 44 (8), 1993.

11. J.S. Edmonds and K.A. Francesconi, Nature, 1981, 289, 602.

12. R.V. Cooney, R.O. Mumma and A.A. Benson, Proc. Natl. Acad. Sci USA, 1978, 75, 4262.

13. R.V. Cooney and A.A. Benson, Chemosphere, 1980, 9, 335.

14. K.J. Irgolic, E.A. Woolson, R.A. Stockton, R.D. Newman, N.R.Bottino, R.A. Zingaro, P.C. Kearney R.A. Pyles, S. Maeda, W.J. McShane and E.R. Cox, Environmental Health Perspectives 1977, 19,61.

15. J.R. Nielsen, Lipids, (1980), 15, 481.

16. No internal standard was used for this spectrum. The signal at δ2 was arbitarily set from a spectrum of a less pure sample previously run in methanol-d4 containing TMS.

EXPERIMENTAL

1. Extraction

Hepatopancreas (96,725 g) from the western rock lobster *Panulirus cygnus* was collected at the Fisherman's Cooperative processing plant, Fremantle. This material was stored at -10° and then thawed and blended to give a mustard yellow suspension.

This suspension was shaken with about half its volume of chloroform. On standing, the chloroform layer was separated from an emulsion and upper aqueous layer. The emulsion was separated from the aqueous layer and centrifuged to give a further chloroform layer, which was combined with the initial chloroform solution, filtered and concentrated under vacuum at <40° to a dark mobile oil (11,140 g; 84 mgAs).

The remaining emulsion was then shaken with an equal volume of chloroform/methanol (2:1). On standing, the lower chloroform layer was separated and the remaining emulsion twice extracted with chloroform/methanol (2:1). The combined chloroform layers were filtered and concentrated under vacuum at <40° to yield a dark viscous oil (8,310 g; 104 mgAs).

Both of the above bulked oils were stored under nitrogen at -10°C.

2. Hexane-Methanol Partition

Samples (~ 1 kg) of the above chloroform extract (11, 140 g; 84 mgAs) were dissolved in (methanol saturated) hexane (2 ℓ) and partitioned with (hexane saturated) methanol (2 ℓ). This was repeated for the total extract. The lower methanol layers were run off and the upper hexane layers further partitioned with (hexane saturated) methanol.

The methanol layers were combined and concentrated under vacuum at < 40° until foaming prevented further concentration to yield a dark oil (1,280 g; 44mgAs). (A)

The upper hexane layers were concentrated to under vacuum at < 40° to give a dark mobile oil (7,430 g; 26mgAs).

Similarly, the chloroform/methanol extract (8,310 g; 104 mgAs) was partitioned between hexane and methanol. The methanol layers were concentrated under vacuum at <40° until foaming began to yield a dark viscous oil (2,125g; 58mgAs). (B)

The hexane layers on evaporation gave a dark oil (4,800 g; 10mgAs).

3. Countercurrent Distribution

The methanol concentrate (A) (1,280 g; 44mgAs) was dissolved in (methanol saturated) hexane and made up to 4 ℓ. This solution was divided into two equal portions and transferred to 5 ℓ separating funnels. Ten further separating funnels were each filled with (methanol saturated) hexane (2ℓ each) and numbered 1 → 12.

(Hexane saturated) methanol (2 ℓ) was then added to 1, shaken with the solution of oil and the lower methanol layer transferred to funnel 2, which contained the remaining solution of oil. Fresh (hexane saturated) methanol was partitioned with 1. The methanol layer of 2 was transferred to 3; the methanol layer of 1 was transferred to 2; fresh (hexane saturated) methanol was again added to 1. This partitioning and transfer process was continued until 12 transfers had been effected.

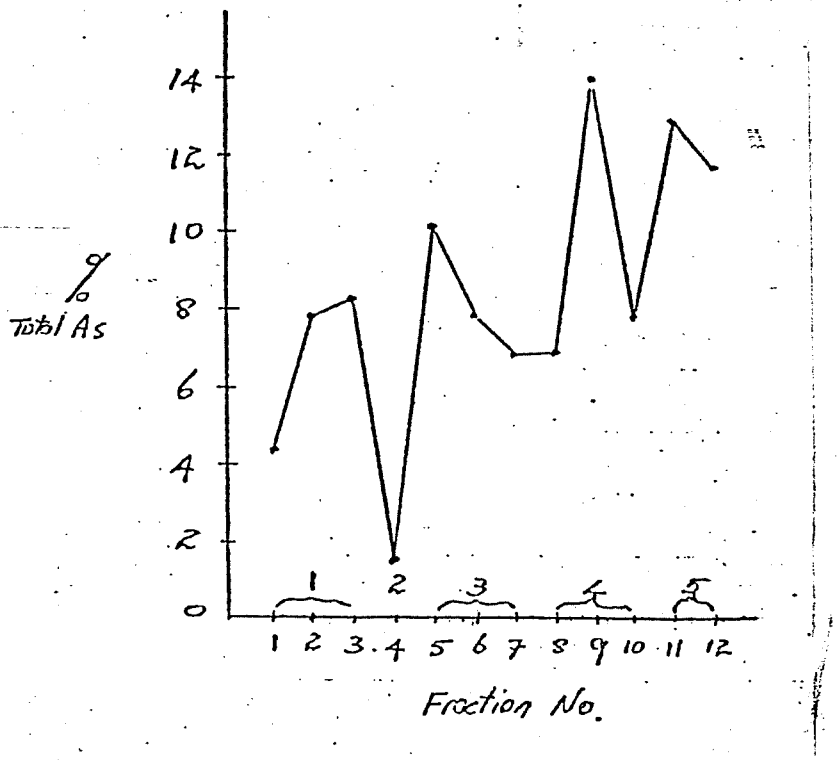
All twelve samples were then concentrated under vacuum to a reduced volume (of single phase) and samples analysed for arsenic content.

On the basis of the arsenic analysis result (see figure 3), five fractions were formed from the twelve generated by countercurrent distribution. (see table 1).

Table 1 Arsenic content of fractions generated from Countercurrent Distribution of oil (A)

Fraction No.	Arsenic (mg)	wt of oil (g)	PPM
1	12.1	400	30
2	0.9	51	17
3	14.6	440	33
4	16.9	199	85
5	14.5	150	97

Figure 3. % Total Arsenic Content vs Fraction No. (Fraction 1 = high hexane partition. Fraction 12= high methanol partition).



4. Acetone Precipitation of methanol concentrate B

The methanol concentrate (B) (2,125 g; 58 mg As) was dissolved in chloroform (2.4 ℓ). Samples (450 mℓ) were transferred to 2ℓ conical flasks and then shaken vigorously as small amounts of acetone were added. Acetone addition (1.5 ℓ) per flask, was stopped after no further precipitation of a dark oil was detected. The samples were left standing overnight.

The solution was decanted off the dark viscous oil, combined with the solution of each flask and concentrated under vacuum at $\leq$ 40° to yield a dark oil (1,518 g; 51 mg As) (C)

The dark viscous precipitate was redissolved in chloroform, combined with those of the other flasks and concentrated under vacuum at < 40° to yield a dark oil (493 g; 37 mg As) (D)

5. Counter Current Distribution

5.1 Partitioning solvents dichloromethane, tetrachloroethylene, methanol and water (31:31:34:8) were shaken in a separating funnel to give equal volumes of an upper 'aqueous' phase and lower 'organic' phase.

5.2 The precipitate (490 g; 36mg As) (D) obtained from acetone precipitation was dissolved in organic phase (1.5 ℓ) and transferred to a 5ℓ separating funnel. Two other separating funnels were each filled with aqueous phase (1.5ℓ) and numbered 1 → 3.

Aqueous phase (1.5ℓ) was added to 1, shaken with the solution of oil and the lower organic phase transferred to funnel 2 and shaken. Fresh aqueous phase (1.5ℓ) was partitioned with 1. The organic layer of 2 was transferred to 3. The organic layer of 1 was transferred to 2 and shaken with the aqueous phase therein.

The organic phases of funnels 2 and 3 were combined and concentrated under vacuum to give an oil (450 g; ——) which still contained a small amount of solvent. (E)

The upper aqueous phase of funnels 2 and 1 were combined, and by introducing small volumes to a rotary evaporator to prevent 'foaming', the phase was reduced to 320 ml (——; 9.3 mgAs). (F)

6. Counter Current Distribution

Half of the soluble oil (1,518 g; 51mgAs) (C) obtained from acetone precipitation of concentrate (B) was dissolved in (methanol saturated) hexane and made up to 2.8 ℓ. Eleven further separating funnels were each filled with (methanol saturated) hexane (2ℓ) and numbered 1→12.

(Hexane saturated) methanol (1.5ℓ) was added to 1, shaken with the solution of oil and the lower methanol layer transferred to funnel 2. Fresh (hexane saturated) methanol was partitioned with 1. The methanol layer of 2 was transferred to 3; the methanol layer of 1 was transferred to 2; fresh (hexane saturated) methanol was added to 1. This partitioning and transfer process was continued until 12 transfers had been effected.

The contents of the funnels were then combined on the basis of an arsenic profile and concentrated under reduced pressure to produce four fractions from the twelve generated by counter current distribution. The remaining half of oil (C) was similarly treated as above and the four fractions combined. (see table 2).

Table 2. Arsenic Content of Fractions generated from Counter Current Distribution of oil C

Fraction No.	Arsenic (mg)	wt. of oil (g)	PPM
1	3	362	8
2	16	807	20
3	18	260	69
4	23	177	130

7. Sephadex LH20 Chromatography (Methanol)

7.1 Column : A glass column (50x600mm) fitted with a teflon tap was clamped upright.

Sephadex LH20 (310g) was swelled in methanol for four hours. The thick slurry was poured into the column giving a bed volume (V_t) of 1.25 ℓ.

Flow Rate : With a head of methanol 45cm, a flow rate of 3.6 mℓ/min was obtained.

Void Volume (Vo) was not determined. However, for the oils chromatographed, no material emerged before a V_e of 430 mℓ.

Sample application : Oil samples (~20g) were dissolved in methanol (50-60 mℓ) and centrifuged. This solution was applied to the top of the Sephadex by careful addtion.

Fraction Collection : A V_e (430 mℓ) was collected, then fractions 1→ 15 were collected as 50 mℓ samples and then fractions 10→25 were collected as 100 mℓ samples. Total elution volume 1.980 mℓ.

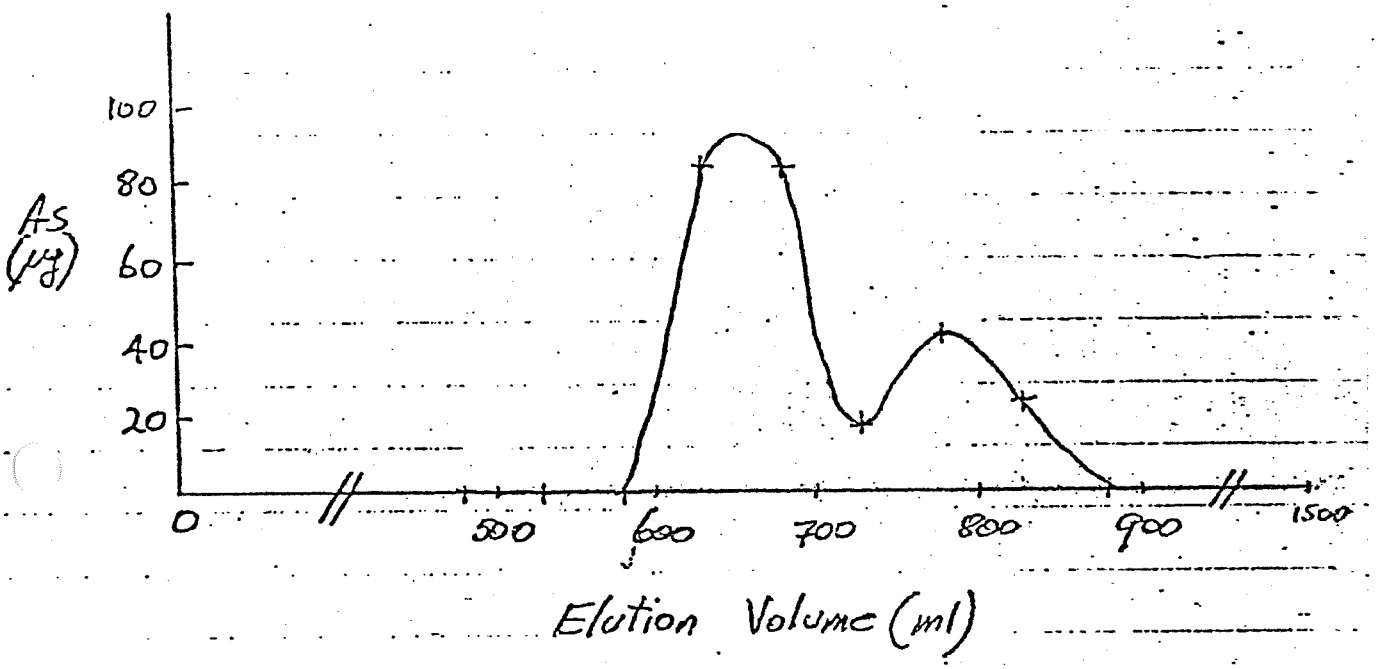
7.2 The oils of Table 1 (150g; 14.5mgAs) and Table 2 (177g;23 mg As) were chromatographed in the above manner. After the first run of each oil, all fractions were analysed for arsenic content, and on the basis of this result, fractions were combined and concentrated under reduced pressure to give two distinct arsenic components. For convenience, oils from tables 1 and 2 were kept separate although their arsenic profiles were very similar (see table 3). A profile of arsenic elution is seen in figure 4.

Table 3. Arsenic Content of the two components from Sephadex LH20 Chromatography in Methanol

Fraction No.	Arsenic (mg)	wt. of oil (g)	PPM
1	9, 6	31, 44	290, 136
2	7, 6	32, 43	219, 140

Figure 4. Arsenic Profile from Sephadex LH20

Column run in Methanol



8. Sephadex LH20 Chromatography (Chloroform/Methanol)

8.1 Column : A glass column (70x700 mm) fitted with a teflon tap was clamped upright.

Sephadex LH20 (420g) was swelled in chloroform-methanol (40:60) for four hours. The thick slurry was poured into the column giving a bed volume (V_t) of 2.23ℓ.

Flow Rate : With a head of solvent of 45 cm, a flow rate of 5 mℓ/min was obtained.

Void Volume (Vo) was not determined. However, for the oils chromatographed, no material emerged before a Ve of 800 mℓ.

Sample Application : Oil samples (≈10g) were dissolved in chloroform methanol (40:60)(60 mℓ) and centrifuged. This solution was applied to the top of Sephadex by careful addition.

Fraction Collection : A Ve (800 mℓ) was collected, and then eight fractions of 50 mℓ each; fractions 9-12 were 100 mℓ each. Total elution volume of 1.6ℓ.

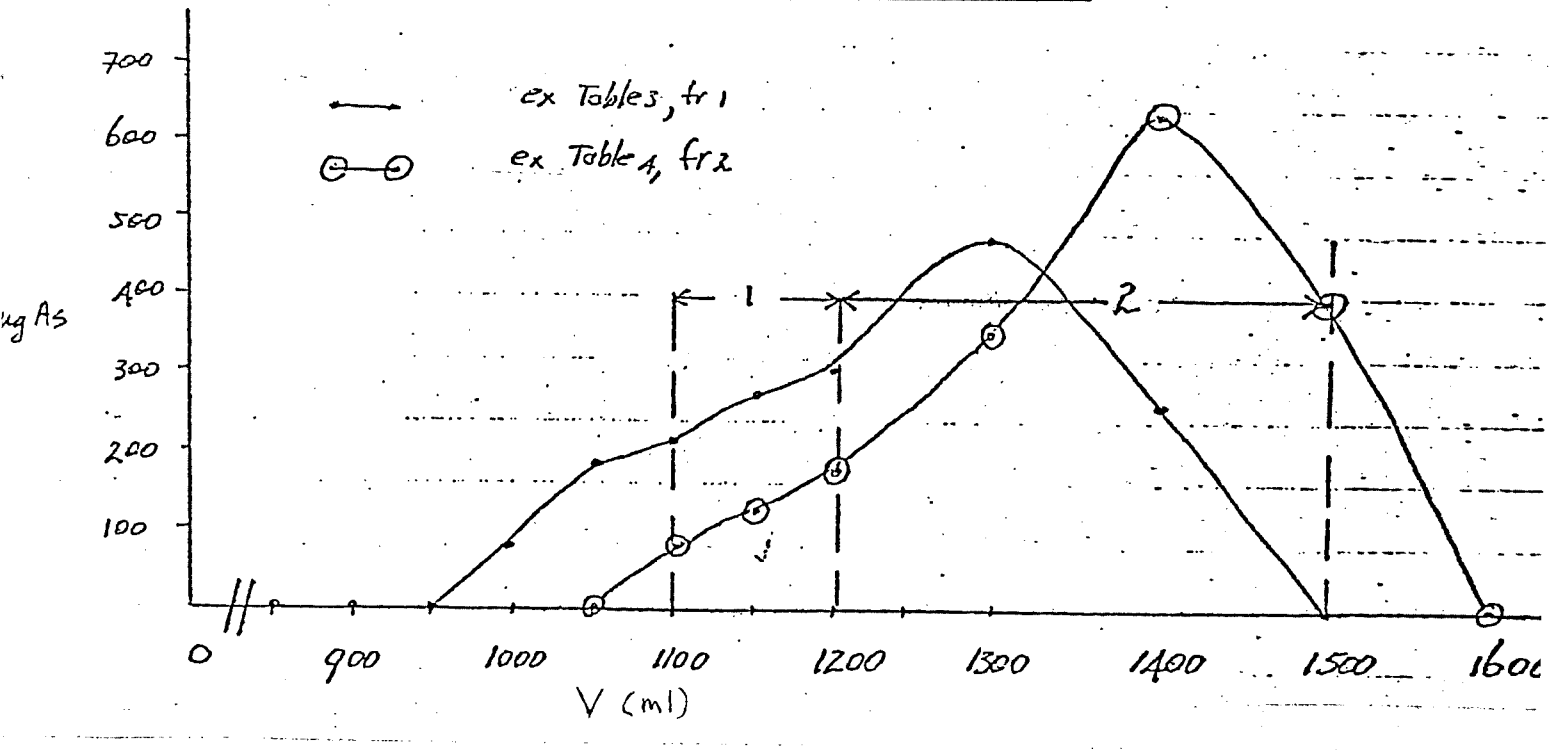
8.2 The four oil samples of Table 3 were each chromatographed as above and fractions were combined on the basis of the profile of arsenic analysis taken after the first run of each of fractions 1 and 2. (See figure 5). Again, for convenience, the fractions originating from Tables 1 and 2 were kept separate (see Table 4).

Table 4. Arsenic Content of the two components from Sephadex LH20 Chromatography in Chloroform-Methanol (40:60)

Fraction No.	Arsenic (mg)	wt. of oil (g)	PPM
1	3.7, 8.68	28, 37	133, 236
2	4.8, 11.40	21, 51	230, 224

Figure 5 Arsenic Profile of Sephadex LH20 eluate

Eluant :- Chloroform-Methanol (40:60)



8.3 The oils E and F of section 5 were similarly chromatographed. Oil F gave two similar fractions as in 8.2,1(20.5g; 3.7mgAs) and 2 (20g; 3.0 mgAs).Oil E, however, was chromatographed and all arsenic fractions combined into one fraction (135g; 1.5mgAs). The substantial loss of arsenic involved in sections 5 or 8.3 cannot be explained. (36mg→9.2mgAs).

9. DEAE Sephadex Chromatography

9.1 Column : A glass column (40x600 mm) fitted with a teflon tap was clamped upright.

DEAE Sephadex (110g) was swelled in methanol overnight. The slurry was poured into the column. The column was then purged with freshly distilled acetic acid, followed by methanol and finally chloroform-methanol (50:50). This gave a bed volume (V_t) of 600 mℓ.

Flow Rate : The flow rate was controlled and maintained at 1 mℓ/min

Sample application : Oil samples (6g) were dissolved in chloroform/methanol (50:50(25 mℓ). This solution was applied to the top of the Sephadex by careful addition.

Fraction Collection : A Ve (175 m) was collected and then fractions 1→5 of 50 mℓ each and fractions 6→8 of 100 mℓ each. A dark green band remained absorbed to the top of the column bed.

9.2 The two oil samples (Table 4, fraction 2) (14g) and (59g; 11.4mgAs) were chromatographed in the above manner. The major arsenic containing fraction (V_t = 525 mℓ) was fraction 6. The profile of arsenic distribution is illustrated for a typical run in figure 6. Results in Table 5.

(Note: A sample of the oil (Table 4, fraction 1) was also run on the above column and the major arsenic component was found in fractions 2 and 3.

Table 5.1 Fractions collected from DEAE Sephadex

Fraction No	Arsenic (mg)	Wt. of oil (g)	PPM
1	0.40	0.16	2583
2	0.30	1.18	251
3	0.17	2.39	72
4	0.48	4.66	102
5	0.78	1.54	507
6	0.03	1.18	25

Table 5.2 Fractions collected from DEAE Sephadex

Fraction No	Arsenic (mg)	Wt. of oil (g)	PPM
1	0.08	0.33	231
2	0.66	0.80	822
3	0.88	6.60	116
4	0.68	9.09	74
5	0.39	7.82	50
6	5.00	3.70	1353
7	0.55	2.59	213
8	0.18	1.67	80

9.3 A second DEAE Sephadex column was prepared as in 9.1.

Column : A glass column (30mmx600mm) fitted with a teflon tap.

DEAE Sephadex (74g) was swelled in methanol and purged with acetic acid, methanol and then chloroform-methanol (50:50).

Flow Rate : The flow rate was controlled and maintained at 1 mℓ/min.

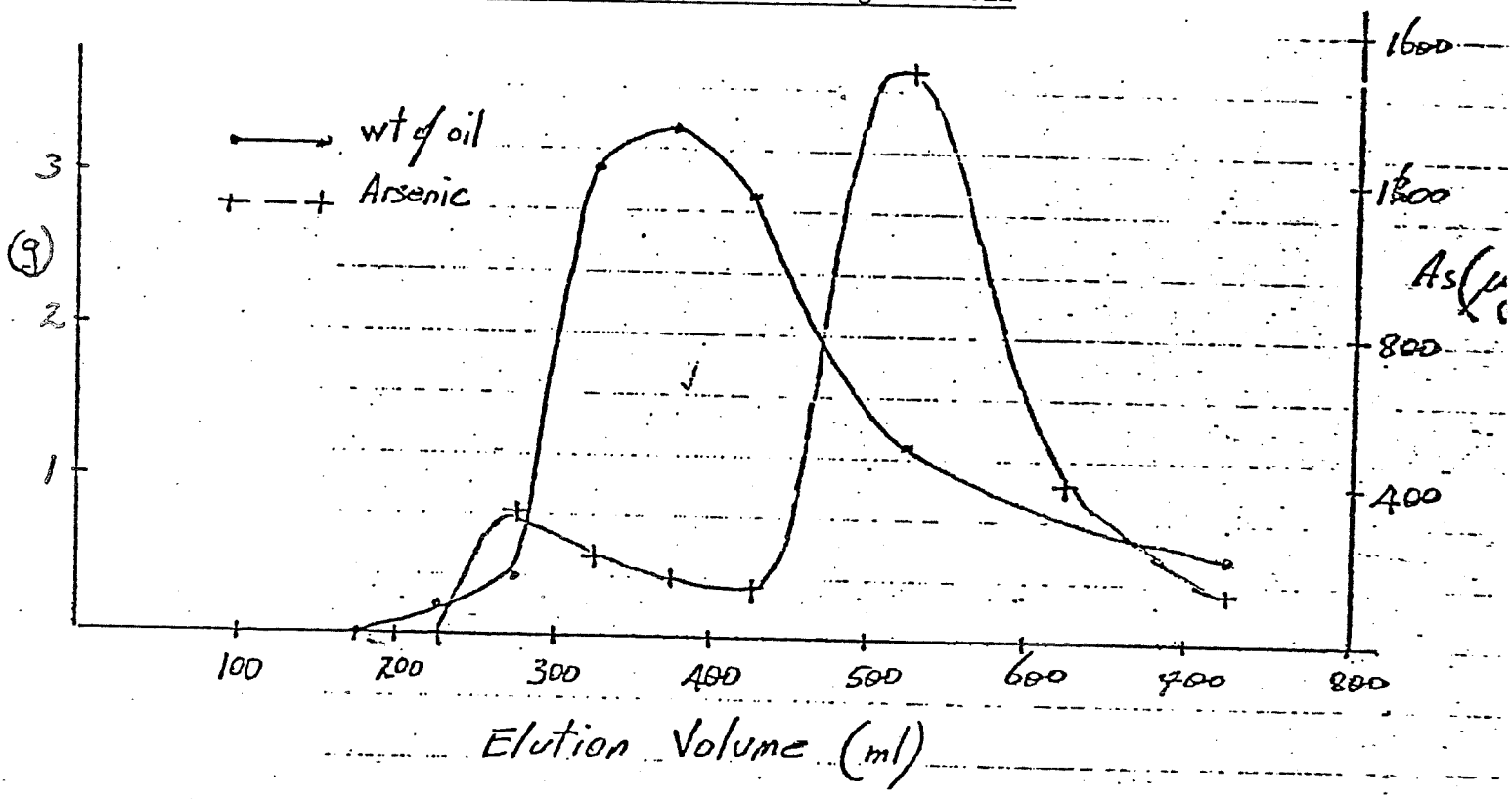
Sample Application : Oil sampes (1.0 - 1.3g) were dissolved in chloroform-methanol (50:50)(5mℓ) and applied to the top of the Sephadex column.

Fraction Collection : A Ve (118 mℓ) was collected and then fractions 1→20 (10mℓ each) and fractions 22→26 (25 mℓ each).

9.4 The major arsenic containing fractions of section 9.2 (fractions band 7) were rechromatographed as above in 9.3 Arsenic analysis of the fractions (samples from each alternate flask) showed the major component to be in fractions 13→20 and these were combined and concentrated under reduced pressure to give a pale yellow viscous oil (2.53g; 3.8mgAs).

This oil was rechromatographed on the column of section 9.3 on a single application and when the arsenic containing fractions were combined and concentrated under reduced pressure, there remained a pale yellow viscous syrup (2.17g; 3.8mgAs).

Figure 6. Arsenic Profile of DEAE Sephadex Elution compared with Weight of Oil



10. Precipitation from Methanol

The arsenic containing oil from section 9.4 (2.17g; 3.8mgAs) was dissolved in warm (40°) methanol (16mℓ) and transferred into two centrifuge tubes. A white solid precipitated on cooling to 0° and the tubes were centrifuged at 0°/10min/5000 RPM. The methanol was removed with a Pasteur pipette. An off-white precipitate remained and this was washed with 1-2 mℓ of methanol in each tube, cooled to -5° and centrifuged at -5°/10min/4000 RPM. The methanol was decanted as before and combined with the other methanol solubles, then concentrated under vacuum to give a pale oil (1.645g).

The precipitate from each tube was dissolved in chloroform, combined and concentrated under vacuum to a white solid (0.265g; 57μgAs).

11. Reverse Phase Chromatography - HPLC

11.1 Waters model 6000 A solvent pump, model U6K injector, semi-preparative, μ Bondapack C_{18} column and a model R401 differential refractometer made up the liquid chromatography system.

Solvent : Methanol

Flow Rate : 1.0 mℓ min^{-1}

Load : approx 80mg in 500μℓ

11.2 The methanol soluble oil (1.645g) from section 10 was dissolved in methanol (10 mℓ). A sample was injected onto the column and, using a single recycle phase gave three fractions of which fraction 2 was the major arsenic containing component. The oil was successively chromatographed to produce, on concentration of the combined fractions (2), a pale yellow oil (1.095g; 3mgAs).

11.3 The arsenic containing fraction from section 11.2 was dissolved in methanol (10 mℓ). This was rechromatographed on the μ Bondapack C_{18} column as for section 11.2. Using the recycle mode, eight fractions were collected. Fraction 2 on concentration gave a pale yellow oil (0.648g; 2.6mgAs).

12. DEAE Sephadex Chromatography

12.1 The DEAE Sephadex column of section 9.3 was reused. Fractions were collected as follows: Ve = 125 mℓ then 5 fractions of 20 mℓ each, 4 fractions of 25 mℓ each and a tenth fraction of 50 mℓ.

12.2 The arsenic containing fraction from section 11.3 (0.65g; 2.6mAs) was applied to the column as a methanol solution (~3mℓ). All of the arsenic was contained in fractions 8 and 9. Fraction 9 was concentrated to a pale yellow viscous oil (0.087g; 1.9mgAs). Fraction 8 was a pale yellow oil (0.104g; 0.8mgAs).

13. Thin layer Cellulose Chromatography

13.1 TLC cellulose plates were prepared at 0.75mm thickness as follows. Cellulose (90g) was shaken vigorously with water (~210mℓ) and then sonicated for 20 minutes. Six plates (20x20) cm were then prepared using a standard spreader.

13.2 The two arsenic containing fractions from section 12.2 were applied to 2 plates each and chromatographed with butanol : acetic acid : water (60:15:25). Four bands were cut from each plate, washed with methanol and the resulting solution analysed for arsenic. The bands (baseline < Rf < 0.5) containing the arsenic were combined to give a pale oil (0.1g; ~0.8mgAs).

13.3 The arsenic containing fraction (0.1g; ~0.8mgAs) was rechromatographed as for section 13.2. Bands (7) were cut according to fluorescence under long wavelength U.V. Fraction five contained all of the arsenic. The solution was concentrated to a pale yellow, viscous oil (0.025g; 0.8mgAs).

14. Cadmium Chloride Complex

14.1 The oil (199g; 16.9mgAs)(see section 3, Table 1, fraction 5) was washed with ethanol (1.6ℓ) and centrifuged. The residue was washed with ethanol (12x100 mℓ), centrifuged, washed again (12x100mℓ) and recentrifuged. The total volume of ethanol used was 4ℓ.

The ethanol extract (in 2x2ℓ flasks for convenience) was stirred. Cadmium chloride (50% aqueous solution) was added dropwise giving a cream precipitate from the deep red solution. Addition was continued until no further precipitation could be seen (2x30mℓ). The suspension was left to stand overnight at -20°.

On returning to room temperature, some 1.8ℓ of ethanol was decanted and the remaining suspension centrifuged. The ethanol supernatant was combined and concentrated to dryness (84g; 2.4mgAs).

The solid residue was dissolved in chloroform (1.8ℓ) and then partitioned with ethylene diamine tetraacetic acid, disodium salt (20g EDTA in 1.2ℓ water) solution. The lower chloroform layer was run off and reextracted with EDTA solution. An emulsion formed to which was added a saline solution (100g NaCl in 500 mℓ H_2O) and the chloroform layer run off. The remaining emulsion was washed once more with saline and the chloroform layers combined, filtered and reduced to a dark oil (51g; 8.2mgAs). No cadmium was detectable on AA analysis.

14.2 The oil of section 8.2, Table 4 fraction 1 (37g; 8.7mgAs) was similarly treated with cadmium chloride solution (50% aqueous) and centrifued. The precipitate was dissolved in chloroform and partitioned with EDTA as before. The chloroform solution was reduced to a viscous oil (11.6g; 2.0mgAs).

DEAE Sephadex Chromatography

9.5 The oil from section 14.1 (51g; 8.2mgAs) was chromatographed in eight lots as outlined in section 9.1, except that after a Ve of 164mℓ , six fractions of 50mℓ and 2 fractions of 200mℓ each were collected. The results are recorded in Table 6.

Table 6. Fractions collected from DEAE Sephadex

Fraction No	Arsenic (mg)	Wt. of oil (g)	PPM
1	0.22	0.59	366
2	1.82	1.30	1403
3	1.43	8.93	160
4	2.60	13.33	195
5	0.83	6.79	122
6	0.80	4.64	172
7	0.00	0.46	0

9.6 The above samples from Table 6 were individually re-chromatographed on the same DEAE Sephadex column and under the same conditions as in section 9.5 Arsenic analyses of the fractions were not checked but the weight distribution, on combining equivalent elution volumes, was similar.

9.7 Two fractions 2 and 3 from Table 5.2, section 9.2 were rerun on DEAE Sephadex and collected as for section 9.6.

9.8 The oil (11.6g; 2.0mgAs) from section 14.2 was similarly chromatographed on the above DEAE Sephadex column and collected as for section 9.6. The results are summarised in Table 7.

Table 7. Fractions collected from DEAE Sephadex

Fraction No	Arsenic (mg)	Wt. of Oil (g)	PPM
1	0.15	0.15	1000
2	0.47	0.46	1021
3	0.37	2.62	141
4	0.40	3.07	130
5	0.14	1.64	85
6	0.08	1.15	70
7	0.08	0.41	195

9.9

Oils collected from fraction 2 of sections 9.6, 9.7 and 9.9 as well as fraction 1 from Table 5.1 section 9.2 were chromatographed on DEAE Sephadex as for section 12.1. The results of the combined common fractions are in Table 8.

Table 8. Fractions collected from DEAE Sephadex

Fraction No	Arsenic (mg)	Wt. of Oil (g)	PPM
1	0.18	0.26	691
2	1.90	0.90	2108
3	1.65	1.05	1565
4	0.43	0.85	506
5	0.00	0.20	0
6	0.00	0.08	0
7	0.00	0.05	0
8	0.00	0.00	0
9	0.00	0.00	0

Heavy Metal Concentrations in the Tail Muscle and Hepatopancreas of the Western Rock Lobster (Panulirus cygnus George)

1. Collection and processing of rock lobsters

Twenty one rock lobsters (Panulirus cygnus George) were caught in pots at the 5 fathom bank off Point Peron in Cockburn Sound, Western Australia. The animals were sacrificed by inserting a Pasteur pipette into the heart sinus and removing the blood. Each animal was then sexed, weighed and measured, the total length and the length of the carapace being recorded.

The carapace was then cut open with secateurs to reveal the intact hepatopancreas. The colour and consistency of the hepatopancreas varied from animal to animal. Each hepatopancreas was collected with the aid of a plastic spatula then placed in a polythene bag and weighed. The organ was homogenized by gently kneading the bag which was then sealed and stored at -10°.

After removing the digestive tract the tail muscle was removed from the shell, weighed, then freeze-dried and weighed again. The dried muscle was finally powdered and stored in a desiccator at -10°.

These results are set out in Table 1.

2. Analytical procedures

(i) Arsenic. Samples were prepared for arsenic analysis by combusting 10-50 mg of material in a 1ℓ round-bottomed flask containing 25 ml of deionized water and filled with oxygen. After combustion the flasks were allowed to cool for 15 minutes before opening, then aliquots of the aqueous solution were taken and analyzed for arsenic by vapour generation atomic absorption spectroscopy using a Pye-Unicam SP190 atomic absorption spectrometer equipped with an As/Se/Hg kit and linked to a Mace Universal Recorder, model FBQ100.

(ii) Selenium. Samples were digested with a mixture of concentrated nitric acid (5 parts) and perchloric acid (2 parts) until fumes of perchloric acid were evolved. The mixture was then heated with concentrated hydrochloric acid (1 ml) for 15 minutes before diluting to the required volume with deionized water. Analysis by vapour generation atomic absorption spectroscopy was then carried out, as in the determination of arsenic.

(iii) Mercury. Samples were digested as in (ii) above then total mercury analyses were carried out by the standard AGAL procedure. Flameless atomic absorption spectroscopy was carried out on the Pye Unicam SP190 instrument equipped with an Hg kit and linked to a Mace Universal Recorder, model FBQ100.

TABLE 1. Measurements of individual rock lobsters used in the determination of levels of trace metals

Animal No.	Sex	Total Length (mm)	Length of Carapace (mm)	Total Weight (g)	Weight of hepatopancreas (g)	Wet Weight of tail muscle (g)	Dry Weight of tail muscle (g)
9	M	196	65	242	10.33	51.40	13.36
14	M	220	74	357	8.9	88.87	23.57
11	M	236	79	412	12.84	108.00	30.13
12	M	240	82	420	18.12	100.17	26.49
3	M	245	83	460	13.98	105.18	26.68
13	M	245	84	480	20.68	106.46	28.06
5	M	256	85	450	14.72	120.70	29.61
8	M	243	85	504	17.04	125.71	27.68
20	M	262	90	552	26.15	150.27	36.80
19	M	277	91	556	23.17	159.68	40.48
17	M	267	92	580	18.31	164.48	40.70
16	M	280	95	687	23.26	198.06	45.75
21	M	280	96	672	31.00	184.30	42.33
18	M	293	100	773	26.37	245.75	58.30
15	F	205	70	270	11.5	65.85	17.54
7	F	218	72	330	16.16	81.97	22.69
10	F	235	77	413	20.98	107.53	26.68
6	F	241	79	447	16.86	124.64	31.56
2	F	240	79	417	18.48	102.35	26.88
1	F	250	85	514	18.40	128.74	33.29
4	F	260	90	535	17.77	145.26	37.56

TABLE 2. Analyses of Heavy Metals in the Hepatopancreas of the Western Rock Lobster

Animal No.	Ag (µg/g)	As (µg/g)	Cd (µg/g)	Cu (µg/g)	Fe (µg/g)	Hg (µg/g)	Se (µg/g)	Zn (µg/g)
9		69.5 ± 1.96						
14	8.8 ± 0.03	26.1 ± 1.10	5.4 ± 0.31	29.2 ± 1.81	39.1 ± 5.20			61.4 ± 1.23
11	4.5 ± 0.04	39.1 ± 3.27	7.9 ± 0.32	10.2 ± 1.15	34.1 ± 4.82			65.8 ± 0.66
12	8.0 ± 0.57	29.1 ± 1.35	8.3 ± 1.21	60.7 ± 0.89	44.3 ± 2.38			44.6 ± 1.03
3	21.1 ± 0.38	26.3 ± 1.45	9.7 ± 0.25	80.7 ± 1.60	63.8 ± 2.10			89.1 ± 0.42
13	6.4 ± 0.03	26.0 ± 1.89	7.8 ± 0.23	47.4 ± 2.96	19.8 ± 2.25			44.7 ± 2.62
5	4.2 ± 0.30	64.9 ± 1.24	3.5 ± 0.22	17.2 ± 2.00	30.7 ± 4.21			70.7 ± 0.74
8	5.8 ± 0.47	19.1 ± 1.79	6.3 ± 0.01	98.4 ± 0.20	26.9 ± 3.00			88.9 ± 2.09
20	11.0 ± 0.48	33.0 ± 0.44	5.7 ± 0.23	101.5 ± 1.68	38.0 ± 0.11	≦ 0.02	2.2 ± 0.29	68.6 ± 4.33
19	11.0 ± 0.20	38.8 ± 1.91	5.2 ± 0.35	94.4 ± 2.52	40.5 ± 0.89	≦ 0.02	2.3 ± 0.22	72.7 ± 1.94
17	5.6 ± 0.31	31.7 ± 0.82	4.0 ± 0.06	70.5 ± 0.19	37.9 ± 5.83	≦ 0.02	3.2 ± 0.22	59.9 ± 0.40
16	9.4 ± 0.27	17.3 ± 1.24	6.2 ± 0.08	90.4 ± 1.68	22.3 ± 1.58	≦ 0.02	1.4 ± 0.28	43.2 ± 0.52
21	5.5 ± 0.80	31.2 ± 2.64	5.6 ± 0.26	109.0 ± 1.24	45.6 ± 5.96	≦ 0.02	2.1 ± 0.20	63.2 ± 1.54
18	11.0 ± 0.02	40.0 ± 3.31	4.2 ± 0.09	68.9 ± 0.70	42.6 ± 0.68			73.7 ± 6.32
15	12.7 ± 0.16	45.2 ± 1.40	6.5 ± 1.08	37.7 ± 0.72	47.8 ± 5.22			73.7 ± 0.18
7	10.0 ± 0.63	28.6 ± 1.97	5.3 ± 0.87	92.0 ± 3.20	31.2 ± 0.35			35.8 ± 1.41
10	8.3 ± 0.03	37.8 ± 2.74	8.2 ± 0.09	83.1 ± 3.00	33.4 ± 1.20			40.1 ± 1.44
6	10.6 ± 0.27	48.5 ± 4.76	3.6 ± 0.30	145.9 ± 7.20	74.7 ± 6.55			84.4 ± 10.30
2	15.1 ± 0.47	74.0 ± 5.60	9.9 ± 0.01	270.6 ± 1.00	51.4 ± 0.20			85.7 ± 0.18
1	6.4 ± 0.09	39.0 ± 2.26	5.7 ± 0.05	17.9 ± 0.70	65.3 ± 3.70			107.9 ± 1.92
4	6.4 ± 0.30	59.8 ± 3.34	6.7 ± 0.52	12.8 ± 0.06	84.4 ± 0.50			98.8 ± 0.14
MEAN ± S.D.	9.1 ± 4.06	39.3 ± 15.98	6.3 ± 1.88	76.9 ± 58.84	43.7 ± 17.03		2.2 ± 0.52	67.8 ± 20.38
RANGE	4.2 - 21.1	17.3 - 74.0	3.5 - 9.9	10.2 - 270.6	19.8 - 84.4		1.4 - 3.2	35.8 - 107.9

TABLE 3. Analyses of Heavy Metals in the Freeze-dried Tail Muscle of the Western Rock Lobster

Animal No.	As (µg/g)	Cu (µg/g)	Hg (µg/g)	Se (µg/g)	Zn (µg/g)	Ag,Cr,Fe,Ni (µg/g)	Cd (µg/g)
1	133.2 ± 6.1	14.9 ± 1.0			71.6 ± 4.37		
3	89.6 ± 1.5	23.1 ± 0.8			74.8 ± 5.15		
5	90.5 ± 6.1	21.6 ± 0.2			76.8 ± 2.8		
10	207.4 ± 10.1	20.3 ± 1.6			70.4 ± 6.17		
11	79.1 ± 4.2	21.2 ± 1.8			71.2 ± 7.09		
16	112.1 ± 4.1		0.113 ± 0.023	1.2 ± 0.14		Not detected	Not detected
17	154.7 ± 0.1		0.16 ± 0.023	1.2 ± 0.0		i.e. ≦ 6	i.e. ≦ 2
19	143.6 ± 7.1		0.124 ± 0.016	1.2 ± 0.28			
20	188.4 ± 19.7		0.147 ± 0.006	1.2 ± 0.21			
21	144.4 ± 14.6		0.145 ± 0.005	1.1 ± 0.14			
MEAN ± S.D.	134.3 ± 42.60	20.2 ± 3.14	0.138 ± 0.020	1.19 ± 0.06	72.96 ± 2.72		
RANGE	79.1 - 207.4	14.9 - 23.6	0.113 - 0.147	1.1 - 1.2	70.4 - 76.8		

TABLE 4. Calculated Values of the Levels of Heavy Metals in the Fresh Tail Muscle of the Western Rock Lobster

Animal No.	As (µg/g)	Cu (µg/g)	Hg (µg/g)	Se (µg/g)	Zn (µg/g)	Ag,Cr,Fe,Ni (µg/g)	Cd (µg/g)
1	34.4 ± 1.6	3.8 ± 0.3			18.5 ± 1.11		
3	22.7 ± 0.4	5.8 ± 0.2			19.0 ± 1.3		
5	22.2 ± 1.5	5.3 ± 0.05			18.8 ± 0.68		
10	51.4 ± 2.5	5.0 ± 0.4			17.5 ± 1.5		
11	22.1 ± 1.2	5.9 ± 0.5			19.9 ± 2.0		
16	25.9 ± 0.9		0.026 ± 0.005	0.30 ± 0.03		Not detected	Not detected
17	38.3 ± 0.1		0.040 ± 0.006	0.30 ± 0.0		i.e. ≦ 1.5	i.e. ≦ 0.5
19	36.4 ± 1.8		0.031 ± 0.004	0.30 ± 0.07			
20	46.1 ± 4.8		0.036 ± 0.001	0.31 ± 0.05			
21	33.2 ± 3.3		0.033 ± 0.001	0.25 ± 0.03			
MEAN ± S.D.	33.3 ± 10.24	5.2 ± 0.84	0.033 ± 0.005	0.29 ± 0.02	18.74 ± 0.60		
RANGE	22.1 - 51.4	3.8 - 5.9	0.026 - 0.040	0.25 - 0.31	17.5 - 19.9		