SUPPLEMENTARY INFORMATION

Enhanced determination of As-phytochelatin complexes in *Chlorella vulgaris* using focused sonication for extraction of water-soluble species

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Methods:

As-GS/PC Extraction

Cells were counted and a known amount of cells transferred, Cells were harvested by centrifugation (5 min, 3000 *g*) washed with deionised water twice and transferred to 15 mL centrifuge tubes. Cells were then washed with desorption solution (1 mM K_2HPO_4, 5 mM MES, 0.5 mM $Ca(NO_3)_2$, pH 6) for 10 min (Sandau, Pulz and Zimmermann, 1996; Abedin, Feldmann and Meharg, 2002). The supernatant was discarded and pelleted cells extracted with 2 mL of 1% formic acid, the mixture was sonicated for 30 s using a Minidelta 8935 generator (FFR ultrasonics, 500 W, 35 kHz) fitted with a 3 mm titanium micro-tip. The micro-tip was rinsed with methanol, then sonicated in 1% formic acid for 10 s between each extraction to avoid cross-contamination. The volume of the extract was determined gravimetrically.

Total arsenic quantification

Total arsenic was quantified using an ICP-MS (X series II, Thermo Scientific, UK) in CCT (Collision Cell Technology) mode with He/H as collision cell gas using 20 µg L^{-1} gallium as an internal standard. The instrument was tuned daily using a 10 µg L^{-1} indium, cerium, cobalt, uranium and lithium solution and the software (Plasmalab) was provided from the manufacturer.

Quantitative speciation analysis

Samples were run immediately after extraction using HPLC-ICP-MS. Separation was achieved in a Discovery C_{18} column (15 x 2.1 mm) fitted with a pre-column Discovery C_{18}. Experimental/instrumental conditions were based on the previous work of (Bluemlein, Rabb and Feldmann, 2009).A gradient elution was used with 0.1% formic acid (eluent A) and 99.9% HPLC grade methanol (eluent B). The detailed elution profile was as follows: 0-20 min linear increase 0-20% B, 20-30 min 20% B, 30-32 min 20-0% B and 32-40 min 0%B. The flow rate was 0.2 mL min^{-1}.

The following parameters were adjusted for O_2 CCT in the ICP-MS according to the manufacturer's instructions: O_2 cell gas flow 0.6-1.45 mL min^{-1}, hexapole bias -9 V, quadrupole bias -14 V, focus voltage -2 V, D2 voltage -100 V. A post column make- up flow was achieved with a tee connector and 0.9 mL min^{-1} indium (20 µg L^{-1} in 2% HNO_3) as internal standard. The injection volume was 50 µL, the

monitored masses were: As, m/z 91 ([$^{75}As^{16}O+$]), S, m/z 48 ([$^{32}S^{16}O+$]) and In, m/z 115. Fresh external standards were prepared for quantification. For arsenic, different solutions of DMA were used and L-cysteine for the quantification of sulphur.

Method quantification limits (MQL) were calculated by multiplying 10 times the standard deviation signal (at the retention time of the quantification species) of 7 consecutive blank samples. Method detection limits (MDL) were calculated by 3.14 times the standard deviation signal of 7 consecutive blank samples to achieve 99% confidence intervals (CI) (at the retention time of the quantification specie).

A correction for methanol content in the mobile phase was performed as follows (Amayo et al., 2011): A blank was injected through the same chromatographic conditions. A post column addition of a solution containing 100 mg L^{-1} DMA and 20 µg L^{-1} indium (as internal standard) was made. The blank was analysed by ICP-MS for arsenic, sulphur and indium at m/z 91 ([$^{75}As^{16}O+$]), S, m/z 48 ([$^{32}S^{16}O+$]) and In, m/z 115.

Kinetics and concentration effect experiments

C. vulgaris cells were cultured for 3-5 days in Bold's media free of arsenic. The cells were then exposed to different concentrations (0-200 mg L^{-1}) of iAs(III), iAs(V) and DMA for 48 h. The concentrations of arsenic used in this study were chosen to elicit a response in *C. vulgaris* cells rather than to reflect natural environment conditions. In other experiments cells were exposed to 50 mg.L^{-1} of iAs(III), iAs(V) or DMA at different exposure times (4, 24, 48 and 72 h). The experiments were performed in triplicates. Cells were supplemented with 0.5% dextrose. GSH, PCs and As-GS/PC complexes were analysed.

iAs(V) exposure experiments results

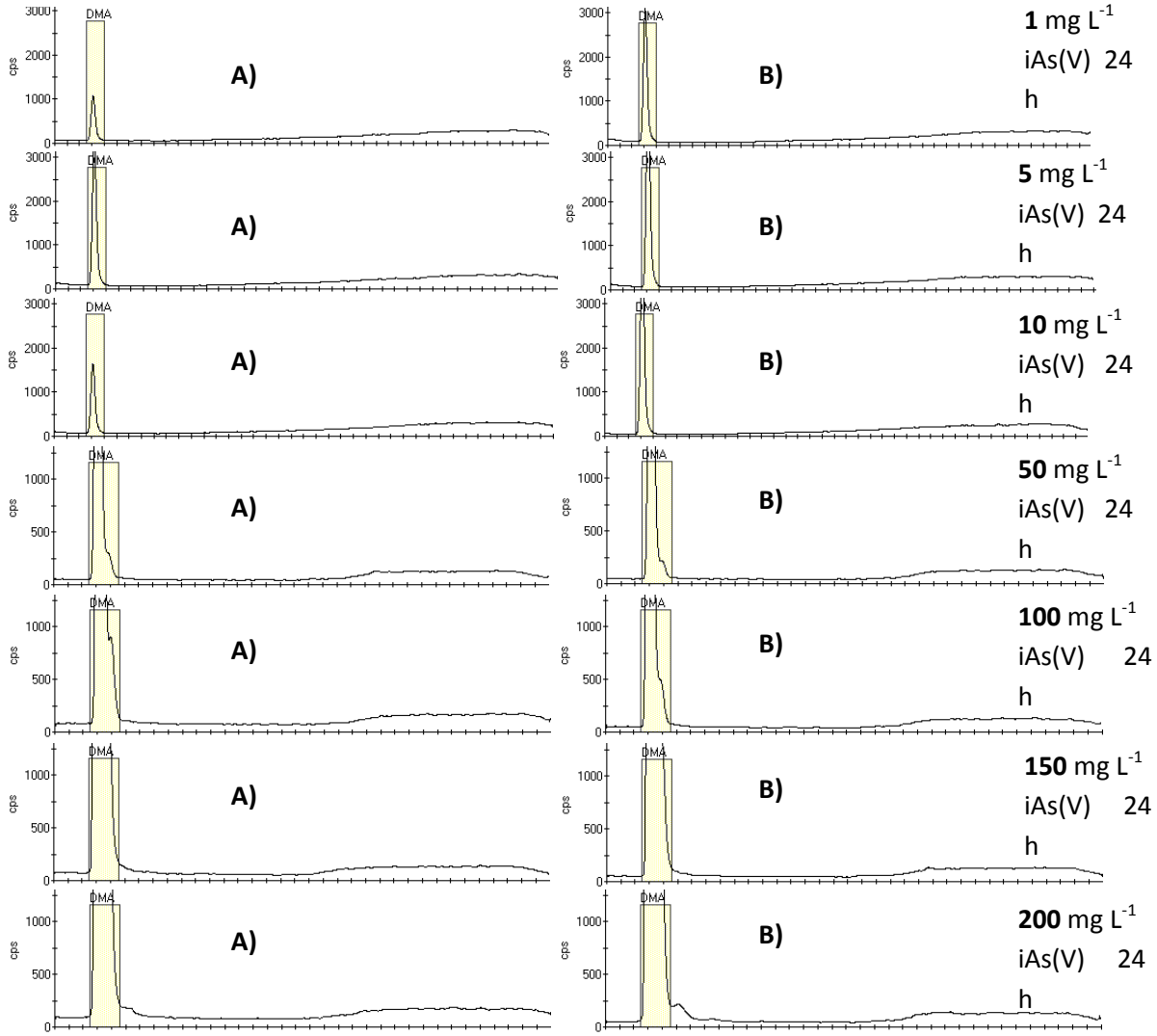

Fig S1 *C. vulgaris* cells exposed to 1, 5, 10, 50, 100, 150 and 200 mg L^{-1} of iAs(V) for 24 h, analysed by HPLC-ICP-MS, two replicates (A and B)

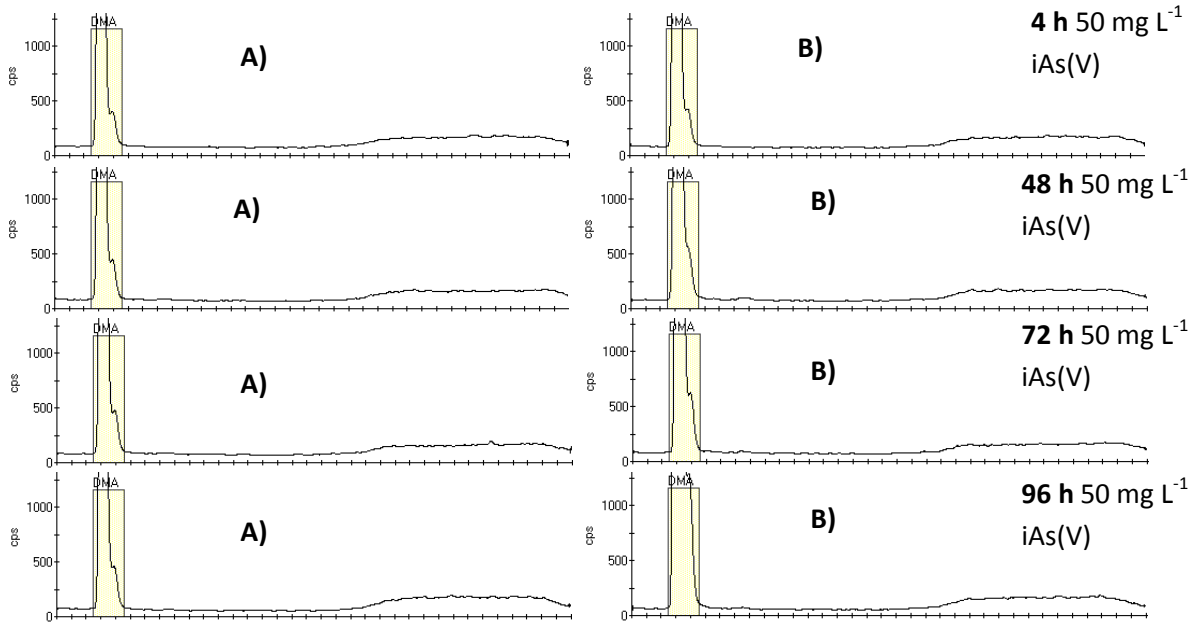

Fig S2 *C. vulgaris* cells exposed to 50 mg L^{-1} of iAs(V) for 4, 48, 72 and 96 h, analysed by HPLC-ICP-MS, two replicates (A and B)

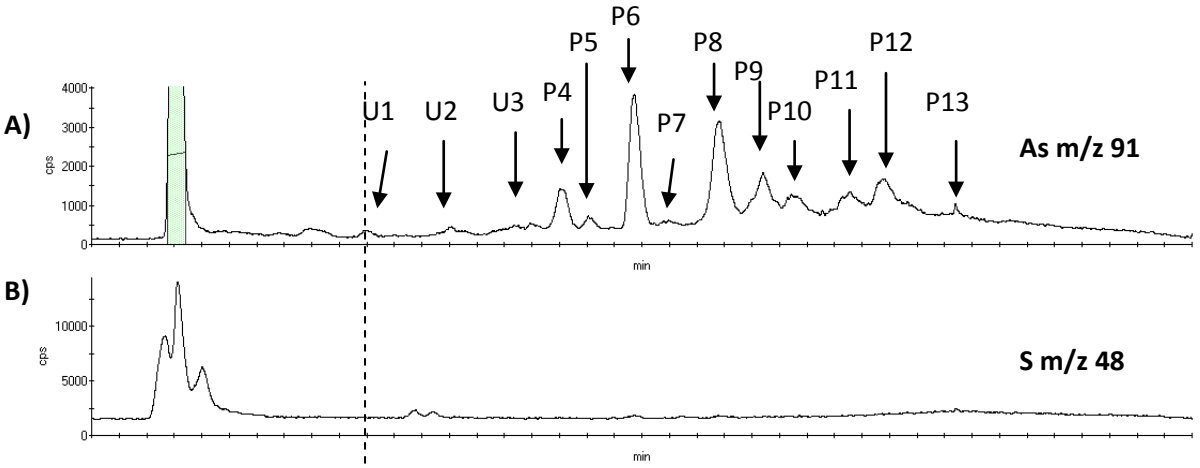

Fig S3 Peak identification chromatogram of *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III) for 48 h, extracted in 1% formic acid 30 s focused sonication. A) signal for arsenic at m/z 91 B) signal for sulphur at m/z 48. Peak identification: **U1-3** Unknowns, **P4** GS-As(III)-PC_2/GS-As(III)-γ-$(Glu\text{-}Cys)_2$, **P5** As(III)-γ-$(Glu\text{-}Cys)_2$, **P6** GS-As(III)-PC_2, **P7** GS-As(III)-γ-$(Glu\text{-}Cys)_2$-Ala, **P8** As(III)-PC_3/ MMA(III)-PC_2, **P9** MMA(III)-PC_2, **P10** As(III)-PC_3/ As(III)-$(PC_2)_2$, **P11** As(III)-$(PC_2)_2$/ As(III)-γ-$(Glu\text{-}Cys)_3$-Ala/ As(III)-γ-$((Glu\text{-}Cys)_2)_2$-Ala/ MMA(III)-γ-$(Glu\text{-}Cys)_2$-Ala, **P12** As(III)-PC_4 and **P13** As(III)-PC_4.

MS/MS analysis

GSH/PC molecules:

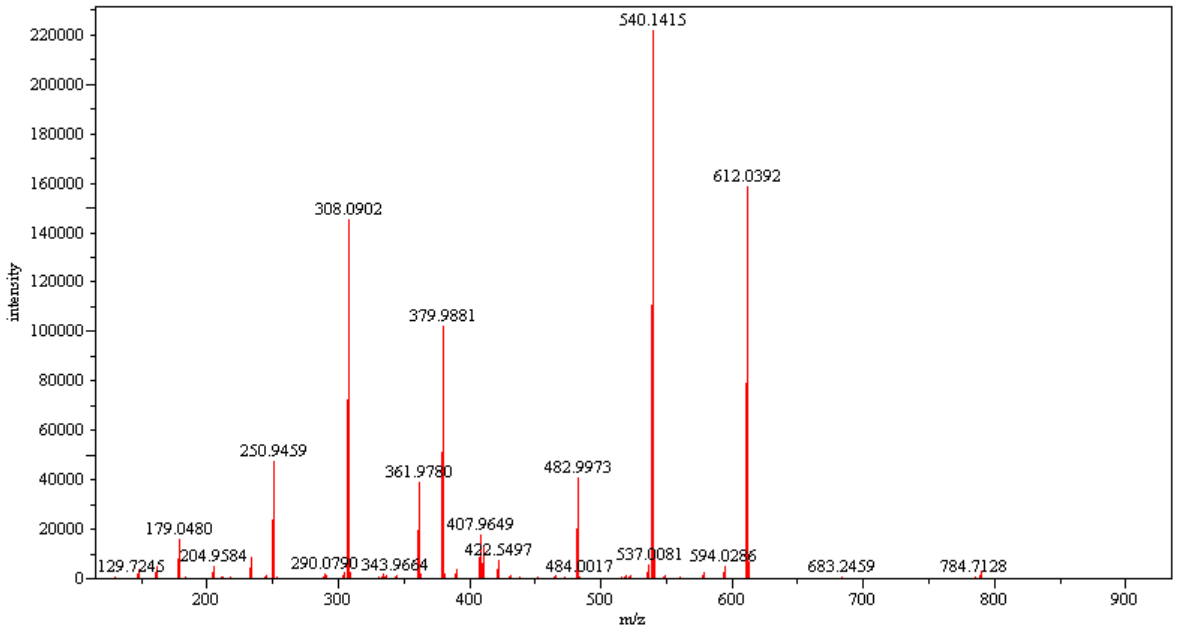

Fig S4 MS/MS spectra for GS-As(III)-PC_2 m/z 460.0663 RT:9.88 min, *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

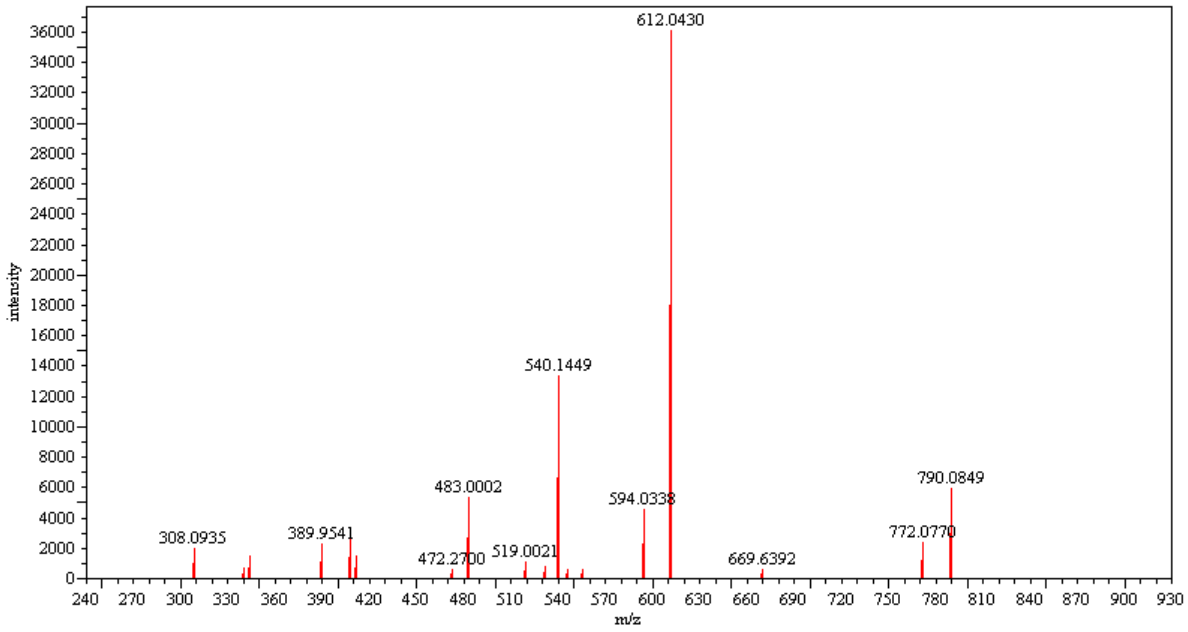

Fig S5 MS/MS spectra for GS-As(III)-PC_2 m/z 919.1247 RT:9.92 min, *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

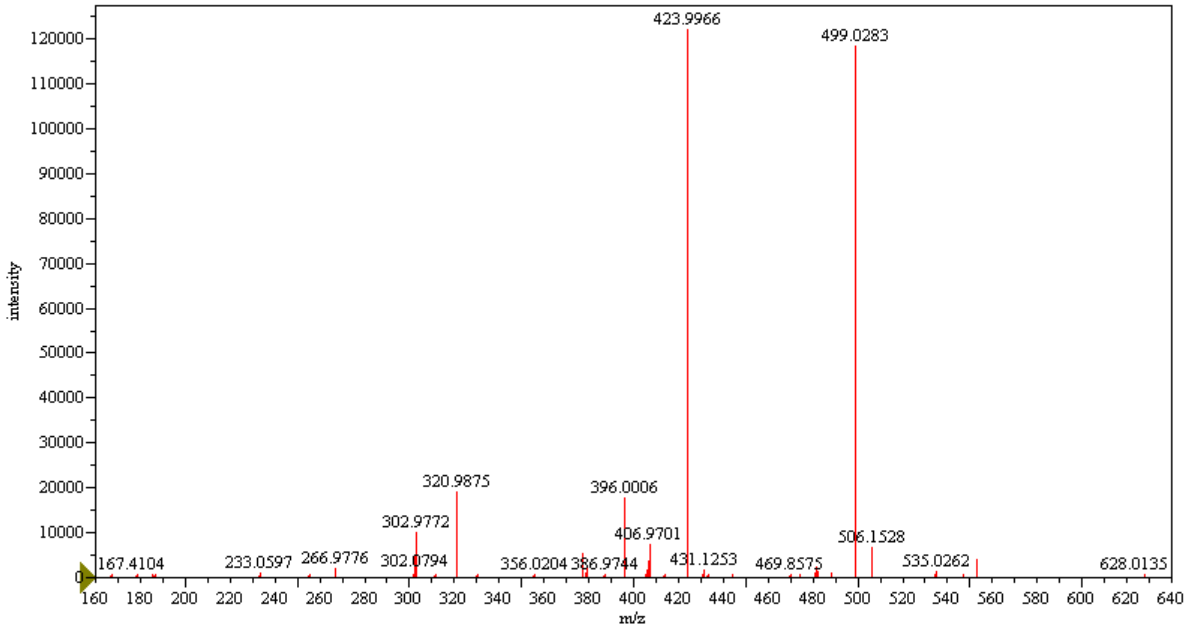

Fig S6 MS/MS spectra for MMA(III)-PC_2 m/z 628.0729 RT:11.31 min , *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

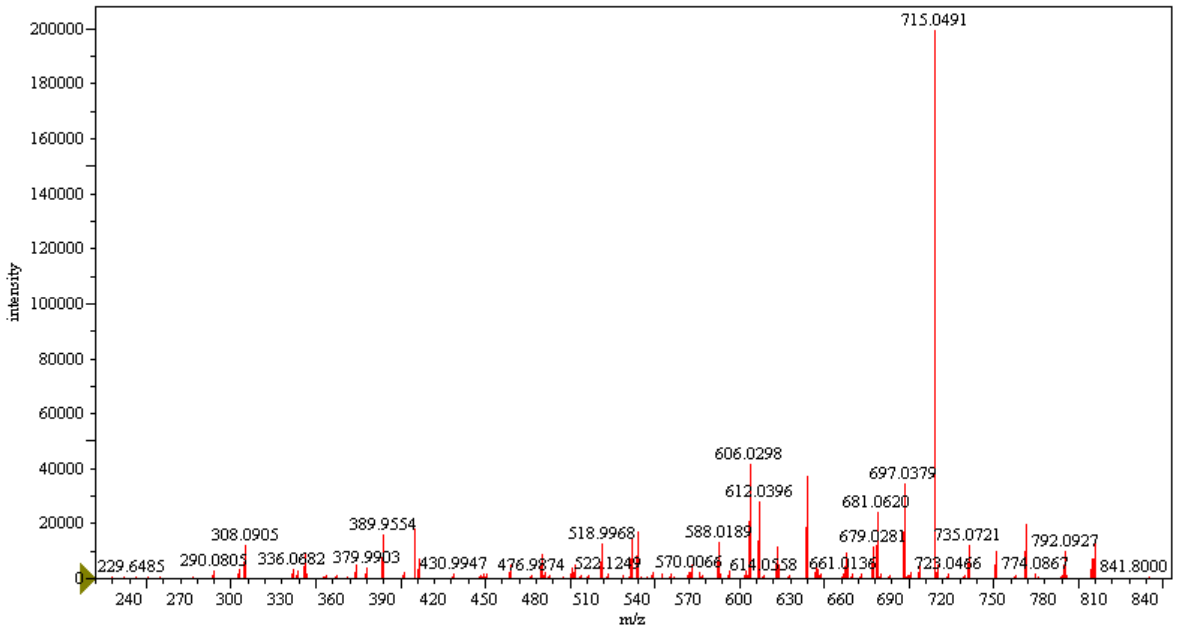

Fig S7 MS/MS spectra for As(III)-PC_3 m/z 844.0931 RT:11.49 min , *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

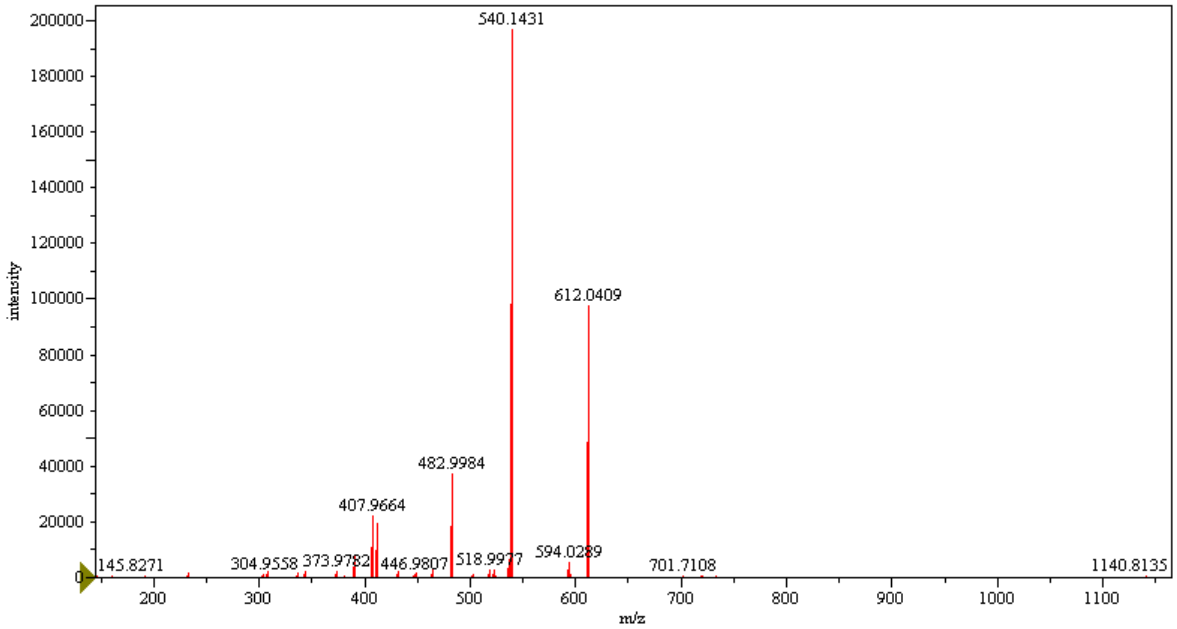

Fig S8 MS/MS spectra for As(III)-$(PC_2)_2$ m/z 576.0923 RT:13.07 min , *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

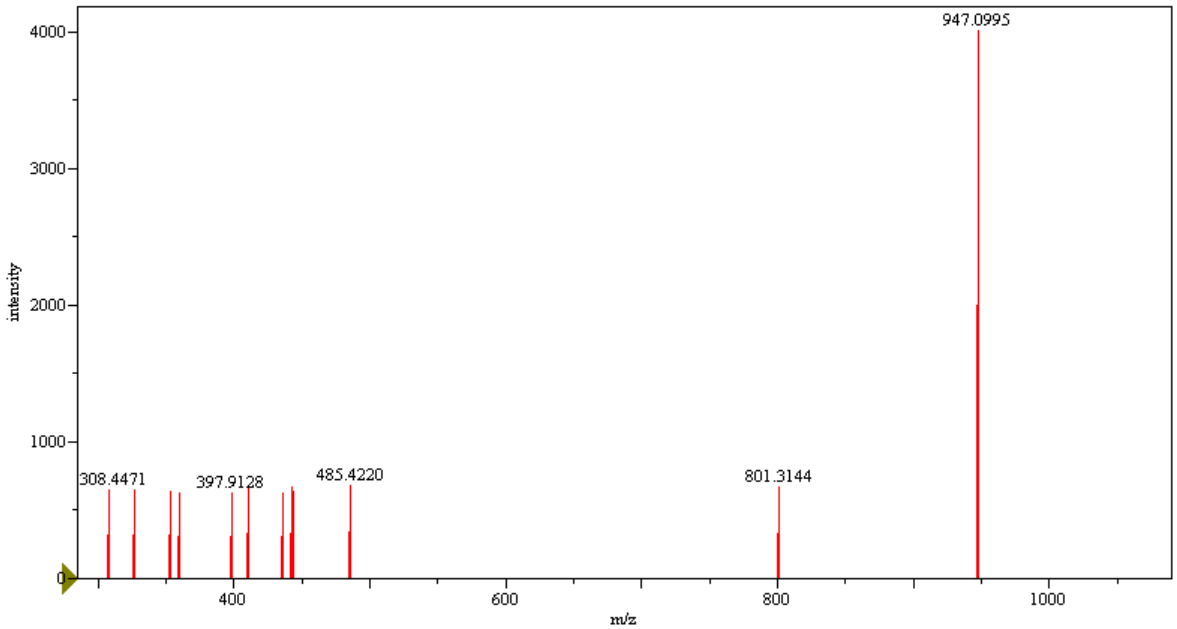

Fig S9 MS/MS spectra for As(III)-PC_4 m/z 1076.1455 RT:15.02 min , *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

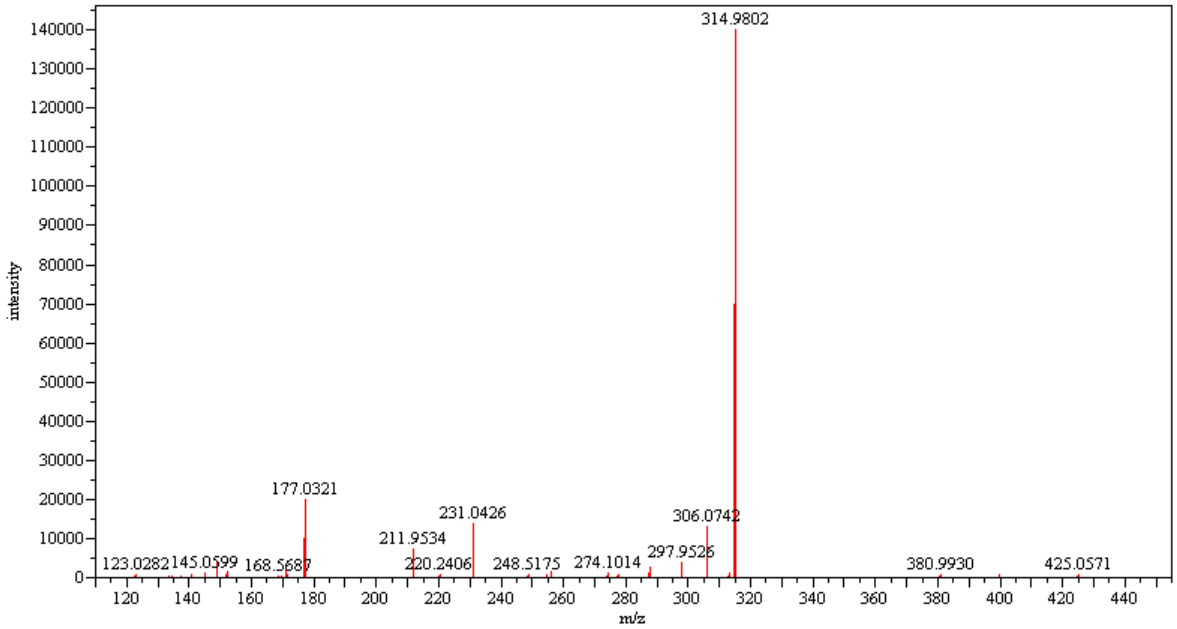

Fig S10 MS/MS spectra for DMAS^{V}-GS m/z 444.0247 RT 4.37 min , *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

hGSH/PC molecules:

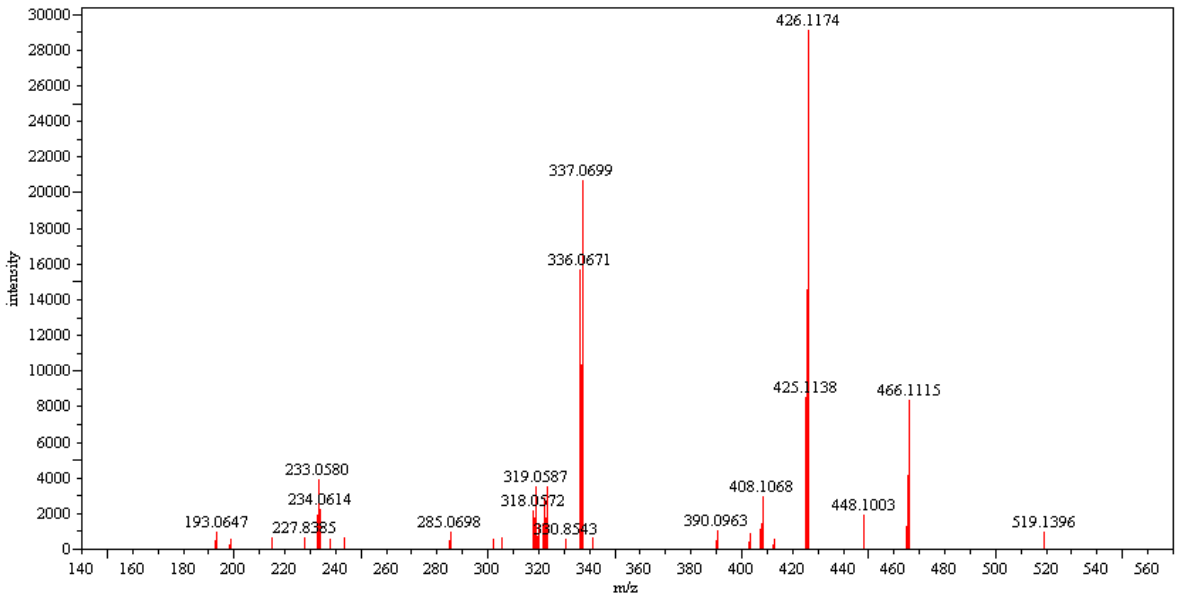

Fig S11 MS/MS spectra for γ-(Glu-Cys)$_2$-Ala m/z 554 RT 6.2845 min *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

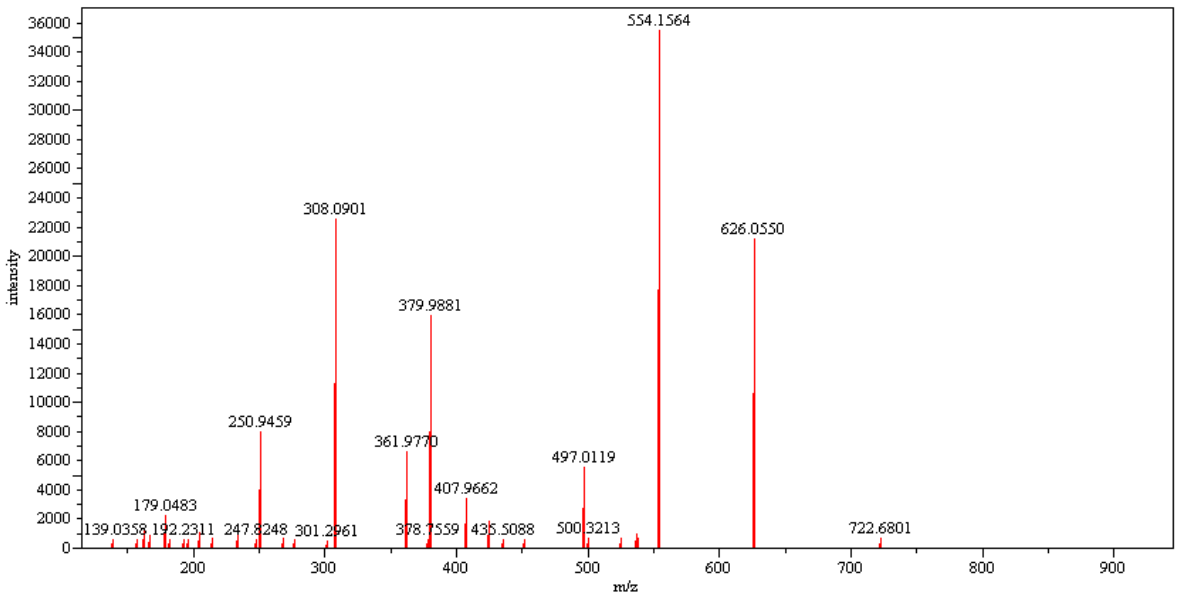

Fig S12 MS/MS spectra for As(III)- γ-(Glu-Cys)$_3$-Ala m/z 858 RT 10.9082 min *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

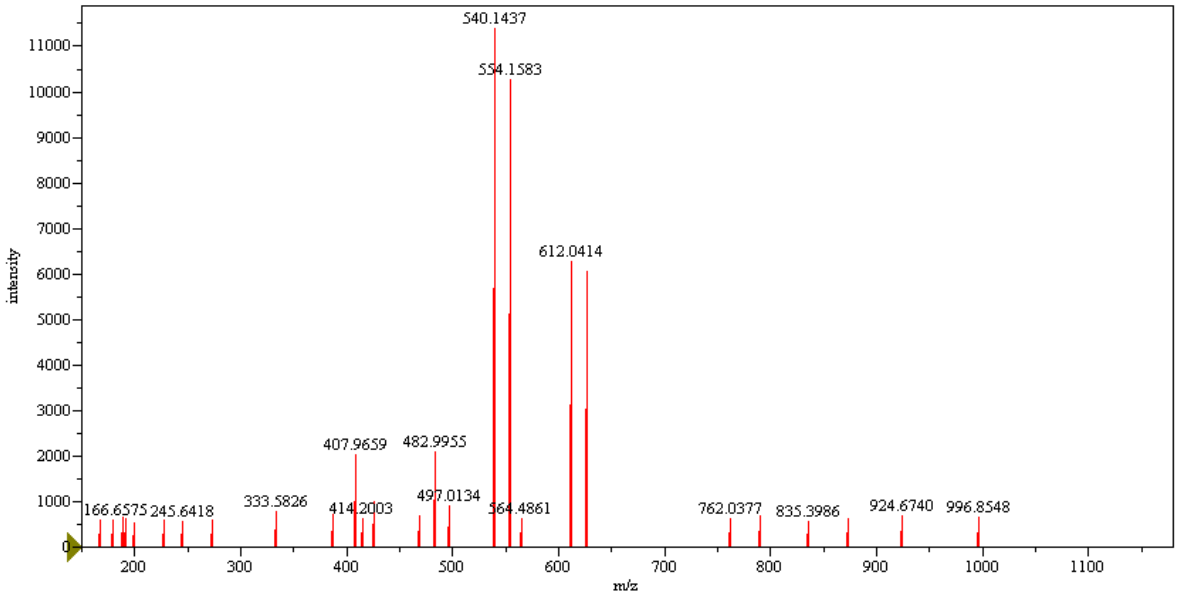

Fig S13 MS/MS spectra for As(III)- γ-((Glu-Cys)$_2$)$_2$-Ala m/z 583 RT 14.6667 min *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

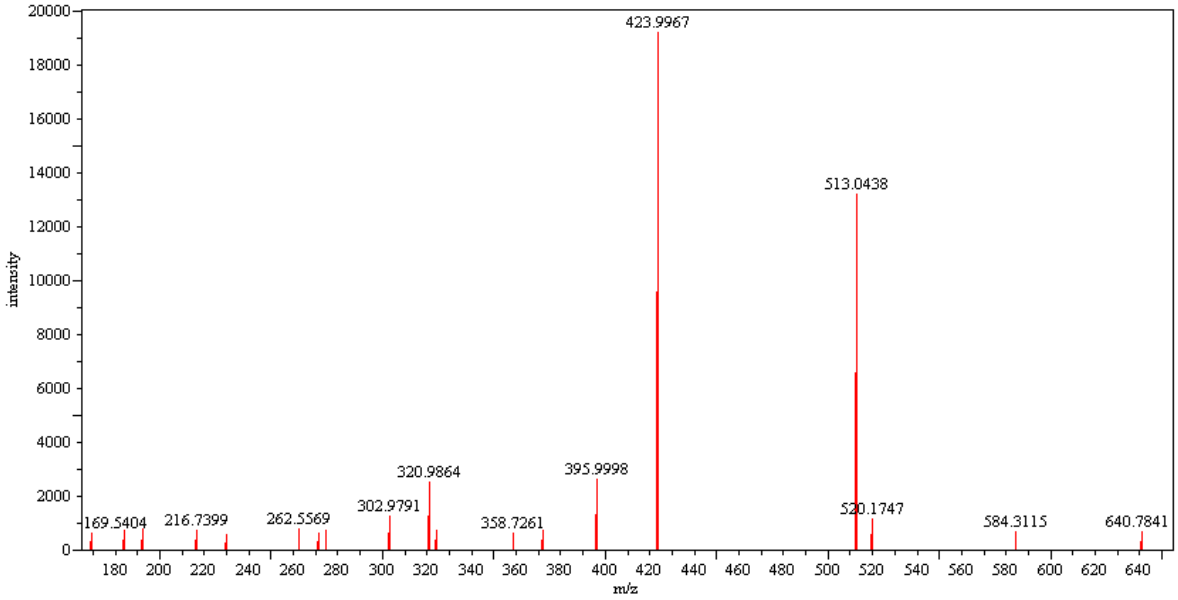

Fig S14 MS/MS spectra for MMA(III)-γ-(Glu-Cys)$_2$-Ala m/z 642 RT 14.8483 min *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

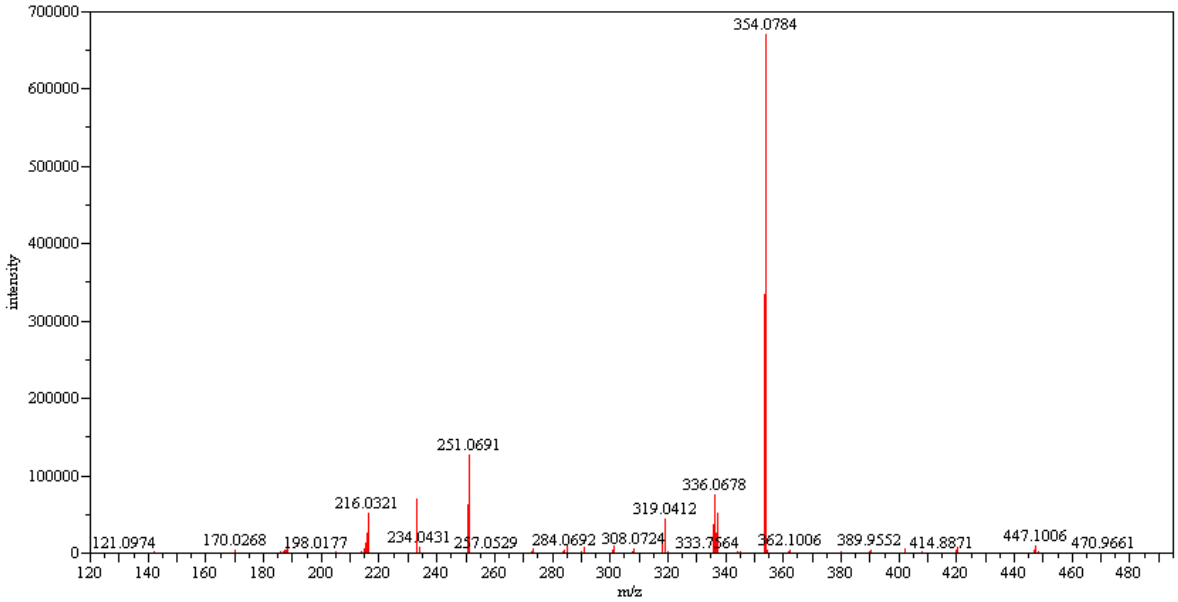

Fig S15 MS/MS spectra for γ-(Glu-Cys)$_2$ m/z 483 RT 4.6997 min *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

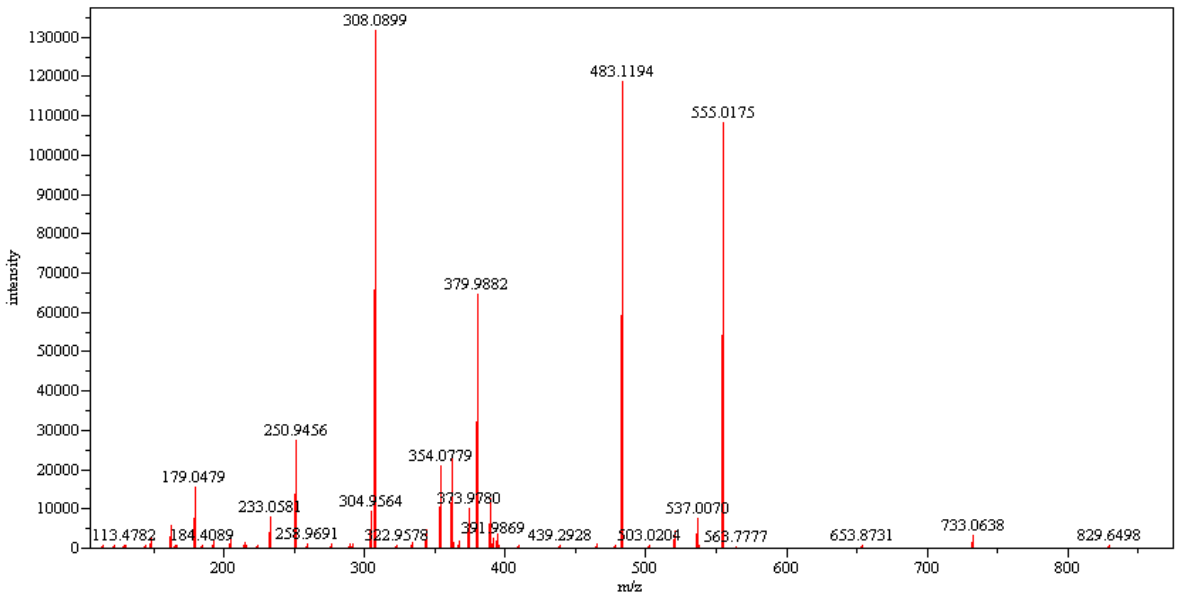

Fig S16 MS/MS spectra for mins GS-As(III)-γ-(Glu-Cys)$_2$ m/z 431 RT 8.0730 *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL)

Table S1 Summary of fragments found in MS/MS analysis *C. vulgaris* cells exposed to 50 mg L^{-1} iAs(III), analysed using ES-MS/MS (Orbitrap Discovery LTQ-XL) (n.o.- not observed,T- Theoretical, F - Found)

Molecule	m/z		b1	y8	b2	y7	b3	y6	b4	y5	b5	y4	b6	y3	b7	y2	b8	y1
Phytochelatins																		
PC_2	540	T	130.1	------	233.1	------	362.1	------	465.1	------	------	411.1	------	308.1	------	179.1	------	76.0
		F	130.1	------	233.1	------	362.1	------	465.1	------	------	411.1	------	308.1	------	179.1	------	76.0
PC_3	772	T	130.1	------	233.1	------	362.1	643.2	465.1	540.1	594.2	411.1	697.2	308.1	------	179.1	------	76.0
		F	130.1	------	233.1	------	362.1	643.2	465.1	540.1	n.o.	411.1	697.2	309.1	------	n.o.	------	76.0
As(III)-PC_3	844	T	130.1	------	233.1	------	362.1	643.2	465.1	540.1	594.2	411.1	697.2	308.1	------	179.1	------	76.0
		F	n.o.	------	n.o.	------	362.0	n.o.	465.1	540.1	594.0	411.1	697.0	308.1	------	n.o.	------	n.o.
GS-As(III)-PC_2	460	T	130.1	------	233.1	------	362.1	------	465.1	540.1	------	411.1	------	308.1	------	179.1	------	76.0
		F	129.7	------	233.1	------	362.0	------	n.o.	540.1	------	422.1	------	308.1	------	179.0	------	n.o.
As(III)-$(PC_2)_2$	576	T	130.1	------	233.1	------	362.1	------	465.1	540.1	594.2	411.1	------	308.1	------	179.1	------	76.0
		F	n.o.	------	233.1	------	n.o.	------	465.1	540.1	594.0	411.1	------	308.1	------	n.o.	------	n.o.
As(III)-PC_4	1076	T	130.1	875.1	233.1	772.2	362.1	643.2	465.1	540.1	594.2	411.1	697.2	308.1	826.2	179.1	929.2	76.0
		F	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	411.1	n.o.	308.4	n.o.	n.o.	n.o.	n.o.
MMA(III)-PC_2	628	T	130.1	------	233.1	------	362.1	------	465.1	------	------	411.1	------	308.1	------	179.1	------	76.0
		F	n.o.	------	233.1	------	n.o.	------	n.o.	------	------	n.o.	------	n.o.	------	178.8	------	n.o.
$DMAS^V$-GS	444	T	136.9	------	231.1	------	------	------	------	------	------	------	------	------	------	176.9	------	------
		F	136.9	------	231.1	------	------	------	------	------	------	------	------	------	------	176.9	------	------
Ala GSH/PC																		
γ-$(Glu\text{-}Cys)_2Ala$	554	T	130.1	------	233.1	------	362.1	------	465.1	------	------	425.1	------	322.1	------	193.1	------	90.1
		F	130.1	------	233.1	------	362.1	------	465.1	------	------	425.1	------	322.1	------	193.1	------	90.1
GS-As(III)-γ-$(Glu\text{-}Cys)_2$-Ala	467	T	130.1	------	233.1	------	362.1	------	465.1	554.2	------	425.1	------	322.1	------	193.1	------	90.1
		F	n.o.	------	233.1	------	362.0	------	n.o.	554.2	------	425.1	------	n.o.	------	192.2	------	n.o.
As(III)-γ-$((Glu\text{-}Cys)_2)_2$-Ala	583	T	130.1	------	233.1	------	362.1	------	465.1	554.2	------	425.1	------	322.1	------	193.1	------	90.1
		F	n.o.	------	n.o.	------	n.o.	------	n.o.	554.2	------	425.1	------	n.o.	------	n.o.	------	n.o.
DesGLY GSH/PC																		
γ-$(Glu\text{-}Cys)_2$	483	T	130.1	------	233.1	------	362.1	------	------	------	------	------	------	354.1	------	251.1	------	122.0
		F	n.o.	------	233.1	------	362.1	------	------	------	------	------	------	354.1	------	251.1	------	121.2
GS-As(III)-γ-$(Glu\text{-}Cys)_2$	431	T	130.1	------	233.1	------	362.1	------	380.0	483.1	------	------	------	354.1	------	251.1	------	122.0
			n.o.	------	233.1	------	362.0	------	380.0	483.1	------	------	------	354.1	------	250.9	------	122.1