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Speciation and Health Risk Considerations of Arsenic in the Edible Mushroom Laccaria Amethystina Collected from Contaminated and Uncontaminated Locations

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Samples of the edible mushroom *Laccaria amethystina,* which is known to accumulate arsenic, were collected from two uncontaminated beech forests and an arsenic-contaminated one in Denmark. The total arsenic concentration was 23 and 77 μg As g^{-1} (dry weight) in the two uncontaminated samples and 1420 μg As g^{-1} in the contaminated sample. The arsenic species were liberated from the samples using focused microwave-assisted extraction, and were separated and detected by anion- and cation-exchange high-performance liquid chromatography with an inductively coupled plasma mass spectrometer as arsenic-selective detector. Dimethylarsinic acid accounted for 68–74%, methylarsonic acid for 0.3–2.9%, trimethylarsine oxide for 0.6–2.0% and arsenic acid for 0.1–6.1% of the total arsenic. The unextractable fraction of arsenic ranged between 15 and 32%. The results also showed that when growing in the highly arsenate-contaminated soil (500–800 μg As g^{-1}) the mushrooms or their associated bacteria were able to biosynthesize dimethylarsinic acid from arsinic acid in the soil. Furthermore, arsenobetaine and trimethylarsine oxide were detected for the first time in *Laccaria amethystina.* Additionally, unidentified arsenic species were detected in the mushroom. The finding of arsenobetaine and trimethylarsine oxide in low amounts in the mushrooms showed that synthesis of this arsenical in nature is not restricted to marine biota. In order to minimize the toxicological risk of arsenic to humans it is recommended not to consume *Laccaria amethystina* mushrooms collected from the highly contaminated soil, because of a genotoxic effect of dimethylarsinic acid observed at high doses in animal experiments. © **1998 John Wiley & Sons, Ltd.**

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1 INTRODUCTION

Arsenic in the chemical forms (species) of arsenous acid [As(III)] and arsenic acid [As(V)] is well known for its toxic properties to humans. Most of the relatively high concentrations of arsenic in seafood are present, however, as organoarsenic compounds of low toxicity, and the inorganic forms usually constitute only a few per cent of the total.[1] The organoarsenic compounds present include dimethylarsinylriboside derivatives ('AsSugars') which are the predominant form of arsenic in shellfish,[2,3] and arsenobetaine (AsB), which is the dominating chemical form in fish and crustaceans.[4–6]

Arsenic is generally present at much lower concentrations in foods of terrestrial origin than in seafood. A calculation of the total intake of arsenic via food in Denmark[7] showed that in spite of a low

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consumption rate, seafood contributed 72% of the total arsenic intake at 118 μg day^{-1}, whereas 12% of the total arsenic was ingested via plant-based foods. In certain industrialized areas, however, atmospheric deposition of arsenic has caused contamination of locally grown leafy vegetables. This led to an increase in the arsenic intake by a factor of 3–5 from this category of foods, but the estimated total intake of arsenic from the diet was still below the provisional tolerable daily intake for inorganic arsenic.[8] Certain mushroom species, including the edible *Laccaria amethystina*, are able to accumulate arsenic. Samples of this mushroom from uncontaminated locations in Denmark[9] showed arsenic concentrations at 58–320 μg As g^{-1} dry weight, and in similar samples from Central Europe[10] the arsenic concentrations were 6–250 μg As g^{-1}. The question arises whether this mushroom is able to bioaccumulate arsenic at even higher concentrations from a contaminated soil environment, and if so what the toxicological implication may be to persons who consume these mushrooms.

Terrestrial fungi (moulds) are able to biosynthesize monomethylarsonic acid (MMA), dimethylarsinic acid (DMA) and trimethylarsine oxide (TMAO) in addition to the gaseous arsines from inorganic arsenic.[5] Recently it was shown that DMA was the dominating arsenic species at 35 μg As g^{-1} (dry weight) in samples of *Laccaria amethystina*.[11] Unexpectedly AsB, which is usually found in marine samples, was detected in other species of arsenic-accumulating mushrooms[12] at 0.25 μg As g^{-1}.

The aim of this paper was to investigate the concentrations and chemical forms of arsenic present in *Laccaria amethystina* collected in uncontaminated beech forest locations, in comparison with samples collected in a similar, but arsenic-contaminated, location. Furthermore, the toxicological implications for humans consuming the mushrooms were evaluated.

2 EXPERIMENTAL

2.1 Samples and sampling

Laccaria amethystina Cooke is a bright greyish-purple mushroom when fresh, with a cap 10–52 mm in diameter. Its distribution is solitary to scattered; it is occasionally gregarious, primarily living under oak or beech or in forests of these trees and of pines.[13]

Pooled samples of the mushroom (cap and stem) comprising 10–100 specimens each were collected in two beech forests 15 and 35 km, respectively, north of Copenhagen. The arsenic content in the uncontaminated forest soils was 2–4 μg As g^{-1}, which is consistent with the Danish background concentration.[8] A similar pooled sample was taken at a contaminated location in a forest near the town of Hillerød, Denmark. The area was formerly used for wood impregnation using a mixture of arsenic pentoxide, cupric oxide and chromium trioxide, and spills from this process caused an intense contamination of the soil. The mushrooms were collected at 'hot-spots' where the arsenic concentration in the soil reached 500–800 μg As g^{-1} in order to illustrate the potential arsenic exposure of humans upon consumption of these mushrooms. During sampling of the mushrooms care was taken not to contaminate the biological material by soil particles. Upon return to the laboratory the samples were freeze-dried and ground in a mortar, then sieved through a 0.5-mm mesh plastic sieve. The powdered samples were stored dry in the dark at room temperature. The water-extractable (bioavailable) arsenic in the contaminated topsoil was present as arsenate.[14]

2.2 Total arsenic determinations

The dry sample (0.1 g) was digested using nitric acid in a high-pressure Teflon-lined steel bomb type DAE II (Berghof GmbH, Tübingen, Germany) which was heated at 160 °C for 4 h. After cooling, the wet-ashed acidic residue was diluted by water; total arsenic was then determined by Zeeman-corrected graphite furnace atomic absorption spectrometry, using platform atomization in pyrolytic graphite tubes. Palladium–magnesium nitrate was used as a chemical modifier to prevent loss by volatilization of arsenic during the furnace temperature programme, and quantification was based on peak area absorbance measurements using the method of standard additions.[6,15]

2.3 Extraction of arsenic species

The dried mushroom sample (0.1 mg) was placed in a 10-ml centrifuge tube and 5.00 ml of a mixture of methanol and water 1 + 9 (v/v) was added. The capped centrifuge tube was inserted into the sample cylinder and positioned in the microwave apparatus (Maxidigest MX 350, Prolabo, Paris, France). Before the continuous focused microwave energy

was applied, 100 ml of cold water (called ballast water) was added to the sample cylinder and surrounded the capped centrifuge tube. The purpose of using ballast water was to absorb part of the microwave energy and thereby to protect the sample from overheating. The microwave apparatus was operated at a range of power and time combinations, and the optimum extraction efficiency was achieved by using four treatments at 75 W power (25% of total power) for 8 minutes each. After each treatment the capped tube, which had reached a maximum temperature of 70 °C, was cooled under running tap-water and the ballast water was renewed. After the fourth and final microwave treatment the sample/extractant mixture was centrifuged and the clear supernatant was injected into the HPLC–ICP–MS system without any further sample pretreatment.

2.4 Arsenic speciation using HPLC–ICP–MS

Aliquots of the sample extracts were diluted with water before the quantitative analysis using anion- and cation-exchange high-performance liquid chromatography (HPLC) systems for the separation of the arsenic species.[6] Arsenic was detected on-line by inductively coupled plasma mass spectrometry (ICP–MS) as described in detail elsewhere.[14] For the organic polymeric strong anion-exchange HPLC column used (ION-120, Interaction Chromatography, CA, USA), a 45 mM ammonium carbonate solution in 97 + 3 (v/v) water-methanol at pH 10.3 at a flow rate of 1 ml min^{-1} served as the mobile phase. For the Ionosphere-C silica-based strong cation-exchange HPLC column (Chrompack International, Middelburg, The Netherlands), a 5 mM pyridinium formate solution in the same solvent mixture at pH 2.7 served as the mobile phase. The ICP–MS instrument (Elan 5000, Sciex Perkin-Elmer, Ont., Canada) was operated at 1300 W radio-frequency power for optimum arsenic signal intensity,[17] which was recorded versus time using the Graphics software facility.

Aqueous solutions of standard substances of As(III), As(V), MMA, DMA and TMAO were chromatographed on the HPLC–ICP–MS systems individually or as mixtures and their retention times (T_R) were recorded. In this way the chromatographic peaks emerging after injection of samples were identified by their retention time values. Based on the recorded signal peak height intensities of injected standards, calibration curves were constructed.[6]

3 RESULTS AND DISCUSSION

3.1 Methylated and inorganic arsenicals

The use of separate anion- and cation-exchange HPLC systems has proved useful and flexible for the separation of 11 anionic or cationic arsenic species occurring in marine biological samples. In the anion-exchange HPLC system, the cationic arsenic species elute unretained with the void volume followed by DMA, As(III), MMA and As(V). In the cation-exchange HPLC system As(III), MMA and As(V) elute, unretained with the void volume, followed by AsSugar **13,** DMA, 2-dimethylarsinylacetic acid, AsB, AsSugar **11,** TMAO, arsenocholine (AsC) and tetramethylarsonium-ion (TMAs) as discussed in detail by Larsen[2]. Typical chromatograms obtained using these two separation techniques with mushroom extracts as samples are given in Figs 1 and 2.

The anion-exchange chromatogram (Fig 1a) showed a large peak at T_R 130–200 s corresponding to DMA, followed by two small peaks which corresponded to MMA and As(V). The peak which eluted unretained with the void volume at T_R 100 s in the same chromatogram indicated that additional neutral or cationic arsenic species were present in the sample extract.

The cation-exchange chromatogram (Fig. 1b) showed a peak at T_R 480 s which corresponded to the protonated TMAO,[17] and a peak corresponding to the protonated form of DMA[14] which eluted at T_R 60–200 s. Furthermore, the identity of a minor peak which eluted at T_R 300 s remains unknown.

3.2 Arsenobetaine in *Laccaria amethystina*

In the cation-exchange chromatogram of sample C from the highly contaminated soil (Fig. 1b), two peaks additionally appeared at T_R 50 s and at T_R 210 s. In order to improve the chromatographic selectivity of these peaks, the mobile phase was diluted 1 + 9 (v/v) with 3% methanol in water.[2] The chromatogram of sample C recorded under these conditions (Fig. 2) showed peaks at T_R 60 s and at T_R 600 s. Previous experiments[2] showed that some dimethylarsinylriboside derivatives elute in the cation exchange system at T_R 60 s. The retention time of the latter peak was identical with that of AsB. In order to substantiate further the presence of AsB in this sample, the mushroom extract was spiked with 140 pg As as AsB. The

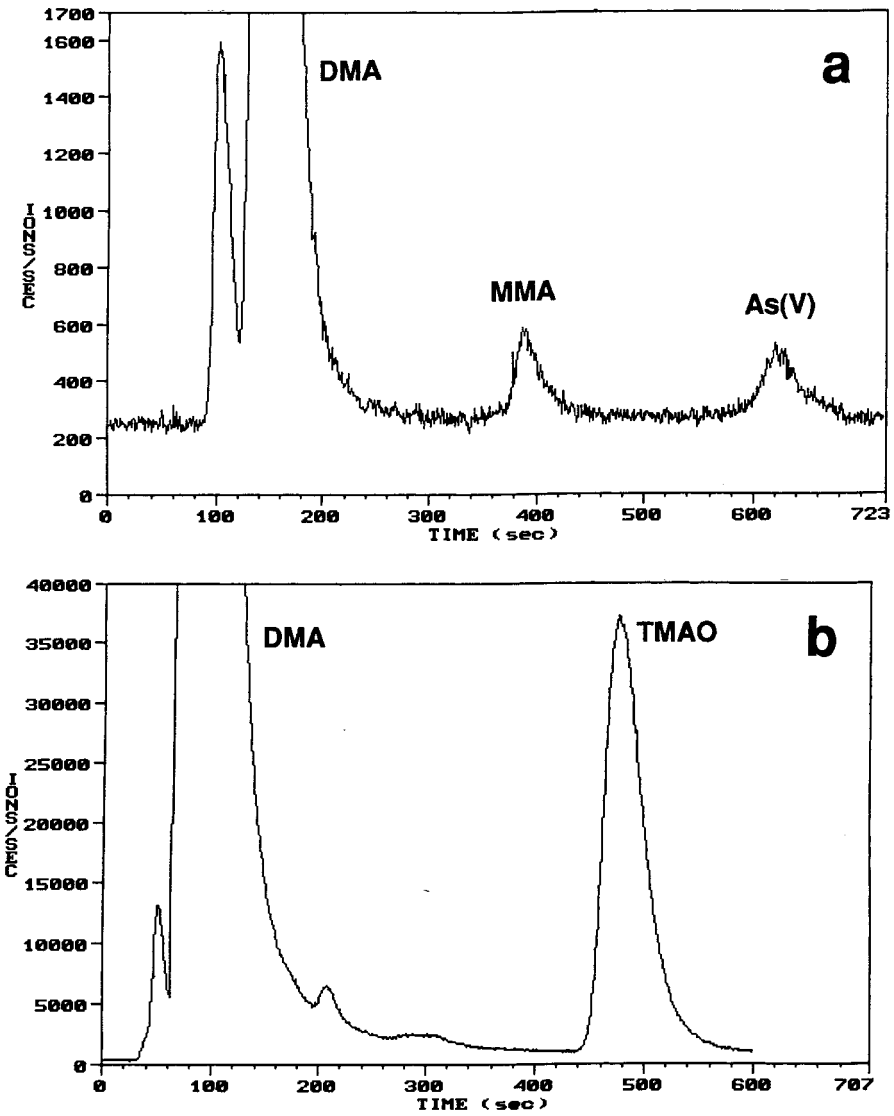

Figure 1 Anion-exchange HPLC–ICP–MS chromatography of (a) sample A using 1 + 20 (v/v) dilution of the extract with water. (b) Cation-exchange HPLC–ICP–MS chromatography of sample C using 1 + 4 (v/v) dilution by water before injection. Injected volume 50 µl. Vertical axis, ICP–MS signal intensity in counts s^{-1} at *m/z* 75 (^{75}As); horizontal axis, time (s). For species acronyms, please see text.

chromatogram in Fig. 2 shows that the spiked standard co-eluted with the AsB originally present in the sample. Although support for the presence of an arsenosugar (e.g. a dimethylarsinylribosyl derivative with a glyceryl phosphate side chain) was provided by co-chromatography with authentic material (Fig. 2), an independent confirmation would be ideal. This is because the arsenic-containing compound which eluted at T_R 60 s is barely separated from the chromatographic void volume (T_R 30 s) and the risk of wrong identification based on retention time alone cannot be ruled out. Unfortunately, the anion-exchange chromatographic system used for the chromatograms in Fig. 1 did not provide any additional information because the arsenosugar co-eluted with DMA in this system.[2] Therefore the extract of mushroom sample C was analysed by anion-exchange HPLC–

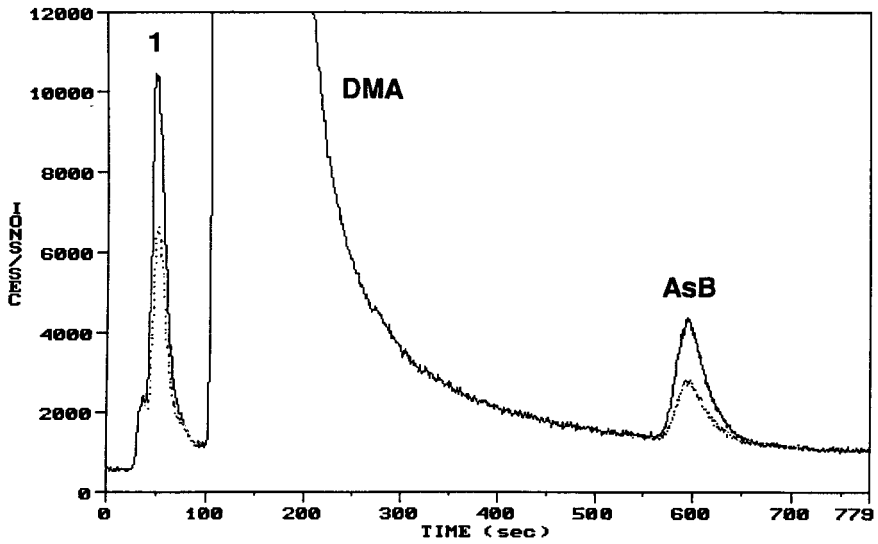

Figure 2 Cation-exchange HPLC–ICP–MS chromatography of sample C using 1 + 4 (v/v) dilution by water. The mobile phase was a 0.5 mM solution of pyridinium formate in 97% water–3% methanol at pH 2.7. The solid line in the chromatogram indicates the arsenic signal intensity recorded after spiking the diluted sample extract with an arsenosugar (peak **1**) and AsB at 165 pg and 140 pg as arsenic, respectively. The dotted trace indicates the signal intensity before spiking. Injected volume 50 µl. Vertical axis, ICP–MS signal intensity in counts s^{-1} at *m/z* 75 (^{75}As); horizontal axis, time (s). For species acronyms, please see text.

ICP–MS using 20 mM phosphate at pH 4.5 as the mobile phase and a Hamilton PRP-X100 strong anion-exchange HPLC column as the stationary phase.[18] In this chromatographic system the retention time of the arsenosugar was established at T_R 360 s using the standard substance. The chromatogram of the sample however, did not show any peak at that retention time. Consequently, the identity of peak **1** remains unknown. Additional work will be carried out to investigate the identity of the unknown substance in *Laccaria amethystina* collected from the highly arsenic-contaminated area.

3.3 Quantitative results

AsB was present in sample C at 0.7 µg As g^{-1} as estimated by the method of standard additions. This value represented 0.05% of the total arsenic content in that sample. AsB could not be detected in any of samples A (<0.6%) or B (<2.0%). The quantitative results which are given in Table 1 show that arsenic as DMA accounted for 68–74% of the total arsenic whereas MMA and the toxic As(V) amount to a few per cent of the total. The likewise toxic As(III) could not be detected in any of the samples. Even though the total arsenic concentration varied markedly, the relative amounts of the arsenic species detected were similar in samples from uncontaminated and contaminated locations. This shows that the mushroom by itself, or in association with soil bacteria, is able to biosynthesize high amounts of DMA also in soil highly contaminated by inorganic arsenic. The high proportion of arsenic as DMA found in *Laccaria amethystina* samples in this study and the low amounts of other inorganic or methylated arsenic species is in accordance with similar results for this mushroom species collected in other parts of Europe.[11,12]

The sum of the extracted species plus unextracted arsenic is in good agreement with the independent total arsenic determination for the sample from the highly contaminated soil (sample C), whereas the similar sum of concentrations unfortunately is somewhat higher for samples A and B. However, the microwave-assisted extraction technique that was used left between 15 and 32% of the total arsenic unextracted in the samples.

3.4 Aspects of arsenic metabolism in terrestrial biota

The metabolism of arsenic in marine life has been proposed to involve the algae-mediated transformation of As(V) to AsSugar via DMA, followed by

Table 1. Quantitative results for four arsenic species and total arsenic concentrations in three samples of *Laccaria amethystina*[a]

Sample	Location	MMA	DMA	TMAO	As(V)	Unextracted[b]	Sum[c]	Total arsenic[d]
A	Uncontaminated	1.0 (1.2%)	61 (68%)	1.8 (2.0%)	3.0 (3.3%)	23 (26%)	90	77
B	Uncontaminated	0.9 (2.9%)	23 (74%)	0.2 (0.6%)	1.9 (6.1%)	4.6 (15%)	31	23
C	Contaminated	4.1 (0.3%)	970 (68%)	20 (1.4%)	n.d.[e]	460 (32%)	1450	1420

[a] All results are given as μg As g^{-1} dry mass, and the relative amounts are given in parentheses as a percentage of the sum.
[b] Concentration of total arsenic remaining after extraction.
[c] Sum of the concentrations of arsenic species and the unextracted arsenic.
[d] Determined in an independent total arsenic determination.
[e] Not detectable, <1.4 μg As g^{-1} dry weight.

several other biosynthetic steps which lead to the formation of AsB.[4] The recent findings of AsB, AsC and TMAs in some arsenic-accumulating mushroom species[12,18–20] indicate that biosynthetic routes similar to those suggested for the formation of AsB and TMAs in the marine environment may also be active in the terrestrial environment. Furthermore, AsB and AsSugars were present in shrimp and mussels collected from deep-sea hydrothermal-vent communities in the Atlantic. In this environment biosynthetically active marine algae are absent, and it was suggested that the formation of the arsenicals was based on the biosynthetic activity of autotrophic bacteria.[21]

However, it remains unknown if the biosynthesis of these organoarsenicals in the terrestrial environment is carried out by soil bacteria associated with the mycelium of *Laccaria* or by the mycelium itself.

3.5 Human health risk considerations

On the basis of epidemiological studies inorganic arsenic has been classified as a suspected human carcinogen of the lung and skin,[22] but in experimental animal studies this compound has not been proved as a carcinogen. Other toxicological animal studies, however, have focused on the possible adverse effects of dimethylarsinic acid, which is the main metabolite of inorganic arsenic.[23] In these experiments mice were orally exposed to a single dose of 1500 mg kg^{-1} body weight of sodium dimethylarsinate. The dose-induced lung-specific DNA damage, *i.e.* DNA single-strand breaks, may be caused by the dimethylarsenic peroxyl radical which is formed during the further metabolism of DMA by reaction between molecular oxygen and DMA.[24] In a study *in vitro*, human alveolar cells were exposed to DMA; this led to a more detailed understanding of the mechanism of the DNA strand breaks and the formation of DNA–protein cross-links.[25] Besides its genotoxic effect *per se*, DMA in mice also promoted the formation of tumours which were initiated by exposure to 4-nitroquinoline 1-oxide, a chemical carcinogen.[26] The genotoxic effects observed in experiments *in vivo* and *in vitro* may indicate a potential pathway for chemical pulmonary carcinogenesis in man.

The genotoxic effects of DMA in animal experiments raise the question of whether ingestion of this compound via consumption of *Laccaria amethystina* with the extraordinarily high DMA concentration of 970 μg As g^{-1} represents any risk to human health. The amount of DMA ingested by an adult (70 kg body weight) via consumption of 50 g (dry weight) of the mushroom from the contaminated location is around 50 mg of arsenic or about 100 mg of DMA. This corresponds to an exposure of 1.4 mg DMA kg^{-1} body weight, which is about three orders of magnitude below the DMA doses used in the animal experiments (1500 mg kg^{-1} body weight). However, since the genotoxic effect observed in the animal experiments may lead to the formation of cancer of the lung and, because no zero-effect exposure level to DMA has been established, we conclude that ingestion of *Laccaria amethystina* grown on the contaminated soil should be avoided in order to minimize the risk of development of cancer in humans.

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REFERENCES

1. J. S. Edmonds and K. A. Francesconi, *Mar. Poll. Bull.* **26,** 665 (1993).
2. E. H. Larsen, *Fresenius' J. Anal. Chem.* **352,** 582 (1995).
3. Y. Shibata and M. Morita, *Appl. Organometal. Chem.* **6,** 343 (1992).
4. K. A. Francesconi and J. S. Edmonds, *Oceanogr. Mar. Biol. Rev.* **31,** 111 (1993).
5. W. R. Cullen and K. J. Reimer, *Chem. Rev.* **89,** 713 (1989).
6. E. H. Larsen, G. Pritzl and S. H. Hansen, *J. Anal. At. Spectrom.* **8,** 1075 (1993).
7. *Food Monitoring in Denmark, Nutrients Contaminants 1983–1987,* Publication No. 195, National Food Agency of Denmark DK-2860 Søborg, Copenhagen, Denmark (1990).
8. E. H. Larsen, L. Moseholm and M. Nielsen, *Sci. Tot. Environ.* **126,** 263 (1992).
9. A. Andersen, S.-E. Lykke, M. Lange and K. Bech, *Trace Elements in Edible Mushrooms*, Publication No. 82, National Food Agency of Denmark, DK-2860 Søborg Denmark (1982) In Danish.
10. T. Stijve and B. Bourgui, *Dtsch. Lebensm. Rundsch.* **87,** 307 (1991).
11. A. R. Byrne, M. Tusek-Znidaric, B. K. Puri and K. Irgolic, *Appl. Organometal. Chem.* **5,** 25 (1991).
12. A. R. Byrne, Z. Slejkovec, T. Stijve, L. Fay, W. Gössler, J. Gailer and K. Irgolic, *Appl. Organometal. Chem.* **9,** 305 (1995).
13. G. M. Mueller, *Nord. J. Bot.* **10**(6), 665 (1991).
14. S. H. Hansen, E. H. Larsen, G. Pritzl and C. Cornett, *J. Anal. At. Spectrom.* **7,** 629 (1992).
15. E. H. Larsen, *J. Anal. At. Spectrom.* **6,** 375 (1991).
16. E. H. Larsen and S. Stürup, *J. Anal. At. Spectrom.* **9,** 1099 (1994).
17. E. H. Larsen, G. Pritzl and S. H. Hansen, *J. Anal. At. Spectrom.* **8,** 557 (1993).
18. D. Kuehnelt, W. Goessler and K. J. Irgolic, *Appl. Organometal. Chem.* **11,** 289 (1997).
19. D. Kuehnelt, W. Goessler and K. J. Irgolic, *Appl. Organometal. Chem.* **11,** 459 (1997).
20. Z. Slejkovec, A. R. Byrne, T. Stijve, W. Goessler and K. J. Irgolic, *Appl. Organometal. Chem.* **11,** 673 (1997).
21. E. H. Larsen, C. R. Quétel, R. Munoz, A. Fiala-Medioni and O. F. X. Donard, *Mar. Chem.* **57**, 341 (1997).
22. *Some Metals Metallic Compounds*, vol. 23 of *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* International Agency for Research on Cancer, Lyon 1980 pp. 39–141.
23. M. Vahter, *Environ. Res.* **21,** 446 (1981).
24. K. Yamanaka and S. Okada, *Environ. Health. Perspect.* **102,** 37 (1994).
25. K. Kato, H. Hayashi, A. Hasegawa, K. Yamanaka and S. Okada, *Environ. Health. Perspect.* **102,** 285 (1994).
26. K. Yamanaka, K. Ohtsubo, A. Hasegawa, H. Hayashi, H. Ohji, M. Kanisawa and S. Okada, *Carcinogenesis* **17,** 767 (1996).

