Protein Standard Absolute Quantification (PSAQ) for improved investigation of staphylococcal food poisoning outbreaks

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Staphylococcal enterotoxins are major causing agents of food-borne diseases. Their detection in food remnants for risk assessment or food poisoning outbreaks investigation suffers from a lack in comprehensive immunological tools. In this study, we demonstrate that the combination of immunocapture and Protein Standard Absolute Quantification (PSAQ) strategy, which uses isotope-labeled enterotoxins as internal standards for MS-based analysis, is powerful to specifically identify and quantify these contaminating agents in food matrices. This approach is believed to significantly improve the elucidation of staphylococcal food poisoning outbreaks.

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Enterotoxin / Food poisoning / Mass spectrometry / Protein standard absolute quantification / Quantification

We have developed an innovative strategy, Protein Standard Absolute Quantification (PSAQ), which uses full-length isotope-labeled proteins as isotope-dilution standards for MS-based quantification of target proteins in complex matrices [1]. As the quantification standards display the same biochemical features as the target proteins, they can be spiked into the samples at early stages of the analytical process. Thus, in contrast with AQUA [2] and QconCAT [3] approaches, which use peptide standards for MS-based absolute quantification, PSAQ allows a highly accurate quantification even if the sample is extensively pre-fractionated before MS analysis [1, 4]. PSAQ also avoids differences in digestion yields between the internal standard and the target protein [1, 5]. Finally, PSAQ offers the largest sequence coverage for quantification, which increases detection specificity and measurement robustness [1]. Taking advantage of these potencies, we combined PSAQ strategy and immunocapture for the detection and absolute quantification of traces of staphylococcal enterotoxin A (SEA), a major agent of food poisoning [6], in contaminated food samples.

Staphylococcal food poisoning (SFP) is a common food-borne disease resulting from ingestion of staphylococcal enterotoxins (SE) preformed in food by Staphylococcus aureus strains [7]. In the United States, SE are responsible for 185,000 annual cases of food poisoning [8]. In France, SE represent the second cause of food-borne diseases after Salmonella [9]. To date, 19 staphylococcal enterotoxins and related toxins (“enterotoxin-like” proteins) have been described [10]. Strains isolated from food involved in SFP mainly pro-
duce SEA and to a lesser extent SED, SEB and SEC [6]. However, due to the lack of specific diagnosis tools against numerous SE and related toxins, many SFP outbreaks remain unsolved. SFP is clinically characterized by gastro-enteritis occurring between 1 to 8 h after food consumption. The biological diagnosis of SFP is conclusive when SE are detected in food remnants [11]. The detection of SE is classically performed using immunological techniques (ELISA) [12]. However, the immunological detection of SE displays major drawbacks. First, due to the high sequence and structural homology between SE, very few specific antibodies are available. Secondly, the complexity of food matrices often generates non-specific reactions [12]. Finally, the well-known IgG-binding staphylococcal protein A is co-secreted in food with SE and can interfere with the assay [12]. Consequently, commercial kits are available only for the detection of five enterotoxins (SEA to SEE) and suffer from serious limitations in terms of sensitivity, specificity and suitability for complex food matrices analysis.

We thus investigated the potential of the PSAQ method as an alternative to ELISA for SFP outbreaks characterization. Semi-hard cow-milk cheese was first chosen as a model, as it represents a high risk for S. aureus growth and SE production. The cheese model was manufactured in the “Lactic Acid Bacteria and Opportunistics Pathogens” laboratory of the French National Institute for Agricultural Research (Jouy en Josas, France). A SEA-producing S. aureus strain was inoculated into the milk before processing. According to the official procedure for dairy products control, a piece of cheese (25 g) was homogenized, depleted from caseins and the extract was concentrated by dialysis against PEG (see reference [12] for more details). This extract was investigated in parallel using a reference quantitative ELISA [12] or using our MS-based PSAQ method. For PSAQ analysis, the cheese extract was spiked with 100 ng of SEA PSAQ standard isotopically labeled with [13C6, 15N2] L-lysine and [13C6, 15N4] L-arginine (for details see reference [1]). The spiked cheese extract was passed through an immunoaffinity column (Biocontrol Systems, Lyon, France). This column is designed to capture five SE (SEA to SEE) but, as warned by the manufacturer, displays cross-reactivity with other staphylococcal toxins such as TSST-1. The eluate was collected and submitted to short-run SDS-PAGE. The region of the gel containing the endogenous enterotoxin and its iso-sorted counterpart (25–30-kDa region) was excised and submitted to digestion with trypsin. This denaturing electrophoresis dramatically improves the digestion of SE, which are protease-resistant proteins, and increases sample decomplexification. The proteolytic peptides extracted from the gel were analyzed in nanoLC-MS using a QTOF mass spectrometer (Waters, Milford, MA). Whereas the low-selectivity immunoaffinity column allows a large-spectrum enterotoxin capture, highly specific assignment of the captured SE is provided by the MS identification of peptides unique to each toxin (i.e. proteotypic peptides). One such proteotypic peptide (peptide NTVQELDLQAR) was detected in the cheese sample, specifically indicating the presence of endogenous SEA. Quantification was performed by comparing the integrated peaks of endogenous/labeled peptide extracted ion chromatograms (MassLynx software, Waters). The endogenous SEA was detected at 2.5 ± 0.2 ng/g of cheese (n = 3) consistently with the ELISA estimate (2.9 ± 0.3 ng/g; n = 3).

To go beyond this proof of concept, we applied the same PSAQ strategy to investigate a naturally contaminated food matrix. A Chinese dessert (coco-pearls), involved in a food poisoning outbreak in France in 2006 (11 patients declared), was collected by the French Agency for Food Safety (AFSSA). From this sample, AFSSA isolated a Staphylococcus aureus strain carrying the gene encoding SEA. An aliquot of this coco-pearl sample (25 g) was homogenized, centrifuged and the supernatant was concentrated by dialysis against PEG. The extract was tested concomitantly using ELISA or PSAQ methodology. For PSAQ quantification, the extract was spiked with 100 ng of SEA PSAQ standard and was processed and analyzed as described above for the cheese sample (see Fig. 1). Two proteotypic peptides specifically indicated the presence of endogenous SEA (peptides YNLNSDVFDGK and NVTVQELDLQAR). Using these peptides, SEA was quantified down to 1.47 ± 0.05 ng/g of food (n = 3). The difference between estimates (mean values) obtained with these two proteotypic peptides was as low as 0.7%. This result was in agreement with the 1.3 ± 0.2 ng/g (n = 3) ELISA estimate obtained from the same sample and was consistent with the symptoms declared (toxic dose = 40 ng according to Ikeda et al. [13]).

In this work, we have harnessed the power of the PSAQ strategy for the specific detection and quantification of SEA in food matrices. The use of isotope dilution with PSAQ standards allows an accurate quantification. Accordingly, the PSAQ analysis and the established ELISA gave comparable estimates and displayed similar sensitivity. On top, PSAQ methodology displayed an unrivalled detection specificity related to proteotypic peptides detection. To perform a relevant comparison with the ELISA estimates, we had to spike the PSAQ standard in the PEG-concentrated food extracts prepared for ELISA testing. However, in contrast to the ELISA approach, PSAQ standards can be added in the food homogenate at the very beginning of the analytical process, enabling to assess the eventual enterotoxin losses during the extraction/concentration procedure. This simple modification would further increase the accuracy of enterotoxin quantification in food.

The PSAQ methodology represents an attractive alternative to immunoassays especially for other previously undetected SE. In this aim, we analyzed 13 SE and defined a panel of 97 theoretical proteotypic peptides potentially allowing the unambiguous MS discrimination of any of these SE (see Supporting Information). From the MS analyses of these 13 toxins using a QTOF mass spectrometer, we further experimentally qualified 38 of these peptides. This panel of qualified proteotypic peptides potentially allows the unambiguous assignment of these 13 SE. At present, we are
Figure 1. PSAQ detection and quantification of staphylococcal enterotoxin A (SEA) in coco-pearls incriminated in a staphylococcal food poisoning outbreak. (A) Coco-pearls involved in a food poisoning outbreak in France in 2006 were collected by the French Agency for Food Safety. A coco-pearl sample (25 g) was homogenized, centrifuged and the supernatant was concentrated by dialysis against polyethylene glycol. The extract was tested concomitantly using quantitative ELISA or PSAQ method. Regarding MS analysis, the concentrated extract was spiked with 100 ng of SEA PSAQ standard, immunoenriched, and submitted to SDS-PAGE and trypsin digestion. The proteolytic peptides were analyzed with nanoLC-MS analysis. Two pairs of proteotypic peptides specifically indicated the presence of endogenous SEA and allowed quantification. Typical raw data obtained with the proteotypic peptides NVTVQELDLQAR (B) and YNLYNSDVFDGK (C) are shown. The relative features of the quantitative ELISA and the MS-based PSAQ strategy for SFP investigation are presented in (D).
synthesizing a library of isotope-labeled full-length SE. These enterotoxin PSAQ standards will be spiked simultaneously in naturally contaminated samples. The SE PSAQ library will thus allow multiplex detections and quantifications of SFP-incriminated enterotoxin(s).

The present experiments, performed on a Q-TOF mass spectrometer, gave a sensitivity limit comparable to that of the established ELISA. However, the use of the multiple reaction monitoring (MRM) method on a triple quadrupole instrument for MS analysis is expected to lower the detection sensitivity by a factor of at least ten. QTOF mass spectrometers present a limited dynamic range that keeps the linearity of the present method within a 1 to 2 log-range. The MRM method should extend the dynamic range of the present assay to up to five logs [14]. Finally, through the choice of targeted transitions, MRM should also dramatically improve the detection of some of the 59 theoretical SE proteotypic peptides that were not observed with QTOF (see Supporting Information), thus increasing the sequence coverage for identification and quantification of SE.

The present PSAQ method overpasses specific technical limitations of existing ELISA for SE characterization but its throughput and cost per analysis compare unfavorably with ELISA. This last method is no doubt the paradigm of the low-cost and high-throughput technique for the detection and quantification of biomarkers down to sub-nanomolar concentrations in large sample cohorts. However, the timescale for ELISA assay development is in the range of a year and high developmental costs, up to the million of dollars, precede systematic ELISA optimization for any potential target or biomarker [14]. This cost also limits ELISA economical pertinence for small panels' characterization such as SFP elucidation or candidate biomarker evaluation. In this regard, the versatility and low developmental cost of the PSAQ methodology position it as good alternative to ELISA for these specific applications.

In conclusion, PSAQ strategy undoubtedly represents the ideal alternative methodology to investigate SFP outbreaks unsolved with the existing immunological tools. This will allow the evaluation of the digestive pathogenicity of poorly characterized SE.

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References