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SUPPORTING INFORMATION

Universal Absolute Quantification of Biomolecules using Element Mass Spectrometry and Generic Standards.

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GENERAL INFORMATION

Phosphorous (P) ICP standard (100 mgL^{-1} P), sodium periodate and bromate IC standard (1000 mgL^{-1} Br) were purchased from Sigma-Aldrich (Steinheim, Germany). Sulfur (S) ICP standard (1000 mgL^{-1} S) was purchased from Merck KGaA (Darmstadl, Germany). Selenium (Se) ICP standard (1000 mgL^{-1} Se) and Arsenic (As) ICP standard (1000 mgL^{-1} As) were purchased from SPEX CertiPrep (Metuchen, NJ, USA).

Methionine, seleno-DL-methionine, BOC-L-Methionine (BOC), tetrabutylammonium bromide and benzeneselenic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Pipobroman was from MedChem Express (Monmouth Junction, NJ, USA).

Venom sample from Naja mossambica from Tanzania was purchased from Latoxan S.A.S. (Valence, France) in lyophilized form and was stored at -20ºC until used.

All solutions were prepared in MilliQ water, obtained from ChemLabor Millipore system, with 0.22 µm filter (Millipak - Millipore). Mobile phase B was prepared in Acetonitrile (ACN) Optima LC/MS, purchased from Fischer Scientific (USA). Formic acid was purchased from Merck KGaA (Germany).

ICP optional gas, Argon:Oxygen (85:15) gas mixture; reaction gas in collision cell, compressed O_2, and Argon:Methane (90:10) gas mixture were purchased from Air Liquide (Madrid, Spain).

capHPLC system was an Infinite Capillary Agilent 1260 Series (Agilent Technologies, Waldbronn, Germany). The reversed phase micro LC columns used were Agilent Zorbax SB C18, 5 µm, 150 mm x 0.3 mm and Sigma BIOShellTM A400 C4, 3.4 um, 150 mm x 0.3 mm. Spark Holland oven (Mistral, The Netherlands) was employed as column heating system to improve chromatographic efficiency. ICP-MS system consisted on a Triple Quad ICP-MS, ICP-QQQ (Agilent 8800, Tokyo, Japan). Capillary LC interface Total Consumption nebulizer (Agilent) was used as interface between the capLC and the ICP systems. The complete capPC-ICPMS configuration is described elsewhere.[1]

The C4 column was used for the absolute quantification of the venom sample, as it provides quantitative recoveries for intact proteins. However, in order to validate the methodology we directly compared absolute quantitative results for three different element species (BOC-L-Methionine, Pipobroman, Bencenoselenic acid) using isotope dilution, as reference. Therefore, it was a relative assessment, and quantitative recoveries from the column were not needed anymore. In this case, we have selected C18 column because retention of the target simple species is higher than using C4, and consequently elute further from the void.

Methane introduction to the plasma was controlled by a Mass Flow Controller PR4000B MKS Instruments, (Andover, USA). Methane gas was mixed on-line with the optional gas used to help organic matter combustion (Ar:O2, 85:15) through a T-connection 5/32" (John Guest, Middlesex, UK) located between the exit of the optional gas from the ICP-QQQ and the entrance to the nebulization chamber.

[1] F. Calderón-Celis, S. Diez-Fernández, J. M. Costa-Fernández, J. Ruiz Encinar, J. J. Calvete, A. Sanz-Medel, *Anal. Chem.* **2016**, 88, 9699–9706

STUDY OF PLASMA BEHAVIOR

In order to systematically study the behavior of plasma ionization and potential correction of signal response variations of the six assayed elements (P, S, Se, As, Br and I), their ICP signals were recorded along a reversed-phase capLC gradient when adding different Ar:CH_4 flows to the plasma. The use of low-flow chromatography (capLC) together with total consumption nebulizers is becoming a cutting-edge alternative[2] in life science speciation to avoid plasma extinction due to the organic content of the mobile phases,[3] as well to avoid nebulization processes (which are conditioned by the physicochemical properties of the matrix) from affecting quantitative results.[4] It is worth mentioning that, in the addition of Ar:CH_4 to the ICP-MS plasma, it produces a dilution effect in the plasma, because the total flow of Ar reaching the plasma increases. However, in the most relevant Ar:CH_4 flow ranges (20 – 100 $mLmin^{-1}$), this extra flow means less than 10% of the total Ar flow (carrier gas + optional gas) in the central channel of the torch, so this dilution effect is not significant. Moreover, because of the use of a total consumption nebulizer, in which the 100% of the sample is nebulized, this variation in the gas flow entering the nebulization chamber through the optional gas inlet produces no effect in the nebulization. Consequently, the effect of carbon in the signal of the assayed heteroatoms can be considered, in this study, to be solely caused by ionization processes.

Inorganic standards of the elements (P, S, Br, As and Se ICP standards, and sodium peroidate standard) were spiked to the mobile phases (A: H_2O/0.2 % formic acid; B: ACN/0.2% formic acid) at the same concentration: 500 μgL^{-1} in the case of P, S, Se and Br; and 300 μgL^{-1} in the case of As and I. Then, chromatographic gradients from 0 to 70% Phase B in 35 min were run at different conditions of methane flows, ranging from 0 to 250 $mLmin^{-1}$, whereas the optional gas was kept constant at the optimal value (8%).

From these analyses, elemental signal along the whole capLC gradient at any methane flow could be evaluated. Thus, by calculating the relative standard deviation (RSD%) of the signal along the gradient it was possible to determine which methane flows provided better correction of signal variations. In addition, from the recorded chromatograms, element signal along the Mobile Phase B gradient could be compared for each methane flow assayed, providing information on signal sensitivity enhancement or depletion due to methane. In the case of the evaluation of signal enhancement, results obtained showed a pattern-like behavior of the elements and it is clearly showed in Figure 2B in the manuscript for P analysis. Signal enhancement was more pronounced at the beginning of the LC gradient (0-30%), i.e., when the ACN content is lower and hence the signal response. In addition, signal enhancement was, in general terms, higher at low-medium flows of Ar:CH_4 (0-150 $mLmin^{-1}$ Ar:CH_4). This results agree with several studies,[5,6] which show that signal enhancement is more pronounced up to a certain value of carbon, which depends on the element. Afterwards, a signal depletion effect is typically observed and becomes more significant the higher the carbon amount in the plasma gets.

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[4] T. Nakazawa, D. Suzuki, H. Sakuma, N. Furuta, *J. Anal. At. Spectrom*. **2014**, 29, 1299-1305.

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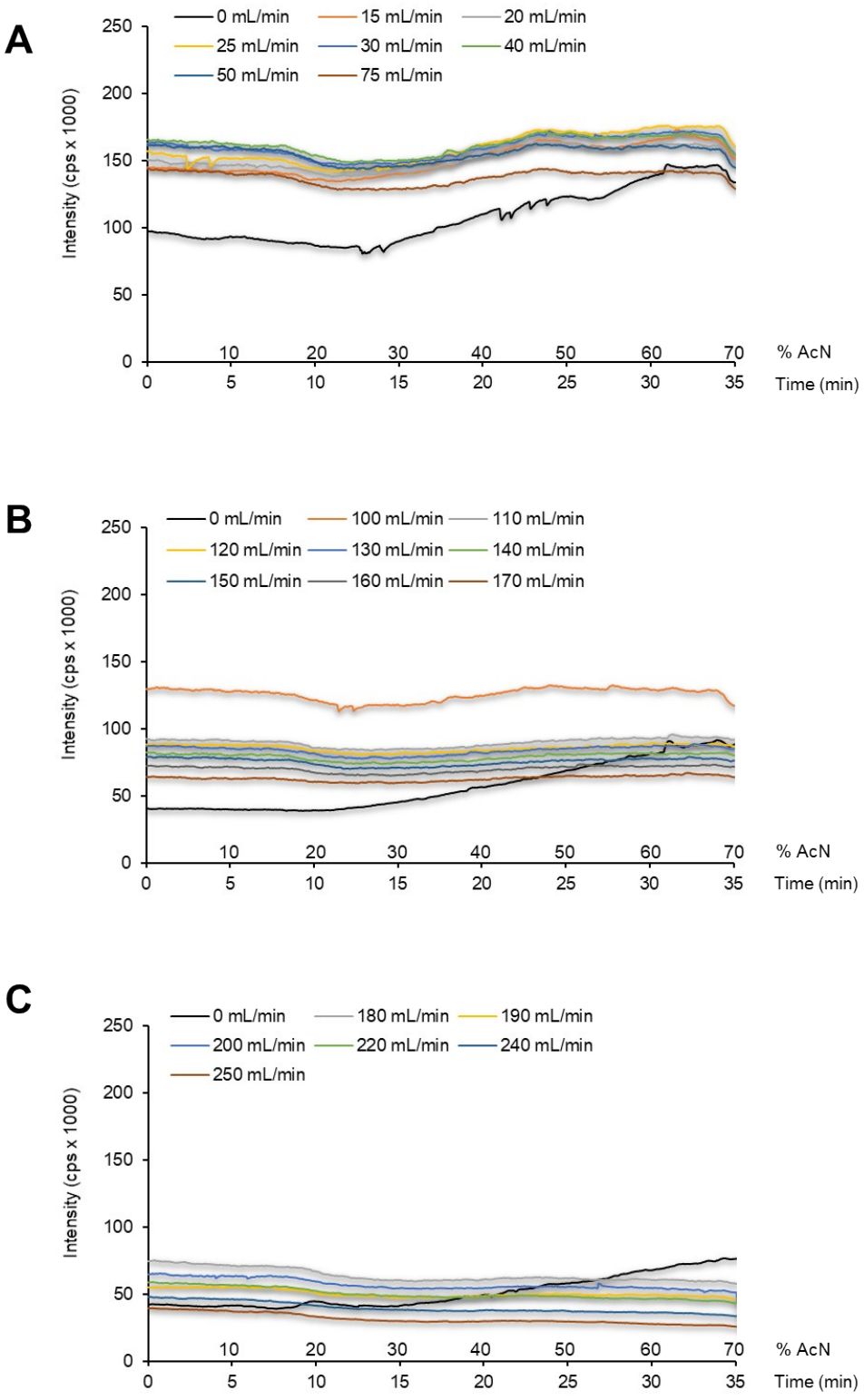
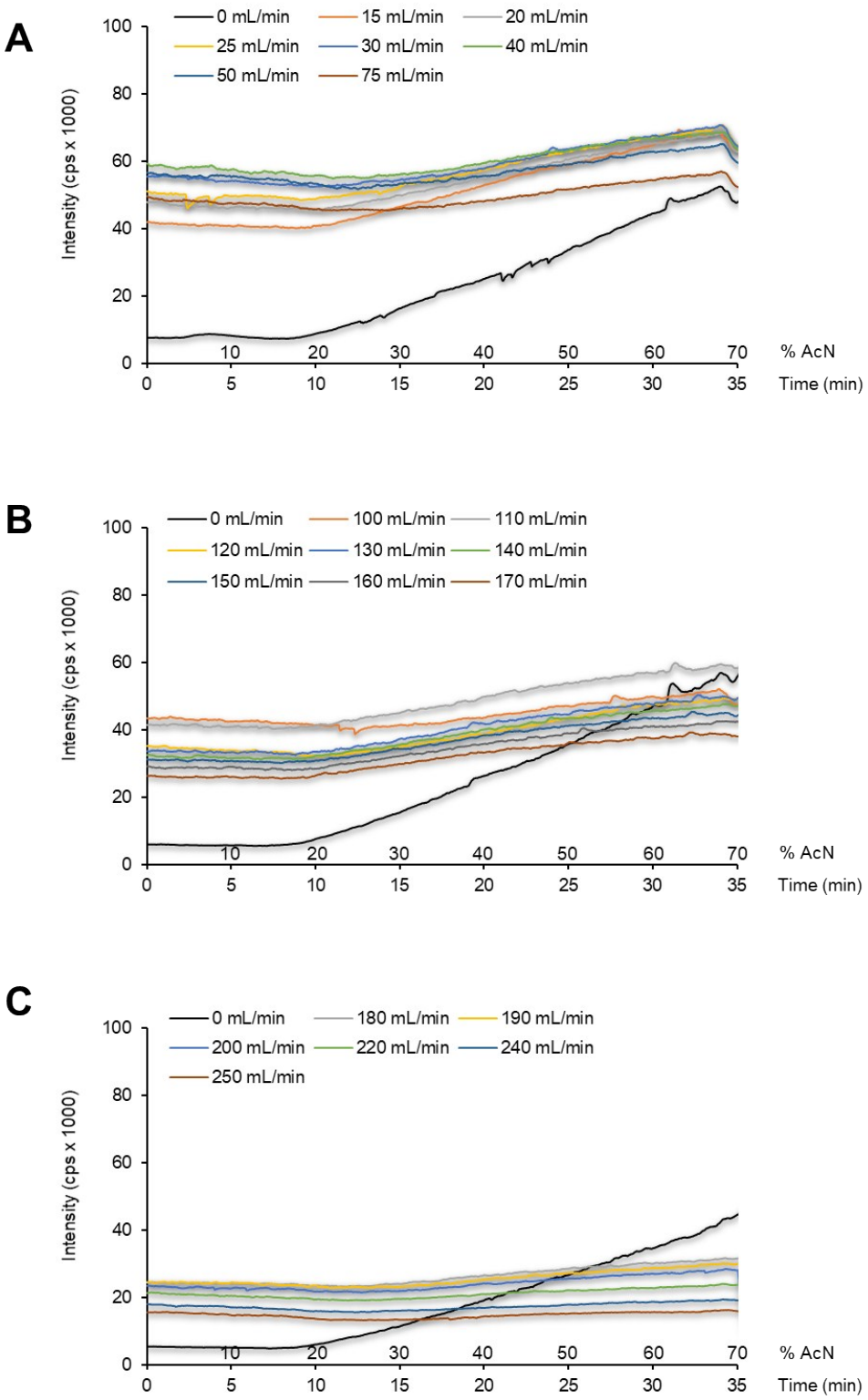
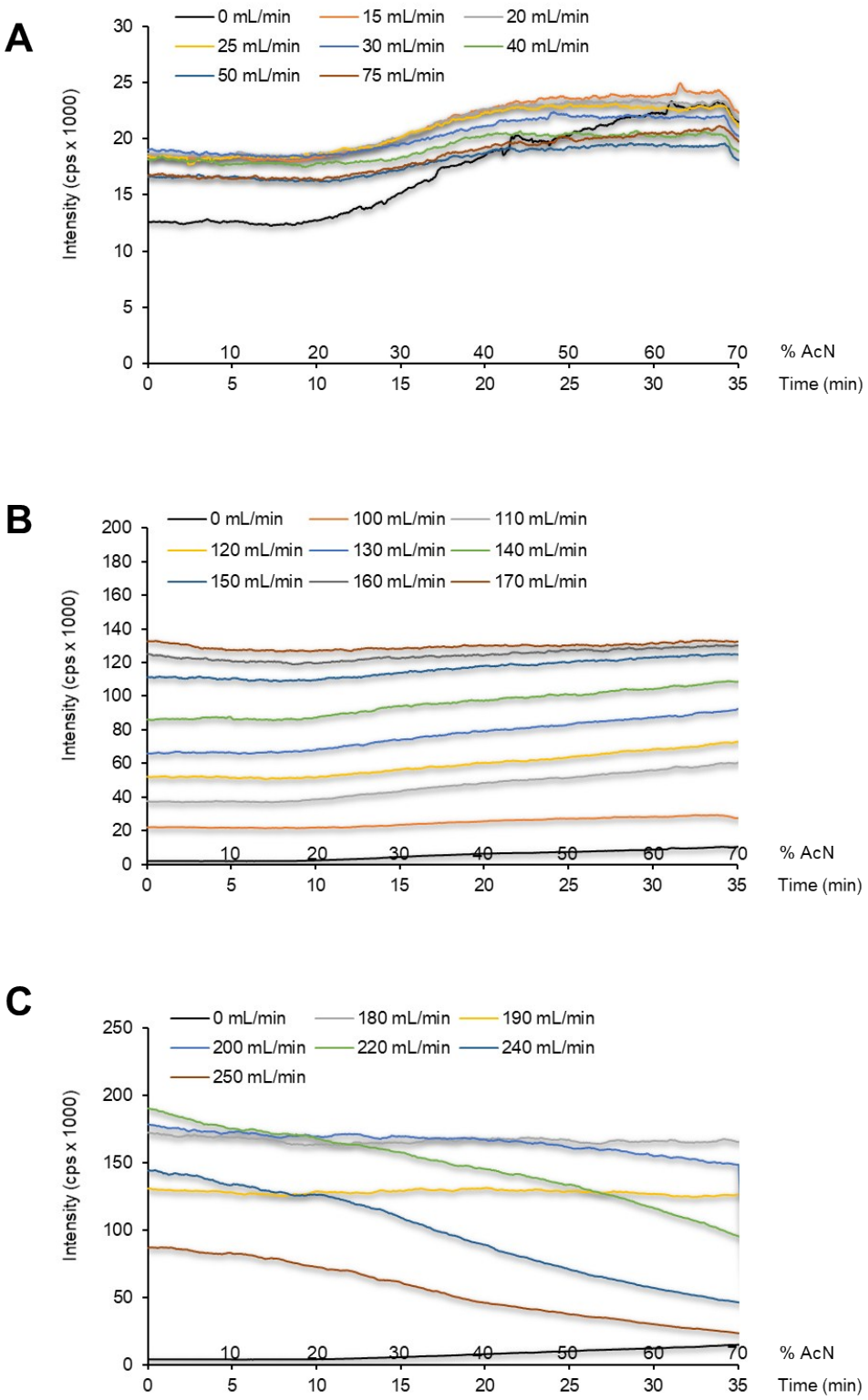
[6] M. Kovačevič, W. Goessler, *Spectrochim. Acta Part B At. Spectrosc*. **2005**, 60, 1357–1362.



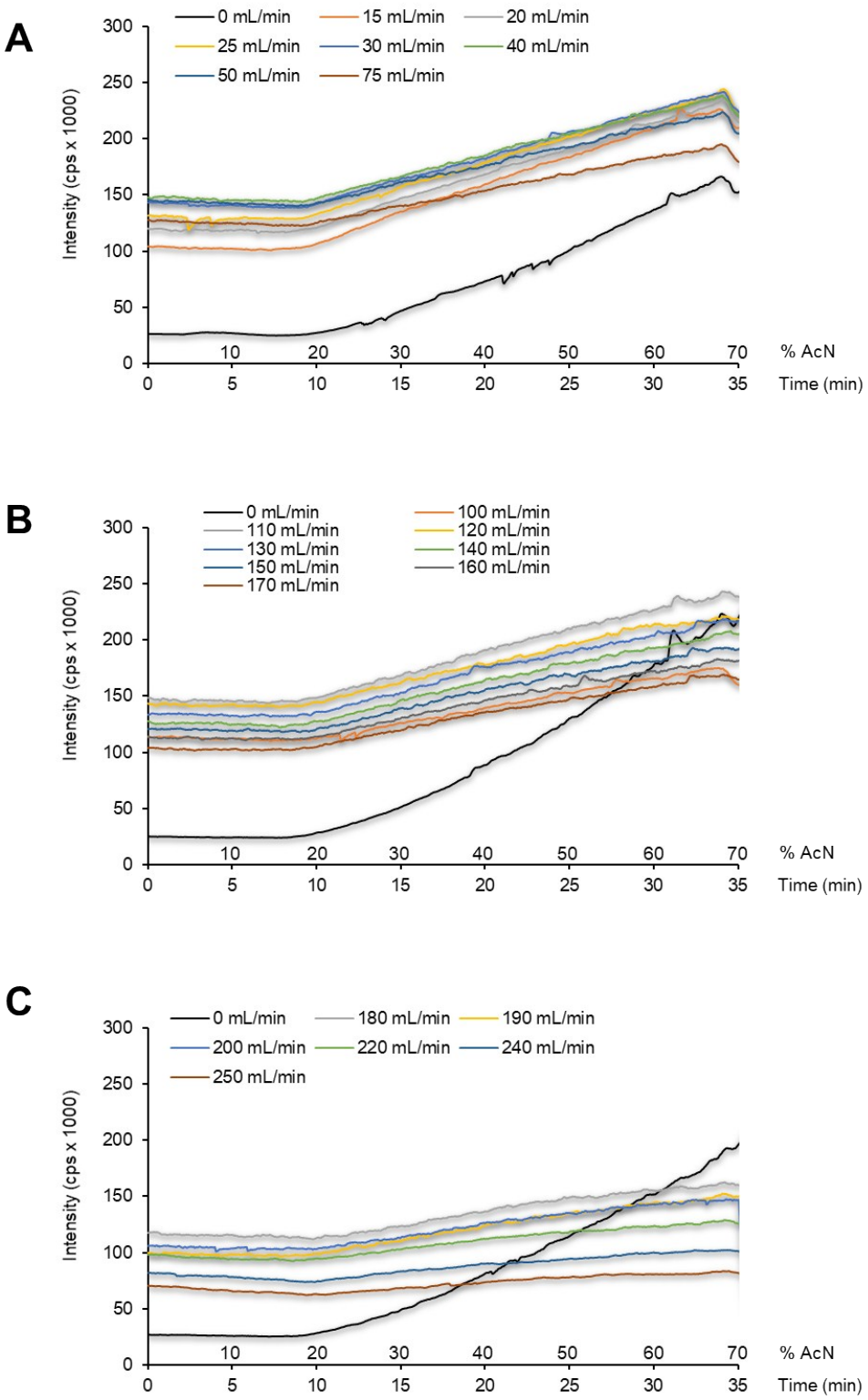
Supplementary Figure S1. Sulfur (S32→48) capLC-ICP-QQQ chromatograms when adding (A) low Ar:CH_4 flows up to 75 $mLmin^{-1}$; (B) middle-range Ar:CH_4 flows between 100 and 170 $mLmin^{-1}$ and (C) high Ar:CH_4 flows up to 250 $mLmin^{-1}$.



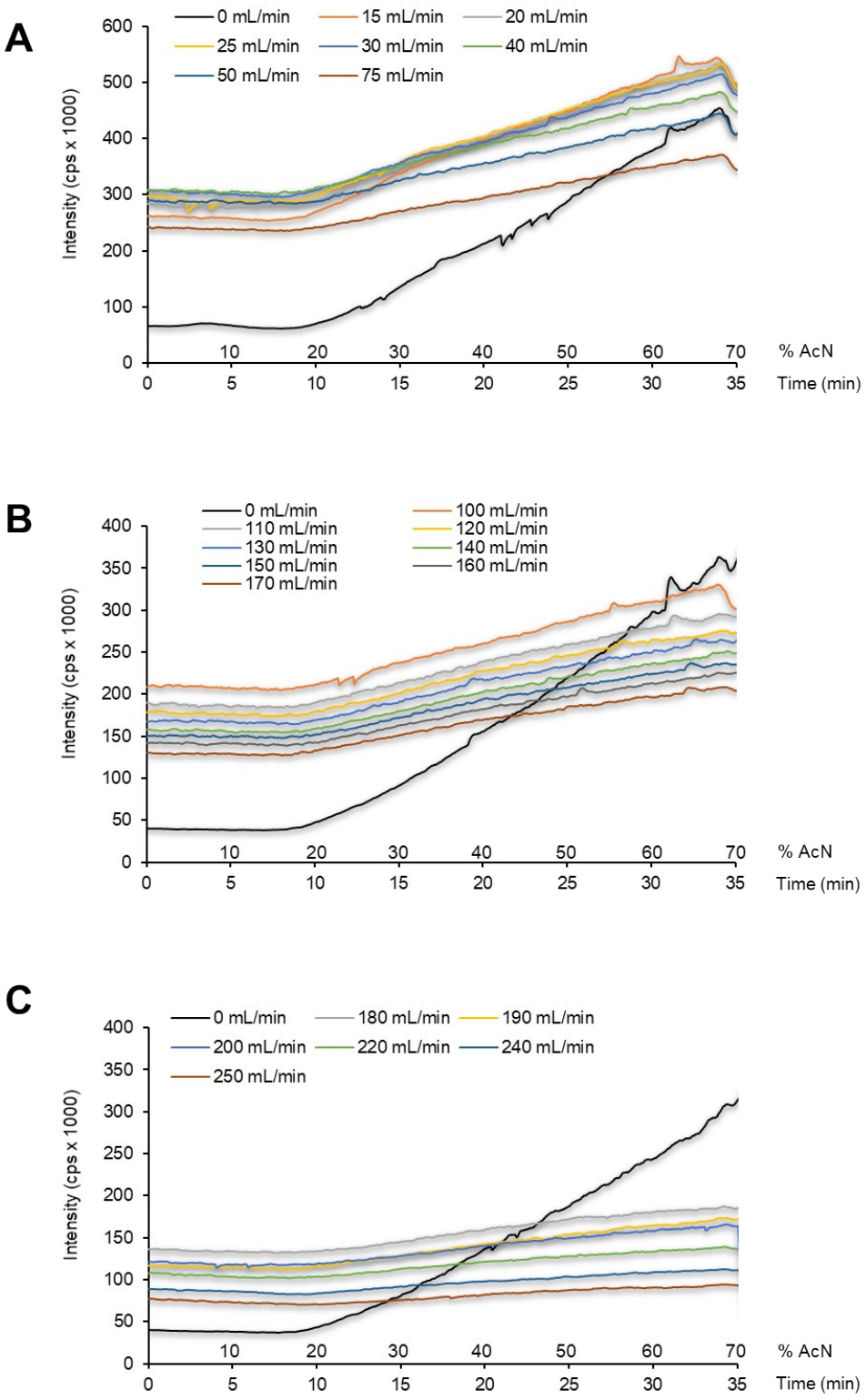
Supplementary Figure S2. Selenium (Se80→96) capLC-ICP-QQQ chromatograms when adding (A) low Ar:CH_4 flows up to 75 mLmin^{-1}; (B) middle-range Ar:CH_4 flows between 100 and 170 mLmin^{-1} and (C) high Ar:CH_4 flows up to 250 mLmin^{-1}.



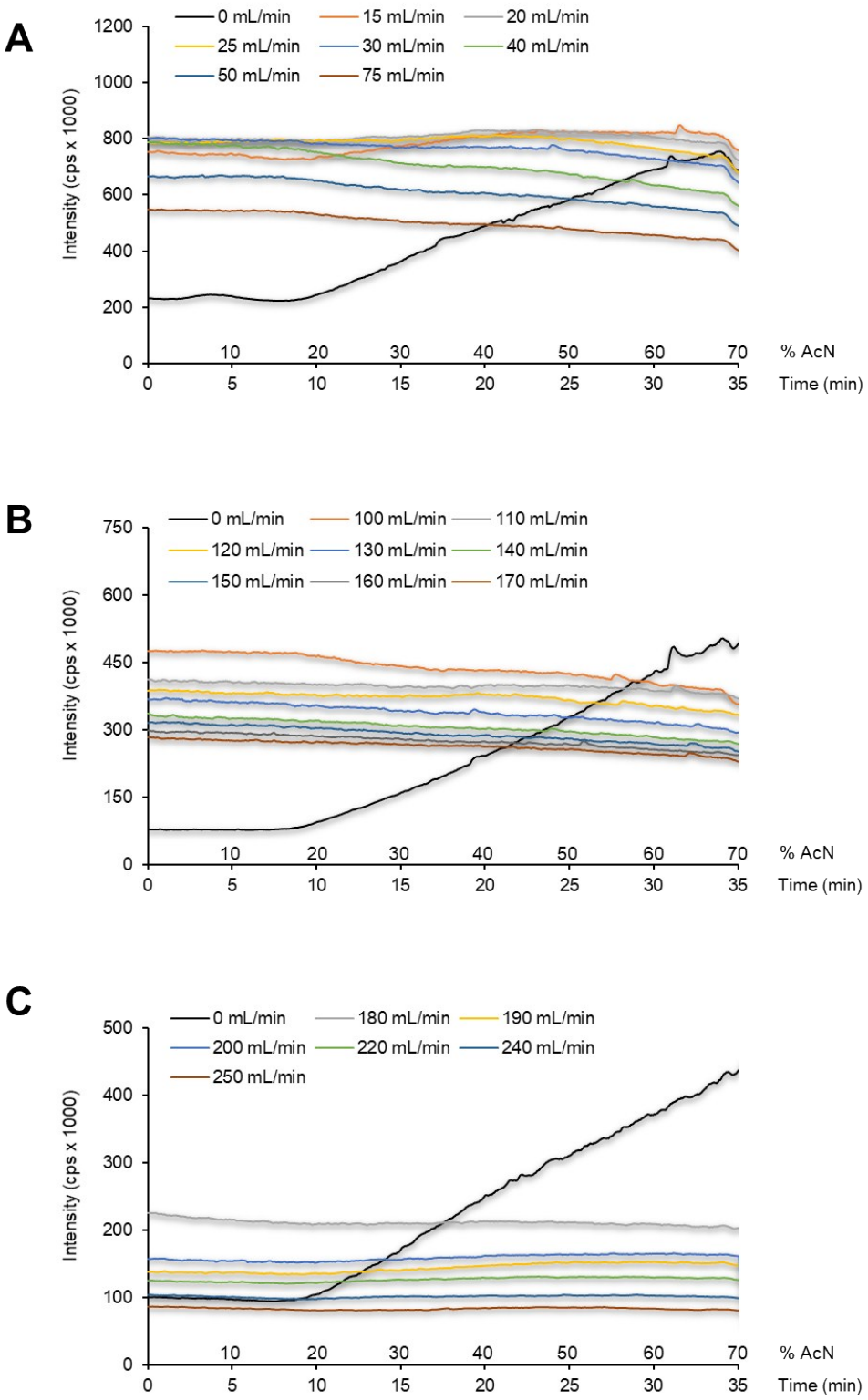
Supplementary Figure S3. Bromine (Br79→79) capLC-ICP-QQQ chromatograms when adding (A) low Ar:CH_4 flows up to 75 $mLmin^{-1}$; (B) middle-range Ar:CH_4 flows between 100 and 170 $mLmin^{-1}$ and (C) high Ar:CH_4 flows up to 250 $mLmin^{-1}$.



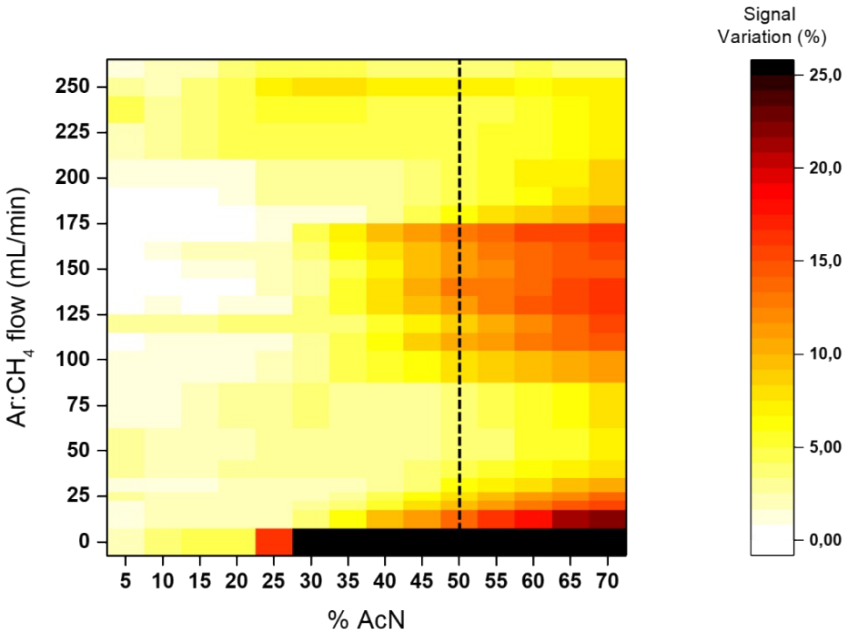
Supplementary Figure S4. Phosphorous (P31→47) capLC-ICP-QQQ chromatograms when adding (A) low Ar:CH_4 flows up to 75 mLmin^{-1}; (B) middle-range Ar:CH_4 flows between 100 and 170 mLmin^{-1} and (C) high Ar:CH_4 flows up to 250 mLmin^{-1}.



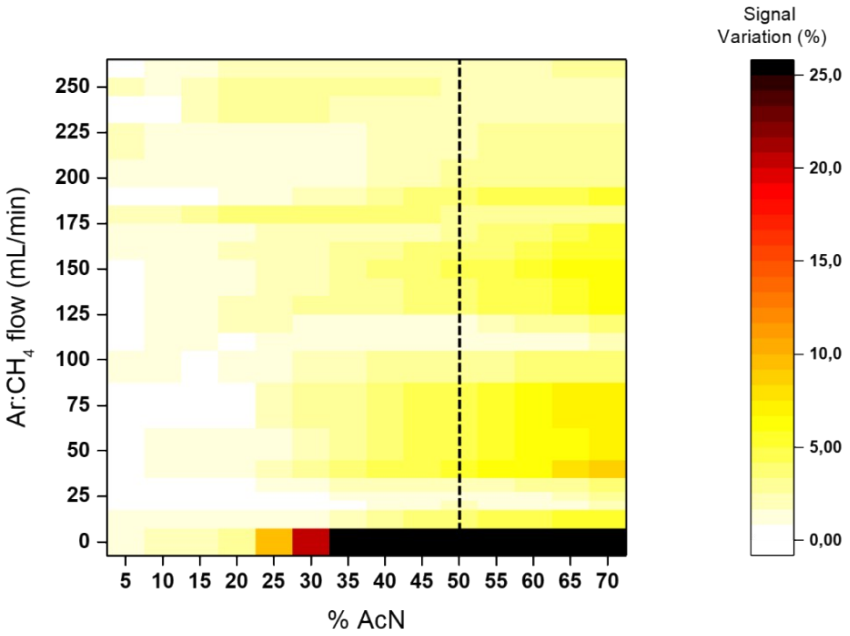
Supplementary Figure S5. Arsenic (As75→91) capLC-ICP-QQQ chromatograms when adding (A) low Ar:CH_4 flows up to 75 $mLmin^{-1}$; (B) middle-range Ar:CH_4 flows between 100 and 170 $mLmin^{-1}$ and (C) high Ar:CH_4 flows up to 250 $mLmin^{-1}$.



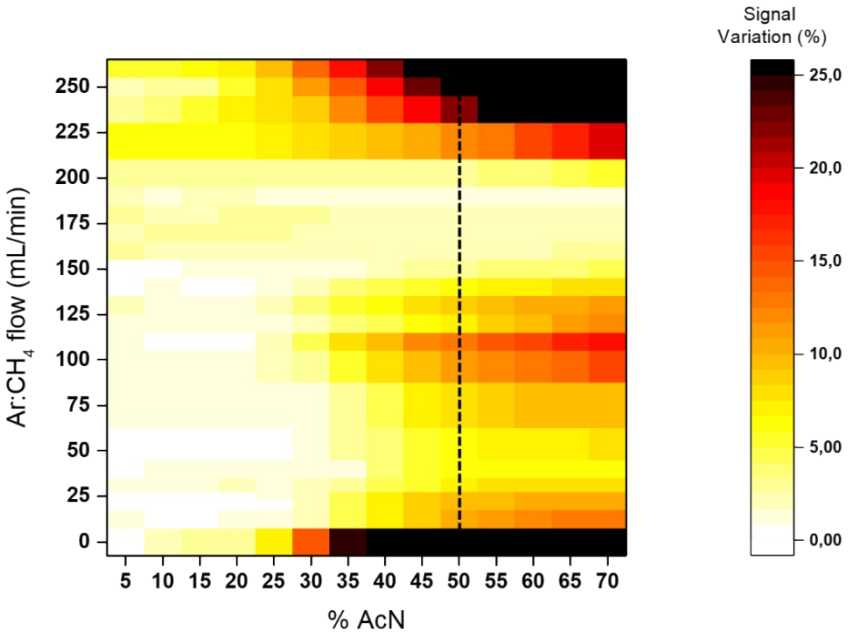
Supplementary Figure S6. Iodine (I127→127) capLC-ICP-QQQ chromatograms when adding (A) low Ar:CH_4 flows up to 75 $mLmin^{-1}$; (B) middle-range Ar:CH_4 flows between 100 and 170 $mLmin^{-1}$ and (C) high Ar:CH_4 flows up to 250 $mLmin^{-1}$.



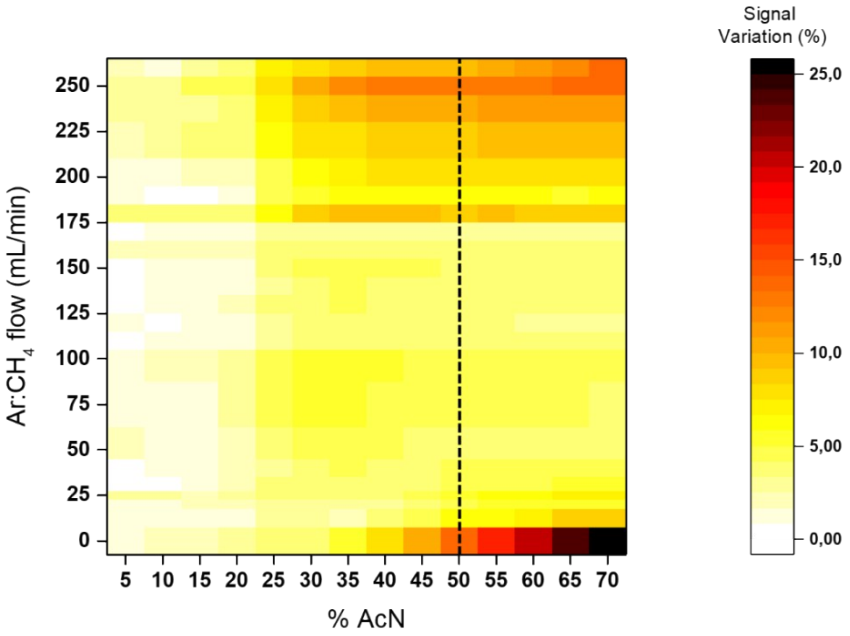
Supplementary Figure S7. Selenium heat map representing the ICP-MS signal variation (%) along the chromatographic gradient for the Ar:CH_4 flows assayed between 0 and 250 $mLmin^{-1}$. Optimal signal correction is accomplished at low (70-75 $mLmin^{-1}$) and high (190-250 $mLmin^{-1}$) methane flows, with signal RSD% below 10% in both cases, providing quantitative errors in the range of 0 to 50% ACN below 6%.



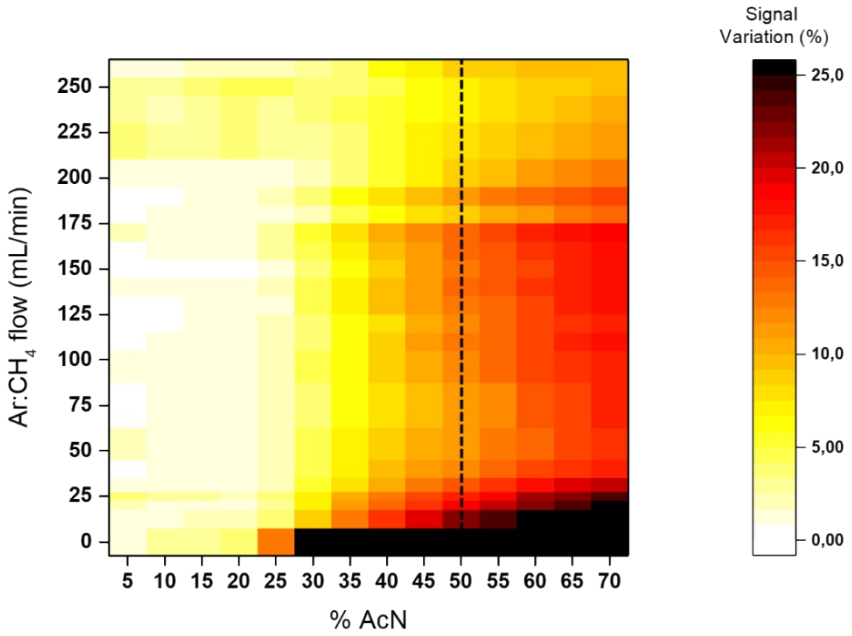
Supplementary Figure S8. Iodine heat map representing the ICP-MS signal variation (%) along the chromatographic gradient for the Ar:CH_4 flows assayed between 0 and 250 $mLmin^{-1}$. Iodine showed good signal stability practically with all methane flows assayed.



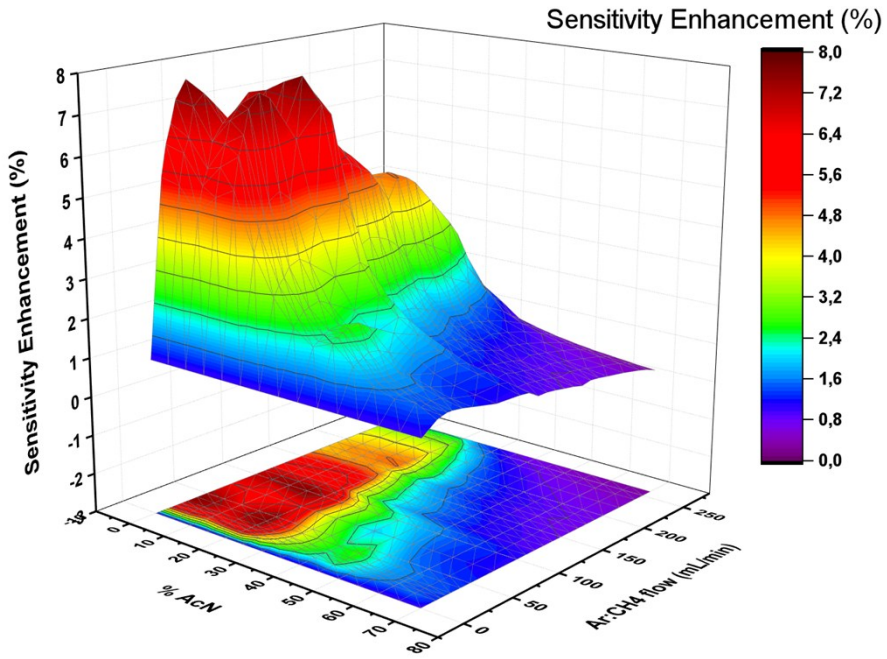
Supplementary Figure S9. Bromine heat map representing the ICP-MS signal variation (%) along the chromatographic gradient for the Ar:CH_4 flows assayed between 0 and 250 mLmin^{-1}. Optimal signal correction is accomplished at low (30-75 mLmin^{-1}) and high (150-200 mLmin^{-1}) methane flows, with quantitative errors in the range of 0 to 50% ACN below 15% and 5%, respectively (in both cases signal RSD% was below 10%).



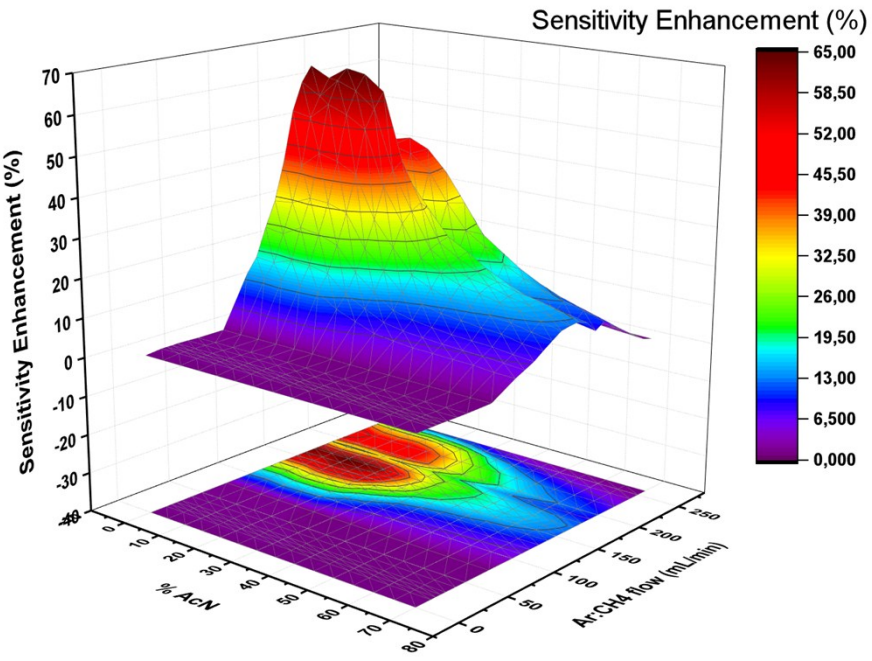
Supplementary Figure S10. Sulfur heat map representing the ICP-MS signal variation (%) along the chromatographic gradient for the Ar:CH_4 flows assayed between 0 and 250 mLmin^{-1}. Correction of signal variations can be appreciated in the range of ~50-150 mLmin^{-1} methane flows, in which signal RSD% along the LC gradient is kept below 5%.



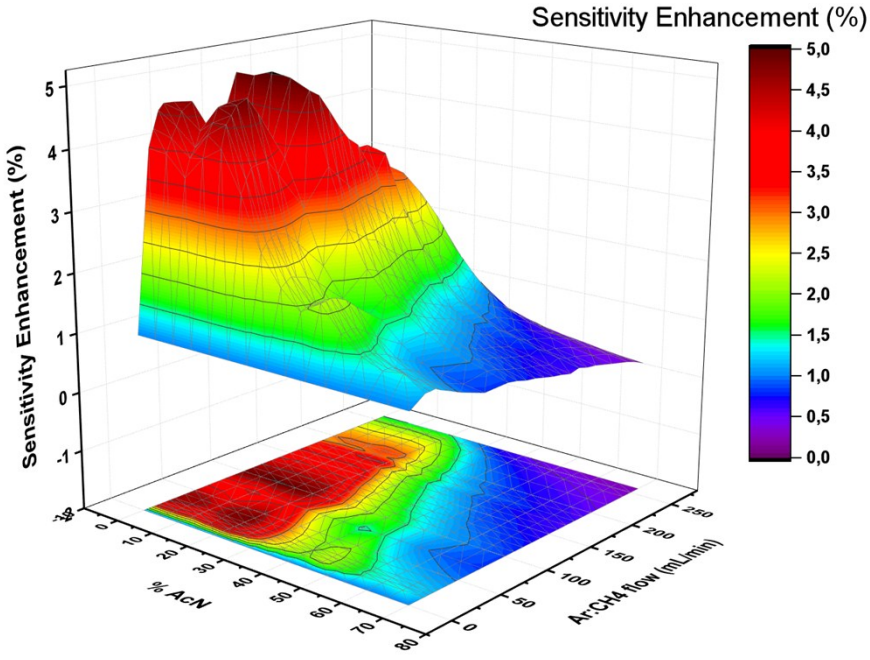
Supplementary Figure S11. Arsenic heat map representing the ICP-MS signal variation (%) along the chromatographic gradient for the Ar:CH_4 flows assayed between 0 and 250 $mLmin^{-1}$. Significant correction of the signal variation (below 10% RSD) during the LC gradient (0-50 % ACN) was accomplished at high Ar:CH_4 flows (220-250 $mLmin^{-1}$). However, at low flows (40-75 $mLmin^{-1}$) signal correction was below 15%, which implies quantitative errors in the order of 30%.



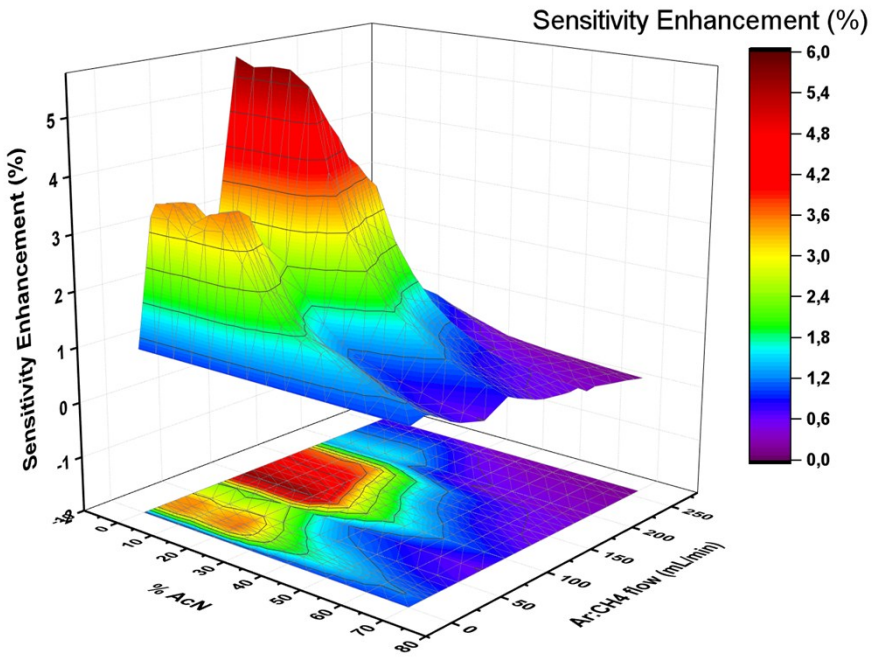
Supplementary Figure S12. Selenium 3D map representing the signal sensitivity enhancement as a function of the Ar:CH_4 (0-250 $mLmin^{-1}$) flow along the reversed phase chromatographic gradient (0-70% ACN).



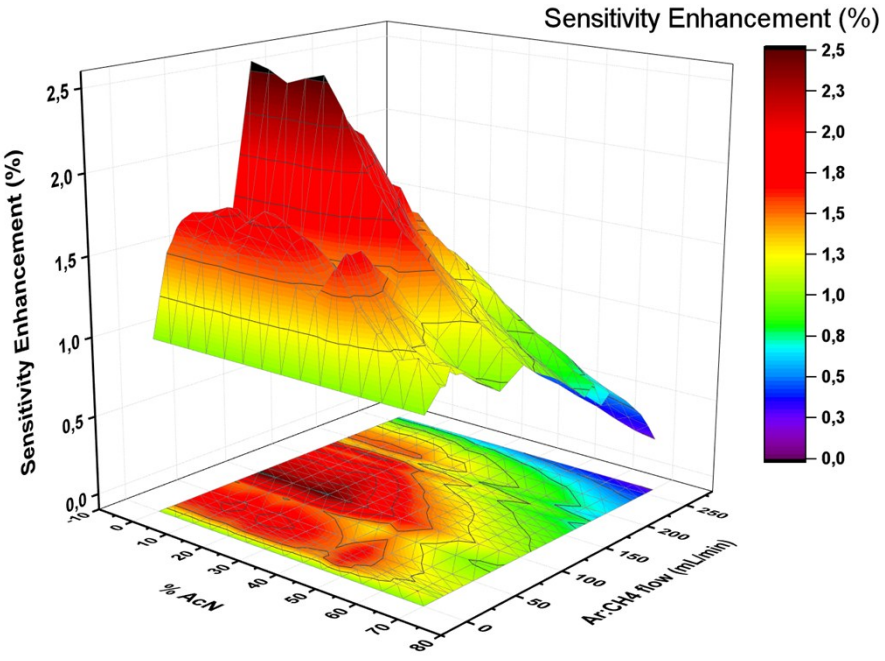
Supplementary Figure S13. Bromine 3D map representing the signal sensitivity enhancement as a function of the Ar:CH_4 (0-250 $mLmin^{-1}$) flow along the reversed phase chromatographic gradient (0-70% ACN).



Supplementary Figure S14. Arsenic 3D map representing the signal sensitivity enhancement as a function of the Ar:CH_4 (0-250 $mLmin^{-1}$) flow along the reversed phase chromatographic gradient (0-70% ACN).



Supplementary Figure S15. Iodine 3D map representing the signal sensitivity enhancement as a function of the Ar:CH_4 (0-250 $mLmin^{-1}$) flow along the reversed phase chromatographic gradient (0-70% ACN).



Supplementary Figure S16. Sulfur 3D map representing the signal sensitivity enhancement as a function of the Ar:CH_4 (0-250 $mLmin^{-1}$) flow along the reversed phase chromatographic gradient (0-70% ACN).

SPECIES-UNSPECIFIC QUANTIFICATION OF S-, Se-, AND Br-CONTAINING BIOMOLECULES

Validation and application of the developed approach was carried out for a series of biomolecules containing S, Se and Br. These elements have more than one stable isotope and therefore results can be compared to parallel experiments in which signal variations are corrected with isotope dilution instead of methane gas. The use of isotope dilution analysis (IDA) is widely accepted, as a mean to correct for signal variations since it uses the "perfect internal standard", i.e. the same target element, but with different number of neutrons, and therefore their behavior in the plasma will be the same. In this study, isotopically-enriched aqueous solution used for post-column IDA consisted on ~ 350 ngg^{-1} of ^{34}S, ^{77}Se and ^{81}Br.

The assayed compounds (BOC-L-Methionine, Bencenoselenic acid, and Pipobroman) were prepared in aqueous solution in concentrations in the range of 1-4 μgg^{-1}. In order to carry out quantification, just before to the chromatographic analysis (C18 capRP-LC) pure standards containing the assayed elements (methionine, seleno-methionine and tetrabutylammonium bromide) at known concentrations were measured by flow injection analysis (capFIA). Concentration of the analytes separated by capLC was determined from these standards by relating the elemental area. The concentrations determined for the analytes when using IDA and methane gas for the correction of signal variations were therefore compared (Table S1). Injection volume in capLC and capFIA analyses was 1 µL, and gradient in the capLC analysis was: 2% mobile phase B for 2 min, 2 to 70% mobile phase B in 35 min, and 70 to 90% mobile phase B in 2 min. Instrumental configuration of the methodology is represented in Figure S17.

Supplementary Table S1. Accuracy (%) of S-, Se-, and Br-containing biomolecules quantification, with and without $Ar:CH_4$, with respect to IDA results. Experimental uncertainties correspond to one standard deviation (n=3).

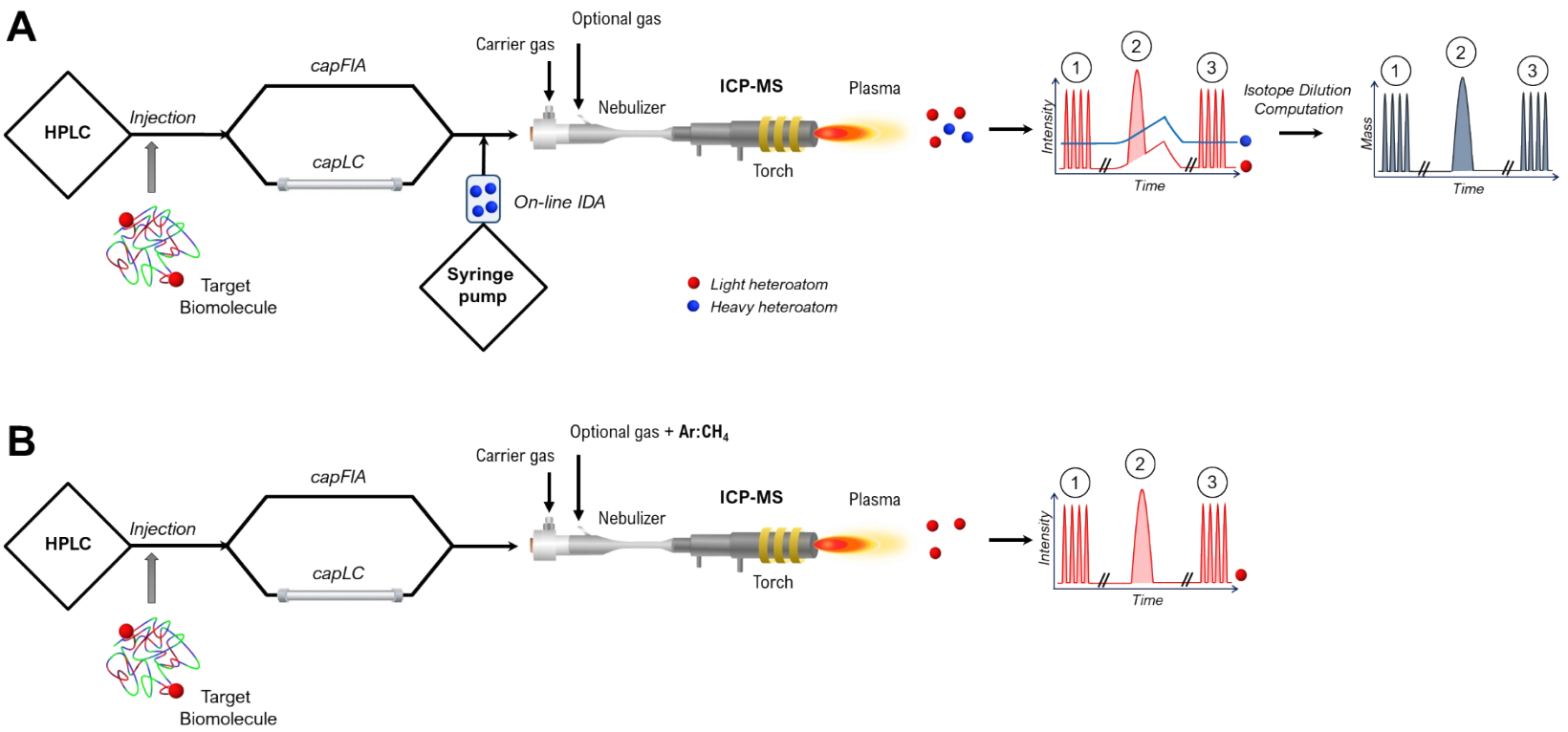
Compound	Standard conditions	50 $mLmin^{-1}$ $Ar:CH_4$
(S) BOC-L-Met	254 ± 8%	102 ± 4 %
(Se) Selenic acid	113 ± 2%	102 ± 2 %
(Br) Pipobroman	153 ± 3%	98 ± 4 %

Supplementary Table S2. Limits of detection (LODs) in elemental fmol for the quantitative analysis of S-, Se- and Br-containing biomolecules in the experimental conditions assayed: 3.5 $\mu Lmin^{-1}$ capLC flow, 1 $\mu Lmin^{-1}$ post-column IDA flow, 1 µL sample injection volume. LODs were calculated from the net peak heights by flow injection analysis (FIA). As expected, the addition of $Ar:CH_4$ proved the best LODs for S and Se because of signal enhancement in the plasma. In contrast, LODs with IDA are worse than with standard conditions, and of course than with $Ar:CH_4$. These worse LODs are most likely due to an increment in the signal background caused by the ^{32}S and ^{80}Se that are present in the isotopically enriched solutions (although at low abundance, <5%) (see Table S2). Moreover, post-column flow required to add continuously the enriched solutions in IDA comes with worse signal to noise ratio as an additional result. In the case of Br, LOD in standard conditions is in the same order that S and Se. However, the addition of $Ar:CH_4$, which contains Br as contaminant, increases signal background covering up the original Br signal enhancement and finally resulting in slightly worse LOD. The reason behind this is again the relationship between isotope abundances in the spike solution and natural ones (Table S3).

Element	Standard Conditions	IDA	50 $mLmin^{-1}$ $Ar:CH_4$
S	188 fmol	223 fmol	104 fmol
Se	82 fmol	337 fmol	36 fmol
Br	130 fmol	2165 fmol	144 fmol

Supplementary Table S3. Natural isotope abundances and isotope abundances in the spike solution of ^{32}S, ^{34}S, ^{79}Br, ^{81}Br, ^{77}Se, and ^{80}Se.

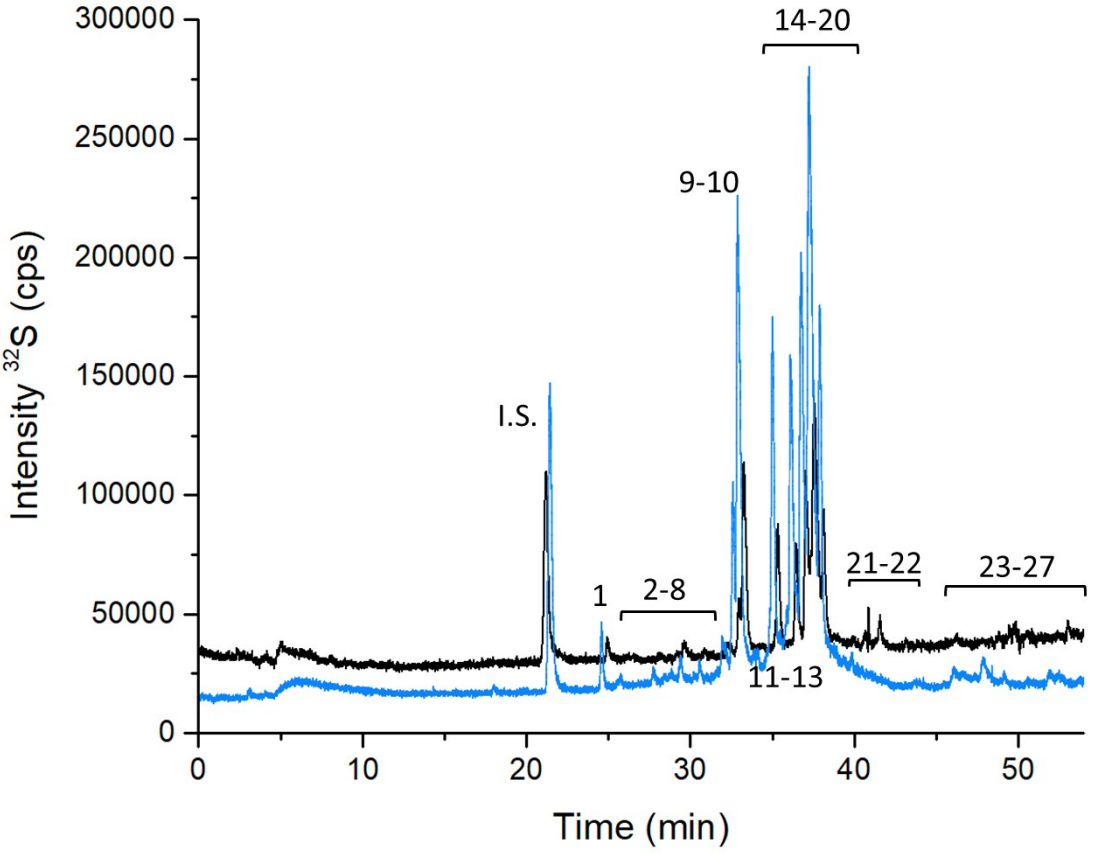
Isotope	Natural Abundance	Abundance in the spike solution
^{32}S	94.99 %	3.34 %
^{34}S	4.25 %	95.45 %
^{79}Br	50.69 %	8.67 %
^{81}Br	49.31 %	91.32 %
^{77}Se	7.63 %	93.14 %
^{80}Se	49.61 %	1.35 %



Supplementary Figure S17. Scheme of the instrumental configuration of the proposed methodology with (A) IDA, and (B) Ar:CH_4 addition. Quantification of compounds can be carried out using standards injected with FIA replicates, before (1) or after (3) of the chromatographic analysis (2). Higher complexity of IDA instrumentation is clearly shown in the figure, as it requires the post-column addition of an isotopic tracer with a syringe pump, and mathematical calculations to compute isotope dilution for signal variations corrections. In contrast, the proposed approach (B) is simpler, as the gas addition is done directly to the ICP through the optional gas inlet. Therefore, capLC can be coupled to either ESI or ICP detector, without requiring any instrumental modification, just by connecting the column exit to the corresponding nebulizer/source.

QUANTIFICATION OF S-CONTAINING BIOMOLECULES PRESENT IN A SNAKE VENOM SAMPLE

Venom sample quantification was carried out as described elsewhere.[1] The sample was spiked with BOC-L-Methionine, as generic internal standard. The same sample of the mixture (i.e., same concentration: 270 μgmL^{-1}, and injection volume: 1µL) was analyzed by capLC(C4 BioShell)-ICP-MS adding 50 $mLmin^{-1}$ Ar:CH_4 to the plasma and monitoring the sulfur present in the proteins and the standard. Correlation of BOC-L-Methionine S peak area, whose corresponding sulfur amount was known, and proteins S peak area provided individual absolute quantities of sulfur corresponding to each protein. Previous capLC-ESI-QToF analysis provided protein identities, enabling to translate sulfur quantities into protein quantities. Quantification was compared with previous IDA analysis, as shows Figure S19.



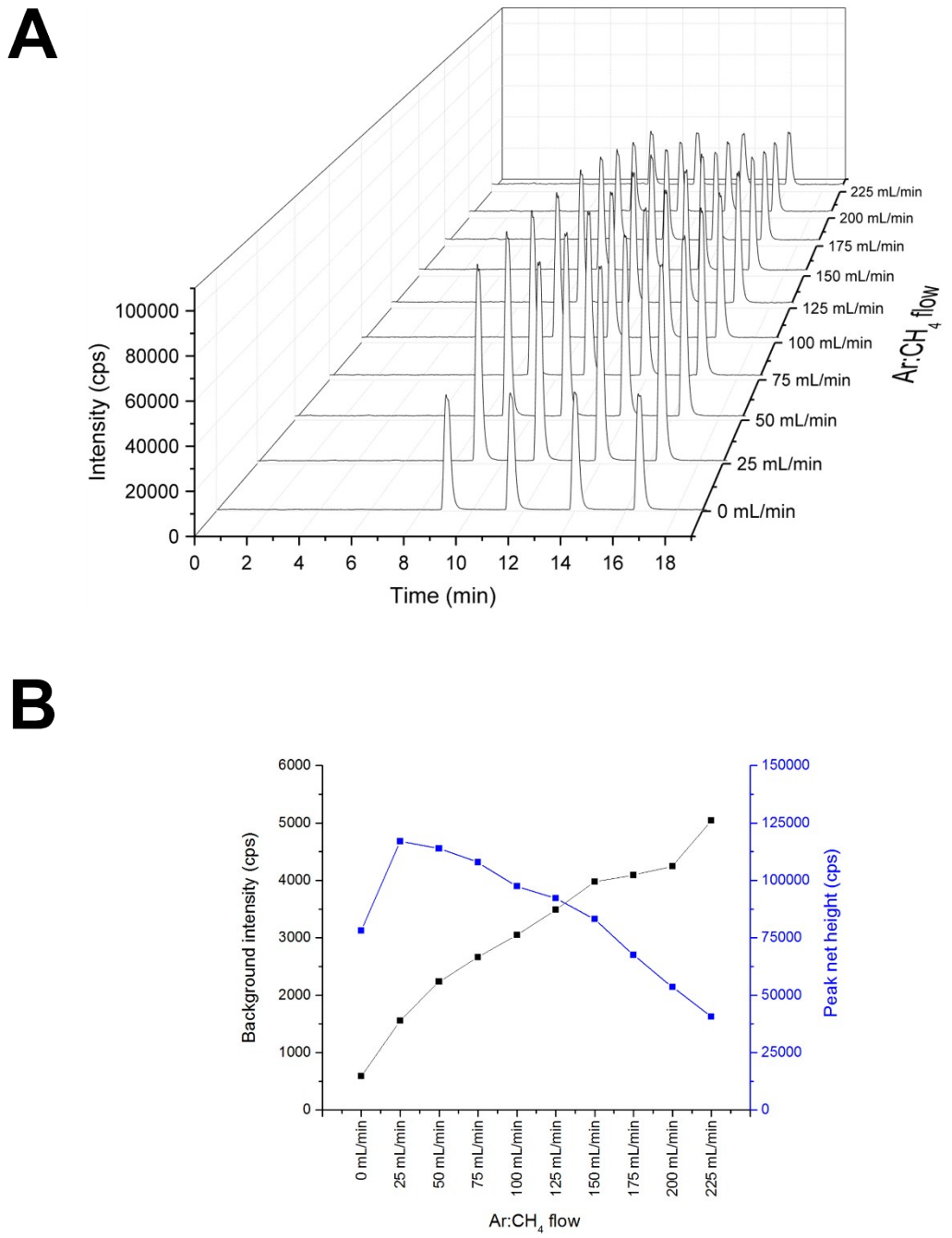
Supplementary Figure S18. Sulfur ICP signal (S 32→48) is plotted versus for IDA (black) and Ar:CH_4 (blue) analysis of *Naja mossambica* venom sample. Experimental conditions were: 1µL sample injection volume, 3.5 $\mu Lmin^{-1}$ LC flow, 1 $\mu Lmin^{-1}$ post-column flow in IDA, gradient of 0.2% formic acid in water (mobile phase A) and acetonitrile (mobile phase B): 2% B for 5 min, 2 - 10% B in 8 min, 10 - 30% B in 28 min, 30-50% in 20 min, and 50 - 90% B in 10 min.

Supplementary Table S4. Quantification results of the individual proteins (in µmol protein per g of venom sample) present in the *Naja mossambica* venom sample analyzed by IDA and with the addition of methane (50 $mLmin^{-1}$ $Ar:CH_4$). Experimental uncertainties correspond to one standard deviation (n=3).

Peak	Family	Closest homolog	MW (Da)	IDA Quantification	$Ar:CH_4$ Quantification
1	3FTx	-	7064.2	1.99 ± 0.06	1.89 ± 0.07
2	3FTx	~P29179	7417.4	0.47 ± 0.07	0.67 ± 0.10
3	3FTx	~P29179	7451.6	0.32 ± 0.04	0.35 ± 0.03
4	3FTx	~P01420	6892.4	1.10 ± 0.13	0.92 ± 0.04
5	3FTx	~Q9W6W6	7786.4	< 0.1	0.39 ± 0.01
6	3FTx	~P01452	7277.3	0.68 ± 0.05	0.93 ± 0.06
7	3FTx	~P01452	7306.3	0.67 ± 0.06	0.49 ± 0.08
8	3FTx	-	7246.2	1.35 ± 0.10	1.68 ± 0.13
9	3FTx	P25517	6832.4	5.09 ± 0.28	5.26 ± 0.29
10	3FTx	P01452'	6704.3	19.0 ± 0.8	19.59 ± 0.79
11	3FTx	-	6686.3	0.18 ± 0.04	0.70 ± 0.14
12	3FTx	-	6829.3	0.22 ± 0.04	0.71 ± 0.04
13	3FTx	-	6687.3	< 0.1	0.38 ± 0.06
14	PLA_2	P00604	13280.9	7.76 ± 0.32	7.64 ± 0.45
15	3FTx	P01470	6882.4	9.54 ± 0.27	9.60 ± 0.40
16	3FTx	P25517'	6813.3	16.2 ± 0.4	16.34 ± 0.62
17	3FTx	P01467	6814.3	27.8 ± 0.8	28.02 ± 1.19
18	3FTx	~P01469	7046.4	5.13 ± 0.26	5.16 ± 0.31
19	PLA_2	P00604'	13237.8	3.40 ± 0.14	3.42 ± 0.18
20	PLA_2	P00002	13196.6	7.35 ± 0.33	7.40 ± 0.40
21	PLA_2	-	13179.7	0.81 ± 0.05	0.81 ± 0.05
22	Minor	-	42000	0.10 ± 0.01	0.14 ± 0.01
23	Endonuclease	-	30000	0.62 ± 0.05	0.75 ± 0.05
24	SVMP	Q10749	46700	0.16 ± 0.01	0.24 ± 0.01
25	SVMP	Q10750	46700	0.26 ± 0.01	0.42 ± 0.01
26	SVMP	Q10751	46700	0.10 ± 0.01	0.13 ± 0.00
27	SVMP	Q10752	46700	0.26 ± 0.01	0.36 ± 0.01

EVALUATION OF BROMINE CONTAMINATION IN THE METHANE GAS

Unconventional results on signal enhancement in the case of bromine were observed. Whereas P, S, Se, As and I showed concordant and consistent results, with signal enhancement between 2 and 6 times at the highest point of enhancement, the case of bromine showed enhancement of up to 60 times. This abnormal behavior can be clearly seen in Figure S3. The case of bromine is the only one among the studied elements in which the addition of methane results in elemental signal over the range of the signal at 0 mLmin-1. In other words, the other elements showed signal enhancement which resulted in better sensitivity, but barely superior to the highest signal recorded when no methane is added at 70% ACN. In order to study such behavior of bromine under the effect of methane, a bromine-containing compound (pure reagent tetrabutylammonium bromide) was analyzed by flow injection (FIA). The peak net intensity of the FIA peaks was studied as a function of the different methane flows added to the plasma, and compared to the intensity of the background. Results are collected in Figure S19A. In this figure the different behavior of peak net height, which increases with methane due to enhanced sensitivity and decreases with higher methane content in the plasma, and background, which constantly increases with methane flow, is appreciated. This results are clear indication of bromine contamination in the methane gas, so that results on signal enhancement cannot be taken into consideration. Nevertheless, addition of methane gas proved signal enhancement as seen in Figure S19B. Furthermore, bromine contamination does not affect the capacity of methane to correct for signal variations along the capLC analysis for bromine. However, it likely affects limits of detection in quantitative results.



Supplementary Figure S19. (A) Bromine ICP signal (Br 79→79) is plotted versus time for the methane flows assayed between 0 and 225 $mLmin^{-1}$. The FIAgram was done at 2% mobile phase B (ACN:formic acid 0.2%) and injection of 1 µL tetrabutylammonium bromide pure reagent was done for quadruplicate. (B) Representation of background intensity and peak net intensity of bromine FIAgram peaks. Peak net intensity increases with the addition of methane due to enhanced sensitivity, and decreases the higher the amount of carbon reaching the plasma is. In contrast, background intensity keeps increasing with the flow of methane, because of the bromine contamination in the methane gas.