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Supplementary Material

Lipids that contain arsenic in the Mediterranean mussel, *Mytilus galloprovincialis*

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Arsenolipid extraction methodology is not standardized for all biological matrices, as it requires adjustments of standard lipid extraction protocols depending on the sample type. In this work we compare the established methodology for arsenolipids using DCM:MeOH with MTBE:MeOH extraction protocols previously reported for non-arsenic lipids. Using *Mytilus edulis* as the source of a homogeneous mussel sample, the work described below established the methodology tested and then adopted in the further works conducted with the study mussel, *Mytilus galloprovincialis*.

Methods:

Chemicals and Standards: as described in the manuscript.

Total arsenic quantification: same procedures as those described on the manuscript.

Arsenolipids: Quantification and Speciation: same procedures as described on the manuscript.

Biological matrix

Mytilus edulis were purchased from the local market in Graz (Austria) (samples origin: Denmark, caught by dredging). Organisms were dissected for the whole tissue and freeze-dried to constant mass. The bulk sample was ground to a powder with a mortar and pestle and sieved (pore: 15 μm). Total arsenic concentration was found to be 10.1 ± 0.7 mg kg^{-1} (n = 8).

Lipid extraction procedures

For all extraction procedures, about 50 mg (weighed to 0.1 mg) of freeze-dried tissue was used. Lipid extraction was conducted in triplicates, using 15 ml propylene tubes (CellStar®Tubes, Greiner bio-one).

DCM:MeOH:

Method I: Lipid extraction was done on one set of triplicates. Sample was weighed into a tube and 5 mL of DCM:MeOH (2:1) solution was added, tubes were vortexed for 10 seconds and shaken on a rotatory shaker arm for an hour at room temperature. After the mixture was centrifuged (3500 g, 20 min), the DCM:MeOH fraction was collected. Another 5 mL DCM:MeOH (2:1) solution was added and procedure was repeated once more, with a longer time in the vortex mixer to allow the total miscegenation of the pellet. The two organic fractions were combined, and phase separation was attained by adding 3.5 mL of water and gently mixing by hand (5 turns). After collecting the water fraction, another 3.5 mL of water was added to the organic phase and the process repeated. The combined water fraction was washed by adding 5 mL DCM:MeOH (2:1), and mixing gently by hand, and the organic fraction was collected and added to the previous combined organic fractions.

MTBE:MeOH:

Method II: Lipid extraction followed the recipe described in Matyash et al. (2008) on one set of triplicates. Sample was weighed directly into to a 15 mL polypropylene tube, 1.5 mL of MeOH was added and the mixture vortexed for 10 seconds before addition of 5 mL of MTBE. The tube was shaken on rotatory shaker arm for an hour at room temperature. Phase separation was attained by adding 1.25 mL of water, without any shaking of tube; after 10 minutes the mixture was centrifuged at 3500 g (20 min). The upper organic phase was collected and the remaining layer and pellet were re-extracted with 2 mL of a mixture of MTBE/MeOH/H_2O (10:3:2.5 v/v/v); after 10 min, the mixture was centrifuged at 3500 g (20 min). The organic phase was collected and combined with the previous one. In a separate experiment, the extraction procedure above was repeated, in triplicate, but on this occasion the solvent volumes were doubled (a 50 mL polypropylene tube was used). No increase in the amount of lipid and arsenolipid extracted was noted. Therefore, all subsequent extractions were performed using the sample/solvent ratio described above, which also corresponded to the volumes used by Matyash et al. (2008).

Method IIa: The water partition when concerns arsenolipids is very important step as it removes water soluble compounds and reduces the void signal on the chromatography results. To infer on the water partition efficiency and effects on the MTBE/MeOH method output, lipid

extraction was conducted on one set of triplicates as described above except for water partition. Water was added and shaking by hand was done as described in the DCM:MeOH recipe (5 turns).

Results:

The methodology developed by Matyash and co-workers (2008) was found to be also suitable for arsenolipids extraction from blue mussels; it gave higher extraction efficiency of total lipids and for arsenolipids (Table S1), particularly for the latter eluting compound (Figure S1), when compared to the method using DCM:MeOH.

In both protocols, an organic phase water-wash resulted in lower extraction yield for lipids, with some of the arsenolipids partitioning into the aqueous phase. The shaking (Method IIa), does not affect the total output of the arsenolipids. However, it shows an increase of arsenolipids in the water fraction. Arsenolipids were thereby found to migrate to the water fraction. It can be hypothesized that MeOH solubility in water imparts "solubility" to lipids, or it assists micelle formation (Moelbert et al. 2004). This migratory event is possibly correlated to the sample matrix and lipid class ratios, since the presence of amphipathic compounds enhances micelle formation (Moelbert et al. 2004). The concentration of these lipids in the water fraction seems to be enhanced also by agitation, given that Method I and IIa had higher values for arsenolipids concentration on the water fraction (Table S1).

The MTBE:MeOH protocol was the more efficient method, it was less time consuming, did not suffer from Cl interference on the arsenic signal, experienced less degradation of arsenolipid compounds and is less toxic than DCM.

Table S1. Comparative extraction of lipids and arsenolipids outputs, using: DCM:MeOH (Method I) and MTBE:MeOH (Method II) and MTBE:MeOH with 1 hour shaking (Method IIa). Averaged values are followed by the standard deviation. Arsenic speciation was done in triplicates for all methods, except the Method DCM, with only one replicate.

	Method	Total Lipids (%)	Total Arsenic (µg As L^{-1})	Void Signal (µg As L^{-1})	Arsenolipids (µg As L^{-1})
Organic Fraction	**I**	16.8 ± 1.8 (n=3)	95.2 (n=1)	3.2 (n=1)	89.3 (n=1)
	II	18.7 ± 1.7 (n=3)	298.4 ± 26.6 (n=3)	118.5 ± 10.1 (n=3)	132.8 ± 11.5 (n=3)

	IIa	13.1 ± 0.5 (n=3)	154.0 ± 6.6 (n=3)	16.4 ± 1.3 (n=3)	131.8 ± 6.3 (n=3)
Water Fraction	**I**	-	232.7 (n=1)	-	15.9 (n=1)
	II	-	225.5 ± 12.1 (n=3)	-	2.5 ± 0.2 (n=3)
	IIa	-	445.4 ± 43.1 (n=3)	-	18.0 ± 0.9 (n=3)

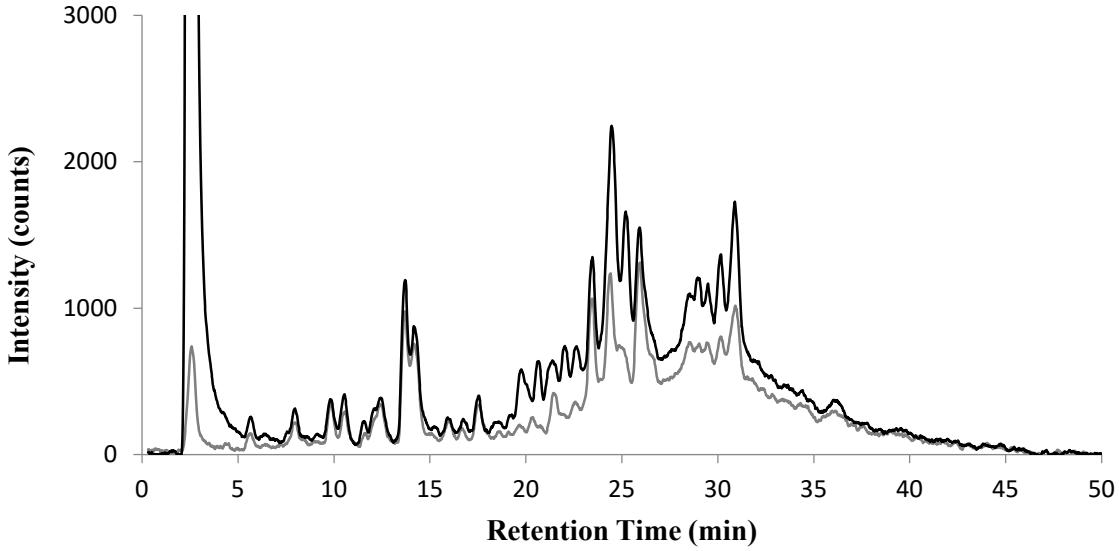

Figure S1. Chromatogram of arsenolipids type-profile (one replica), with Method I (DCM:MeOH) with a grey line, and Method II (MTBE:MeOH) with a black line.

MTBE:MeOH: Standard extraction and Spiked samples

Using Method II, mussels´ sample were spiked with the same a mixture of standards aiming for a 2 µg L^{-1} increment of each compound (II-Spk). Additionally, lipid extraction was conducted on 1 mL of a solution of the 6 in-house arsenolipid standards, each with a concentration of 10 µg As L^{-1} (II-StD). These two procedures were each done with three replicates.

Spiked samples showed that sample matrix does not alter the retention times (Figure S2) and the mean recovery was 98 ± 12% for all the spiked compounds. Extraction of the 6 standard compounds, using the Method II, was successfully accomplished, although the recovery was lower ranging from 64 - 85% (Table S4). Degradation of these compounds was observed as a

front peak was detected, with the averaged concentration of 0.50 ± 0.06 µg L^{-1} (peak not observed for method blanks).

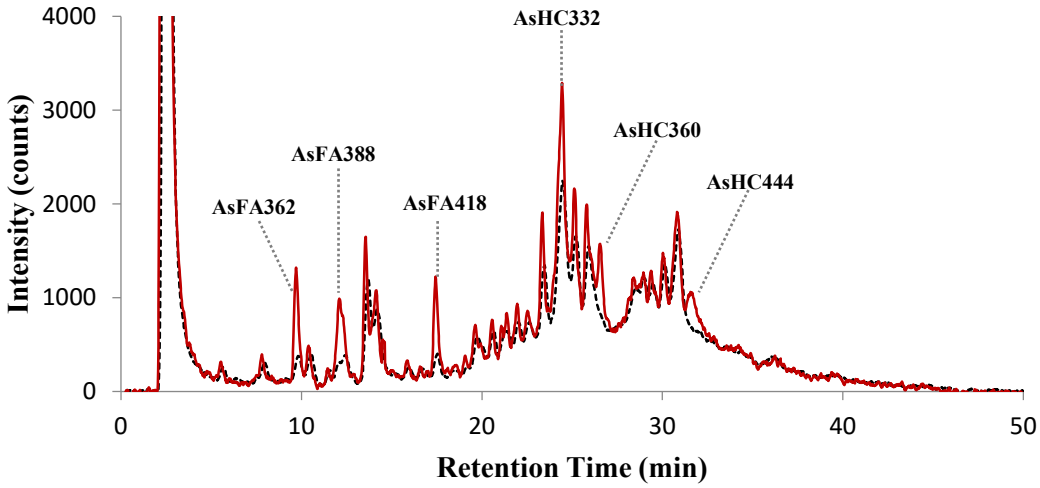

Figure S2. HPLC-MS chromatograms of arsenolipid profiles, with Method II (black dashed line) and for spiked samples (red line).

Table S2. Concentrations (µg L^{-1}) of the standard arsenolipids compounds present at the biologic sample matrix with the different methods: DCM:MeOH (Method I, n = 1) and MTBE:MeOH (Method II, IIa, II-Spk and II-StD, n = 3). nd: not detected. Averaged values are followed by the standard deviation. Arsenolipids equivalent to 2 µg As L^{-1} were added to the spiked samples.

Method	AsFA362	AsFA388	AsFA418	AsHC332	AsHC360	AsHC444
I	0.8	1.3	1.1	1.4	nd	nd
II	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	3.1 ± 0.1	nd	nd
IIa	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	2.6 ± 0.5	nd	nd
II-Spk	2.7 ± 0.2	2.9 ± 0.2	2.9 ± 0.0	5.0 ± 0.2	2.4 ± 0.0	2.6 ± 0.3
II-StD	8.5 ± 0.2	7.9 ± 0.2	7.3 ± 0.1	6.2 ± 0.4	6.4 ± 0.3	6.7 ± 0.3

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