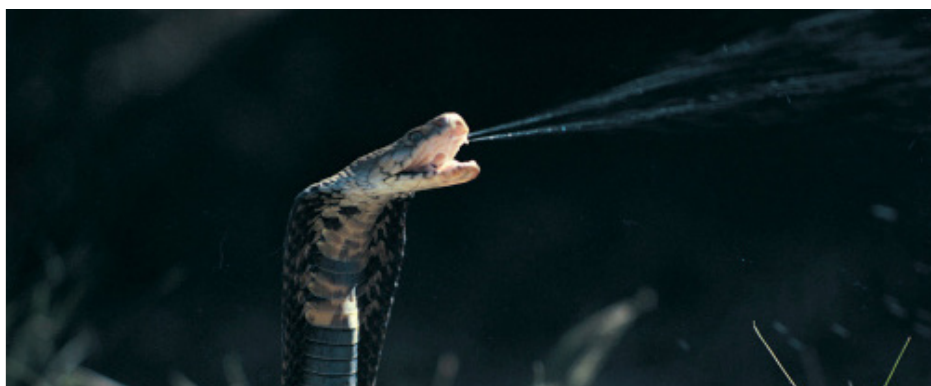


Absolute Quantification of Proteins in Snake Venom

Using capLC-ICP-QQQ and online isotope dilution analysis



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Introduction

Venoms are complex biological fluids that contain unique mixtures of peptides and proteins. Identifying and quantifying the composition of venoms is of increasing scientific interest. It is especially important to characterize the toxins responsible for the severe biological effects of the venom on humans. Better understanding of the symptoms of envenoming would help with the development of effective therapies. Also, venoms are being investigated as potential sources of new compounds in drug development.

In this study, an absolute quantification method suitable for the direct quantification of intact proteins was applied to the analysis of the venom of the Mozambique spitting cobra (*Naja mossambica*). This cobra is one of the most dangerous snakes in Africa. The cobra's venom is mainly toxic to cells (cytotoxic), causing swelling of the bite wound that may evolve into tissue necrosis and gangrene [1, 2]. The cytotoxic components of the cobra venom have been identified mainly as members of the three-finger toxin (3FTx) and phospholipase A2 (PLA2) protein families [3, 4].



The methodology was based on capillary liquid chromatography (capLC) coupled to a triple quadrupole ICP-MS (ICP-QQQ). Absolute protein quantification was achieved by measuring the sulfur heteroelement in the proteins, calibrated using online isotope dilution analysis (IDA). ICP-QQQ uses MS/MS mode to control reaction chemistry in the collision/reaction cell (CRC), giving consistent removal of spectral interferences using reactive cell gases [5]. Efficient removal of spectral overlaps using MS/MS allows access to multiple isotopes of biologically important elements such as iron, sulfur, and selenium. ICP-QQQ enables the quantification of metalloproteins and peptides using IDA, without the need for compound-specific calibration standards [6, 7].

Most proteins (> 95%) contain sulfur from methionine and cysteine residues [8, 9], but sulfur determination is difficult by conventional single-quadrupole ICP-MS. The challenges are due to the element's high ionization potential (10.4 eV) that leads to low sensitivity, and the occurrence of spectral interferences from multiple polyatomic ions that overlap all isotopes of sulfur. ICP-QQQ provides low backgrounds and high sensitivity, and removes multiple matrix interferences using MS/MS, freeing up the three most abundant S isotopes for accurate measurement using IDA.

In this study, capLC-ICP-QQQ was used for the quantitative analysis of intact proteins, isolated, and present in simple mixtures. The method was also applied to the analysis and quantification of the major toxins comprising the venom proteome of the Mozambique spitting cobra.

Experimental

Reagents and samples

Methionine and BOC-Met-OH (Sigma-Aldrich, Germany) were used as standards. Bovine serum albumin (BSA), transferrin, β -casein, and cytochrome C (Sigma-Aldrich, Germany); and intact monoclonal antibody (mAb) Mass Check Standard (Waters, USA) were used as protein standards in the recovery study. Other reagents included sulfur (1000 mg/L S) ICP standard (Merck KGaA, Germany); solid isotopically enriched ^{34}S (Isoflex USA); and sodium hydroxide (VWR Chemicals Belgium). Lyophilized *Naja mossaambica* venom was obtained from the specialist venom supplier Latoxan S.A.S., France. The venom was collected from a snake from Tanzania, and was stored at $-20\text{ }^{\circ}\text{C}$ before use. All solutions were prepared in Milli-Q water (ChemLabor Millipore system, with $0.22\text{ }\mu\text{m}$ filter, Millipak - Millipore). Mobile phase B was prepared in acetonitrile (ACN) Optima® LC/MS (Fischer Scientific, USA). Formic acid (FA) was bought from Merck KGaA (Germany).

Instrumentation

Capillary LC separation was performed using an Agilent 1200 Series HPLC system fitted with a BIOShellTM A400 C4, $3.4\text{ }\mu\text{m}$, $150\text{ mm} \times 0.3\text{ mm}$ reversed-phase column (Sigma-Aldrich, Germany) and autosampler. Chromatographic column and post-column connections comprised Agilent PEEK-coated fused silica capillaries $200\text{ mm} \times 100\text{ }\mu\text{m}$ id (ICP and syringe connection) and $50\text{ }\mu\text{m}$ (column connection), and a $0.03''$ (0.8 mm) Agilent zero-dead volume T-connector. Post-column flow was provided by a syringe pump system kdScientific (Holliston, MA, USA). The column was heated using a Spark Holland oven (Mistral, The Netherlands) to improve chromatographic efficiency.

Sulfur isotope measurements were carried out using an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ). The capLC column was connected to the ICP-QQQ using the Agilent capillary LC interface kit (Agilent product number G3680A). The kit consists of a total consumption nebulizer with single pass spray chamber. Enriched ^{34}S was added to the LC eluate solution post-column. Sulfur isotopes were measured by ICP-QQQ using an oxygen mass-shift MS/MS method and the S content of the proteins was determined by IDA [10]. BOC-Met-OH was used as the internal standard (IS) to correct for any injection errors. capLC-ICP-QQQ operating conditions are given in Table 1.

Table 1. capLC-ICP-QQQ operating conditions.

ICP-QQQ		
RF power (W)	1550	
Sampling depth (mm)	8.0	
Carrier gas flow rate (L/min)	0.85	
Makeup gas flow rate (L/min)	0.00	
O ₂ cell gas flow rate (mL/min)	0.16	
Data acquisition ion pairs, Q1 (S ⁺) » Q2 (SO ⁺) mass (m/z)	32 → 48 34 → 50	
capLC		
Chromatographic flow rate ($\mu\text{L}/\text{min}$)	4.5; 3.5*	
Mobile phase A	H ₂ O/0.2% FA	
Mobile phase B	AcN/0.2% FA	
Temperature ($^{\circ}\text{C}$)	80	
Chromatographic gradient	Time range (Min)	% mobile phase B
BSA and Intact mAb standards	0	2
	2	2
	16	60
	18	90
<i>Naja mossaambica</i> sample	0	1.5
	8	1.5
	10	10
	40	30
	47	90

* Conditions for measurement of venom sample

Results and Discussion

Absolute protein quantification

Absolute quantification of proteins was achieved through the measurement of sulfur by capHPLC-ICP-QQQ, with post-column IDA. Two individual protein samples (BSA and mAb) were spiked with BOC-Met-OH IS. Quantitative ID (mass purity) results for BSA, $95 \pm 5\%$ ($n=3$), compared well with the sample purity data provided by the manufacturer ($\geq 98\%$). The ID mass purity result for Intact mAb was $77 \pm 4\%$ ($n=3$).

To confirm the accuracy and specificity of the ICP-MS/MS analysis, digests of the two protein standards were quantified using external calibration of sulfur. The results obtained by external calibration were $96 \pm 1\%$ for BSA and $79 \pm 2\%$ for Intact mAb. These results are in good agreement with the ID mass purity results for BSA and Intact mAb shown above.

Quantitative analysis of snake venom proteome

The method was applied to the analysis and quantification of the proteins present in a snake venom sample. Before analysis, chromatographic recovery was calculated by measuring the sulfur mass of a sample eluting from the chromatographic column and comparing this to the sulfur mass recorded directly by flow injection (FI). Chromatographic recovery for a series of protein standards (cytochrome C, β -casein, transferrin, BSA, Intact mAb) was higher than 98%. For the *Naja mossambica* protein content, it was $99 \pm 1\%$ ($n=3$). The total sulfur mass content corresponded almost exactly to the sum of the sulfur contained in the different venom protein peaks detected via their sulfur heteroelement content (Figure 1). The excellent recovery obtained for the complex sample shows that quantitative protein recoveries for the chromatographic column are species (individual protein) independent.

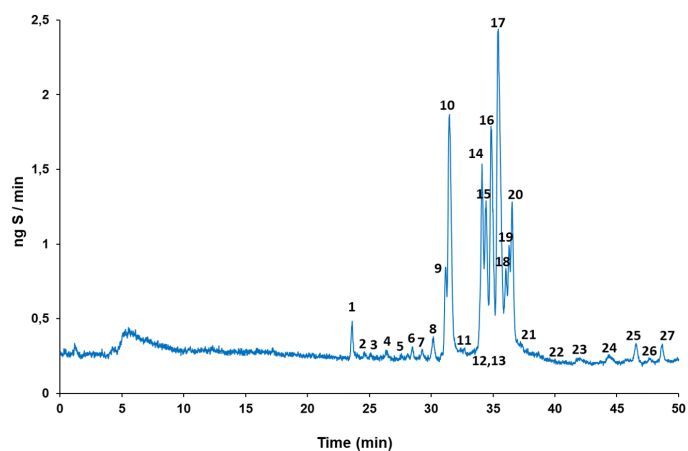


Figure 1. capLC-ICP-QQQ mass flow chromatogram of *Naja mossambica* venom. All the venom protein species eluted between 20 and 50 min (S detection). The 27 sulfur-containing peaks are numbered—see Table 2 for more information.

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Parallel capLC-ESI-MS analysis was used to identify the proteins from their molecular weight, according to database information (Table 2). By identifying the proteins, it was possible to know the S-to-protein stoichiometry of each peak. This information was then used to translate sulfur mass into individual protein quantities, in μmol protein per gram of venom sample. The quantified results are summarized in Table 2.

Table 2. Matching of the masses of protein peaks from *Naja mossambica* venom to known protein families. Closest available protein species, estimated exact mass, and calculated concentration are listed. Uncertainty corresponds to one standard deviation ($n=3$).

Peak	Family	Closest homolog	Mol wt (Da)	$\mu\text{mol protein/g venom sample}$
1	3FTx	–	7064.2	1.99 ± 0.06
2	3FTx	~P29179	7417.4	0.471 ± 0.066
3	3FTx	~P29179	7451.6	0.325 ± 0.040
4	3FTx	~P01420	6892.4	1.10 ± 0.13
5	3FTx	~Q9W6W6	7786.4	< 0.1
6	3FTx	~P01452	7277.3	0.680 ± 0.050
7	3FTx	~P01452	7306.3	0.668 ± 0.057
8	3FTx	–	7246.2	1.35 ± 0.10
9	3FTx	P25517	6832.4	5.09 ± 0.28
10	3FTx	P01452	6704.3	19.0 ± 0.8
11	3FTx	–	6686.3	0.183 ± 0.035
12	3FTx	–	6829.3	0.220 ± 0.039
13	3FTx	–	6687.3	< 0.1
14	PLA ₂	P00604	13280.9	7.76 ± 0.32
15	3FTx	P01470	6882.4	9.54 ± 0.27
16	3FTx	P25517	6813.3	16.2 ± 0.4
17	3FTx	P01467	6814.3	27.8 ± 0.8
18	3FTx	~P01469	7046.4	5.13 ± 0.26
19	PLA ₂	P00604	13237.8	3.40 ± 0.14
20	PLA ₂	P00002	13196.6	7.35 ± 0.33
21	PLA ₂	–	13179.7	0.805 ± 0.049
22	Minor	–	42000	0.102 ± 0.009
23	Endonuclease	–	30000	0.619 ± 0.050
24	SVMP	Q10749	46700	0.165 ± 0.009
25	SVMP	Q10750	46700	0.264 ± 0.013
26	SVMP	Q10751	46700	0.097 ± 0.006
27	SVMP	Q10752	46700	0.257 ± 0.006

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Conclusions

The capLC-ICP-QQQ method uses measurement of sulfur by IDA to enable the absolute quantification of intact proteins without the need for protein-specific standards.

Agilent ICP-QQQ instrumentation is especially suited for IDA analysis of S as it uses MS/MS to remove multiple spectral interferences from several S isotopes using an oxygen mass-shift method. By adding enriched ^{34}S post-column and spiking each sample with a generic S-containing internal standard, multiple S isotopes can be measured as SO^+ product ions. MS/MS ensures that each S isotope mass enters the CRC in isolation, so no interfering product ions can be formed from the other isotopes of S. This approach allows the sulfur content of the proteins to be determined by IDA.

The method was applied successfully to the quantification and mass purity confirmation of protein standards. If quantitative chromatographic recoveries can be assured, it is even possible to quantify nonpure protein samples using this method. The potential of the methodology for the quantification of intact proteins present in relatively complex samples was demonstrated by analyzing 27 proteins in snake venom.

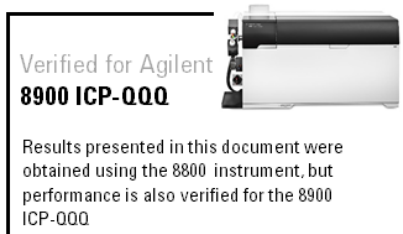
References

1. D. A. Warrell, *Lancet*, **2010**, 375, 77–88
2. J.M. Gutiérrez, D. Williams, H.W Fan, D.A. Warrell, *Toxicon*, **2010**, 56 (7), 1223-1235
3. D. Petras, L. Sanz, Á. Segura, M. Herrera, M. Villalta, D. Solano, M. Vargas, G. León, D. A. Warrell, R. D. G. Theakston, R. A. Harrison, N. Durfa, A. Nasidi, J. M. Gutiérrez, and J. J. Calvete, *J. Proteome Res.* **2011**, 10 (3), 1266–1280.
4. I. Méndez, J.M. Gutiérrez, Y. Angulo, J.J. Calvete, B. Lomonte, *Toxicon*, **2011**, 58 (6–7), 558–564.
5. L. Balcaen, G. Woods, M. Resano, and F. Vanhaecke, *J. Anal. At. Spectrom.*, **2013**, 28, 33–39
6. Y. Anan, Y. Hatakeyama, M. Tokumoto, and Y. Ogra, Analysis of selenoproteins in rat serum by Triple Quadrupole ICP-MS, Agilent publication, 2013, 5991-2750EN
7. P. De Raeve, J. Bianga, Fast and accurate absolute-quantification of proteins and antibodies using Isotope Dilution-Triple Quadrupole ICP-MS. Agilent publication, 2016, 5991-6118EN
8. M. Wind, A. Wegener, A. Eisenmenger, R. Keller, W.D. Lehmann, *Angewandte Chemie International*, **2003**, 42:3425–3427
9. H. Zhang, W. Yan, R Aebersold, *Current Opinions Chem. Biol.* **2004**, 8 (1) 66–75
10. F. Calderon-Celis, S. Diez-Fernandez, J. M. Costa-Fernandez, J. R. Encinar, J.J. Calvete, and A. Sanz-Medel, Elemental Mass Spectrometry for Absolute Intact Protein Quantification without Protein-Specific Standards: Application to Snake Venomics, *Anal. Chem.*, **2016**, 88 (19), 9699–9706.

More Information

The full results of the study were published in:

Francisco Calderón-Celis et al., Elemental Mass Spectrometry for Absolute Intact Protein Quantification without Protein-Specific Standards: Application to Snake Venomics, *Anal. Chem.*, **2016**, 88 (19), 9699–9706.



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