TECHNICAL BRIEF

A proteomics assay to detect eight CBRN-relevant toxins in food

Benoit Gilquin[1,2,3,4], Michel Jaquinod[1,2,3], Mathilde Louwagie[1,2,3], Sylvie Kieffer-Jaquinod[1,2,3], Alexandra Kraut[1,2,3], Myriam Ferro[1,2,3], François Becher[5] and Virginie Brun[1,2,3]

[1] Université Grenoble-Alpes, Grenoble, France
[2] CEA, BIG, Biologie à Grande Echelle, Grenoble, France
[3] INSERM, U1038, Grenoble, France
[4] CEA, LETI, Clinatec, Grenoble, France
[5] CEA, iBiTec-S, Laboratoire d'Etude du Métabolisme des Médicaments, Gif-sur-Yvette, France

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A proteomics assay was set up to analyze food substrates for eight toxins of the CBRN (chemical, biological, radiological and nuclear) threat, namely ricin, *Clostridium perfringens* epsilon toxin (ETX), *Staphylococcus aureus* enterotoxins (SEA, SEB and SED), shigatoxins from *Shigella dysenteriae* and entero-hemorragic *Escherichia coli* strains (STX1 and STX2) and *Campylobacter jejuni* cytolethal distending toxin (CDT). The assay developed was based on an antibody-free sample preparation followed by bottom-up LC-MS/MS analysis operated in targeted mode. Highly specific detection and absolute quantification were obtained using isotopically labeled proteins (PSAQ standards) spiked into the food matrix. The sensitivity of the assay for the eight toxins was lower than the oral LD50 which would likely be used in a criminal contamination of food supply. This assay should be useful in monitoring biological threats. In the public-health domain, it opens the way for multiplex investigation of food-borne toxins using targeted LC-MS/MS.

Keywords:
Bioterrorism / Food / Mass spectrometry / Quantification / Technology / Toxin

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The geopolitical situation and recent terrorist attacks have led the international and various national authorities to reinforce monitoring for unconventional warfare agents [1]. Monitoring for biological threats, i.e., the deliberate spread of infectious microorganisms or toxins through water or food supplies or the air requires rapid and reliable methods for early identification of bioterrorism agents [2].

Over the last decade, MS-based methods have emerged as powerful analytical solutions to detect toxins linked to the CBRN (chemical, biological, radiological and nuclear) threat [3]. MS-based assays have been developed to detect CBRN-related protein toxins in various matrices, including water, food and biological fluids. To ensure highly sensitive detection in complex matrices, most of these assays were based on an antibody-based enrichment step to extract the targeted toxins prior to protein digestion and subsequent targeted LC-MS/MS analysis. However, as interferences can occur when multiple antibodies with different specificities are used, multiplexing by these methods is limited to a few toxins

Correspondence: Dr. V. Brun, Unité de Biologie à Grande Echelle, CEA/DRF/BIG/INSERM/UGA 1038, 17 avenue des Martyrs, 38054 Grenoble cedex 9, France
E-mail: virginie.brun@cea.fr
Fax: +33 4 38 78 50 51

Abbreviations: **CBRN**, chemical, biological, radiological and nuclear; **CDT**, cytolethal distending toxin; **ETX**, *Clostridium perfringens* epsilon toxin; **PSAQ**, protein standard absolute quantification; **SEA**, *Staphylococcus aureus* enterotoxin A; **SEB**, *Staphylococcus aureus* enterotoxin B; **SEC**, *Staphylococcus aureus* enterotoxin C; **SED**, *Staphylococcus aureus* enterotoxin D; **STX1**, shigatoxin 1; **STX2**, shigatoxin 2

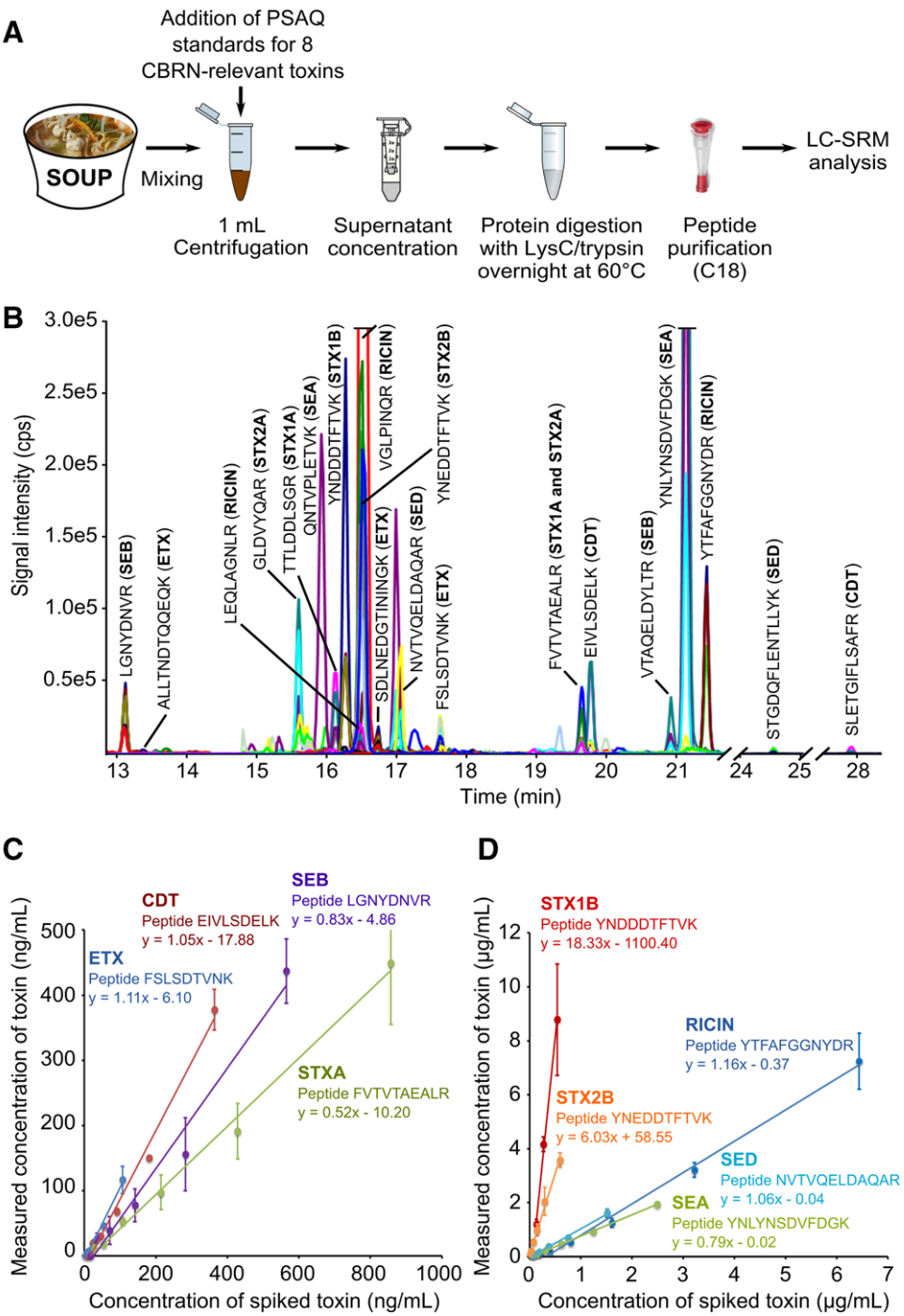

Figure 1. Detection and quantification of CBRN-relevant toxins in food matrices using PSAQ standards and targeted proteomics. (A) Workflow for processing soup samples. A detailed protocol is available in the Supporting Information. (B) Extracted ion chromatogram obtained from an LC-SRM analysis screening for the eight CBRN-relevant toxins in soup matrix ("Chinese" soup). (C) Calibration curves obtained for ETX, SEB, STXA (STX1A and/or STX2A) and CDT. (D) Calibration curves obtained for ricin, SEA, SED, STX1B and STX2B. For each analyte, the calibration curve included a zero and five non-zero calibration points, each performed in three technical replicates.

(<5) at a time [4–6]. The goal of this study was to develop an antibody-free proteomics assay to improve the multiplexing capacity for CBRN-related and food-borne toxins. Soup-based matrices were selected as surrogates for gastric fluid, which might be investigated in the context of food poisoning. Two different varieties were chosen—"Chinese" or "mushroom" soup—because of their high complexity: presence of lipids, proteins originating from different species, extensive dynamic range for proteins. Eight toxins triggering severe gastrointestinal disorders in humans were simultaneously investigated in soup matrices. Five of these toxins were selected from those registered by the Center for Disease Control (CDC) as potential biological weapons (http://emergency.cdc.gov/agent/agentlist.asp): ricin from *Ricinus communis;* epsilon toxin (ETX) from *Clostridium perfringens; Staphylococcus aureus* enterotoxin B (SEB) and shigatoxins (STX1 and STX2) from *Shigella dysenteriae* or shigatoxin-producing *Escherichia coli* strains.

Staphylococcus aureus enterotoxins (SEA and SED) that are frequently implicated in food poisoning [7, 8] were also included in the toxin panel. Finally, *Campylobacter jejuni* cytolethal distending toxin (CDT) was also investigated. CDT is a genotoxin considered to be a major virulence factor for Gram-negative bacteria. When expressed by *Campylobacter jejuni* strains, it is associated with severe gastroenteritis [9]. A variant of CDT (called CDT-V) was found to be involved in the high virulence of shigatoxin-producing *Escherichia coli* strains [10]. There is currently no method to directly detect CDT, which can only be identified by bacterial strain genotyping or cytotoxicity assays after strain isolation.

We started by developing an LC-SRM method to detect the eight targeted toxins in soup matrix. The eight toxins were either purchased or produced in the laboratory in recombinant forms, either as full-length versions or, for safety considerations, as protein subunits (Supporting Information Table S1). To improve detection specificity in this complex matrix and ensure reliable assay results, we also synthesized isotopically labeled versions of the targeted toxins to serve as quantification references (protein standards for absolute quantification, PSAQ standards) (Supporting Information Table S1) [11]. The two different soup matrices were spiked with unlabeled and labeled toxins, and the optimal processing conditions for toxin extraction, digestion and LC-SRM analysis were determined (Fig. 1). The biochemical protocol retained was based on blending the soup, centrifugation to remove solid matter and concentration of the remaining liquid using a 3-kDa cutoff ultrafiltration device (Fig. 1A). SEB is very resistant to proteolysis, and digestion conditions therefore had to be optimized. The optimal protocol was double-enzyme digestion with overnight incubation at 60°C (see the Supporting Information for the detailed sample processing and digestion protocol). To ensure highly specific and sensitive detection of the eight CBRN-related toxins, LC-SRM methods were designed to target the two or three most-responsive signature peptides for each toxin. Thus, 19 signature peptides were selected for the LC-SRM analysis by using a 6500QTrap hybrid triple quadrupole/ion trap mass spectrometer (AB Sciex). Each peptide (in labeled and unlabeled forms) was monitored based on at least three fragment ions, resulting in an inclusion list consisting of 118 SRM transitions (Supporting Information Table S2). To further improve detection sensitivity, SRM acquisition was scheduled, but as the nature of the matrix can significantly impact LC retention time, relatively broad acquisition windows (120 s) were applied. The signature peptides included in the final LC-SRM screening method and their relative characteristics are summarized in Table 2. By applying this protocol, this group of toxins can be identified within 20–24 h (including overnight digestion). For faster identification of bioterrorism toxin agents, the assay duration could potentially be significantly decreased through the use of microwave-assisted proteolysis [12] or pressure cycling technology [13].

Once the biochemical procedure and the LC-SRM method were optimized to screen for the eight CBRN-related toxins in soup matrices, we checked whether our assay was sufficiently sensitive to detect toxins at relevant toxic concentrations in food. To do this, a multiplex calibration experiment was

Table 1. List of monitored CBRN-related toxins and their characteristics

Toxin	Species	Structure	Reported or extrapolated oral LD50	Amount considered to be highly toxic in a human being (70 kg)	Amount to be detected per mL of soup[c)]
Ricin	*Ricinus communis*	Heterodimer of A and B chains	10 mg/kg [14]	700 mg	1.3 mg of ricin A chain
ETX	*Clostridium perfringens*	Secreted as a single chain	70 ng/kg [17][a)]	4.9 μg	20 ng
SEA	*Staphylococcus aureus*	Monomer or homodimer	>0.71 μg/kg [18][b)]	>50 μg	>200 ng
SEB	*Staphylococcus aureus*	Monomer or homodimer	>0.71 μg/kg [18]	>50 μg	>200 ng
SED	*Staphylococcus aureus*	Monomer or homodimer	>0.71 μg/kg [18][b)]	>50 μg	>200 ng
STX1	*Shigella dysenteriae, Escherichia coli EHEC*	AB5 multimer (A subunit + pentamer of B subunits)	8 μg/kg [14][a)]	560 μg	2.24 μg
STX2	*Shigella dysenteriae, Escherichia coli EHEC*	AB5 multimer (A subunit + pentamer of B subunits)	0.29 μg/kg [14][a)]	20.3 μg	81 ng
CDT	*Campylobacter jejuni*	Heterotrimer of A, B and C chains	Not determined	Not determined	Not determined

a) Oral LD50 in humans was not reported and was extrapolated from intraperitoneal LD50 in mouse.
b) Oral LD50 in humans was not reported and was extrapolated from SEB oral toxicity.
c) For a 250 mL portion of soup.

Table 2. Analytical performance characteristics of the multiplexed proteomics assay

Toxin	PSAQ standard	Signature peptide monitored by LC-SRM[a)]	Peptide specificity	Range of concentrations tested (ng/mL of soup)	Linearity (R^2)	Accuracy (trueness)[b)] (%)	LLOQ[c)] (ng/mL of soup)	Precision at LLOQ (CV in %)
Ricin	Ricin chain A	VGLPINQR	Specific (chain A)	201–6441	0.99	114	805	12
		YTFAFGGNYDR	Specific (chain A)					
		LEQLAGNLR	Specific (chain A)					
ETX	ETX	ALLTNDTQQEQK	Specific	3–106	0.99	109	13	20
		FSLSDTVNK	Specific					
		SDLNEDGTININGK	Specific					
SEA	SEA	QNTVPLETVK	Specific	77–2490	0.99	78	78	18
		YNLYNSDVFDGK	Specific					
SEB	SEB	**LGNYDNVR**	Specific	17–564	0.99	83	141	24
		VTAQELDYLTR	Specific					
SED	SED	STGDQFLENTLLYK	Specific	47–1519	0.99	105	48	8
		NVTVQELDAQAR	Specific					
STX1		TTLDDLSGR	Specific (A subunit)					
	STX1B	**YNDDDTFTVK**	Specific (B subunit)	17–544	0.98	ND	136	14
STX2		GLDVYQAR	Specific (A subunit)					
	STX2B	**YNEDDTFTVK**	Specific (B subunit)	19–598	0.99	ND	37	19
STX1 and STX2	STX1A and STX2A	**FVTVTAEALR**	Shared between STX1A and STX2A	26.5–858	0.99	ND	212	27
CDT	CDT C subunit	**EIVLSDELK**	Specific (C subunit)	11–336	0.99	104	23	14
		SLETGIFLSAFR	Specific (C subunit)					

ND: Not determined
a) Peptides indicated in bold were used to establish the multiplex calibration curve.
b) Trueness corresponds to the slope value (%) of the calibration curve for the peptide considered. For shigatoxins, accuracy could not be determined.
c) LLOQ was determined by applying the FDA guidelines for bioanalytical method validation, i.e., accuracy between 80 and 120%, precision $\leq$ 20% and signal-to-noise ratio $>$5. When accuracy was $<$80% or $>$120%, or precision was $>$20%, LLOQ was determined based on a signal-to-noise ratio $>$10.

designed to determine the performance of the overall analytical process. Unlabeled toxins were added to the "Chinese" soup matrix at a range of concentrations covering their reported or extrapolated oral LD50 (Table 1). PSAQ standards were spiked into samples in fixed amounts before sample processing and LC-SRM analysis. To enhance quantification sensitivity, only the most responsive peptide(s) for each toxin in the two varieties of soup matrix were monitored (Table 2). STX1 and STX2 were quantified using signature peptides for STX1B and STX2B subunits, respectively, but peptide FVTVTAEALR—which is shared between STX1A and STX2A—was also retained in the inclusion list. Figure 1 (C and D) shows the calibration curves obtained for the different toxins monitored. The sensitivity of the assay developed was excellent, with results below the toxin concentrations expected to be detected in the event of intentional food poisoning. For four of the toxins targeted (ricin, ETX, SED and CDT), the assay displayed excellent quantification performance conforming to the stringent guidelines of the health authorities, i.e., accuracy between 80 and 120% and precision $\leq$ 20% (Table 2). The assay did not quite meet these requirements for SEA and SEB quantification, but could do so if an increased number of technical replicates were performed (only three technical replicates were performed in this study). Shigatoxins were considerably more challenging to assay than the other toxins. First, the concentrations of unlabeled STX1 and STX2 seemed to be overestimated by the supplier (Supporting Information Fig. S1). Likely, the tested concentration ranges of shigatoxins were much lower than initially presumed. Second, their native structure and that of the quantification standards were different (unlabeled STX1 and STX2 were AB5 protein multimers, while PSAQ standards corresponded to A or B subunits). Differential behavior of these different forms during sample processing and digestion affected quantification accuracy. Notably, attempts were made to "equalize" the structure of native STX and PSAQ standards using urea denaturation and reduction/alkylation treatment during sample processing. However, as this protocol led

to the loss of SEB signature peptides, we decided to favor multiplexing capabilities over STX quantification accuracy.

In conclusion, we have successfully developed a multiplex proteomics assay which sensitively detects and quantifies eight CBRN-relevant toxins present in a food matrix. To our knowledge, the sole assay that displays a superior multiplexing power was described by Jenko and coworkers in 2014 and consisted in an ELISA microarray targeting ten CBRN-related toxins, including ricin, SEB, STX1, STX2 and six variants of the *Clostridium botulinum* toxin [14]. The assay described here circumvents the need for immunological reagents and as such, has the potential to allow even greater multiplexing. Thus, it could easily be extended to the detection of abrin [15] and additional staphylococcal enterotoxins, such as SEC, SEE and SEH [16]. In the case of highly homologous proteins (such as staphylococcal enterotoxins), exquisite detection specificity is guaranteed by the monitoring of unique signature peptides. Interestingly, our assay was the first described to target CDT, an extremely potent genotoxin produced by Gram-negative pathogenic bacteria. No direct assay is currently available for this toxin. Thus, our assay which was initially developed to improve the biodefense analytical arsenal might also be useful for public health monitoring and food quality control. We will now test whether this assay is compatible with clinical samples (serum/plasma/urine/stool), with the goal of improving the medical management of victims in the event of a bioterrorism attack.

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The authors have declared no conflict of interest.

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