Diagnostic development and public health surveillance require technologies that provide specific identification and absolute quantification of protein biomarkers. Beside immunologically related techniques (e.g. enzyme-linked immunosorbent assay), MS is gaining increasing interest due to its high sensitivity and specificity. Furthermore, MS-based analyses are extremely accurate quantitatively, provided that suitable reference standards are available. Recently, the use of chemically synthesized isotope-labeled marker peptides for MS-based absolute quantification of proteins has led to major advances. However, we show here that the use of such peptides can lead to severe biases. In this work, we present an innovative strategy (Protein Standard Absolute Quantification) that uses in vitro-synthesized isotope-labeled full-length proteins as standards for absolute quantification. As those protein standards perfectly match the biochemical properties of the target proteins, they can be directly added into the samples to be analyzed, allowing a highly accurate quantification of proteins even in prefractionated complex samples. The power of our Protein Standard Absolute Quantification methodology for accurate absolute quantification of biomarkers was demonstrated both on water and urine samples contaminated with *Staphylococcus aureus* superantigenic toxins as typical biomarkers of public health interest. *Molecular & Cellular Proteomics* 6:2139–2149, 2007.

Mass spectrometry MS has greatly contributed to the maturation of proteomics (1). It is now possible to characterize hundreds of proteins in an hour time frame and compare protein abundances in pairs of samples. The next frontier lies in accurate absolute quantitation. Although label-free spectral counting approaches (2, 3) are attracting considerable interest, robust absolute quantitative methodologies typically rely on the well-established isotope dilution principle (4), in which internal standardization is achieved with isotope-labeled homologs of specific proteolytic peptides from the target protein(s) (5, 6). The Absolute Quantitation (AQUA) approach uses chemically synthesized isotope-labeled peptides which are spiked into the samples in known quantities before MS analysis (5–8). Recently, the synthesis and metabolic labeling of an artificial concatemer of standard peptides (QconCAT), which can be spiked into the samples before trypsin digestion, was introduced to extend the number of quantified proteins (9–12). Although AQUA and QconCAT approaches have significantly advanced the quantitative measurement of proteins in biological samples, calibration with AQUA peptides and QconCAT constructs suffer from the following limitations: 1) a failure to take into account the actual efficiency of the proteolysis step required before MS analysis; 2) an incompatibility with sample prefractionation, which is often necessary when dealing with biological samples (13); and 3) a poor protein sequence coverage, limiting the statistical reliability of the quantification. We propose here an original Protein Standard Absolute Quantification (PSAQ) strategy for the absolute quantification of trace proteins in complex samples. This strategy is based on the use of in vitro-synthesized isotope-labeled full-length proteins as standards for quantification. To validate our methodology, we compared it with the two alternatives, AQUA and QconCAT strategies, using *Staphylococcus aureus* superantigenic toxins in water and urine samples as typical biomarkers of public health concern (14, 15).

*S. aureus* superantigenic toxins (enterotoxins and toxic shock syndrome toxin-1 [TSST-1]) are virulence factors responsible for severe diseases in humans among which the highly lethal staphylococcal toxic shock syndrome (14, 15). Staphyloccocal toxic shock syndrome is related to the ability of enterotoxins and TSST-1 to polyclonally and extensively activate T cells at picomolar concentrations, a property referred to as superantigenicity. Besides their superantigenic activity, staphyloccocal enterotoxins also display specific emetic properties and constitute a major cause of food poisoning (15). For all these reasons, staphyloccocal enterotoxins constitute a significant threat as biological weapons. The Center for Disease Control has registered the staphyloccocal

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1 The abbreviations used are: AQUA, Absolute Quantification; AAA, amino acid analysis; MRM, multiple reaction monitoring; PSAQ, protein standard absolute quantification; QconCAT, concatemer of standard peptides for absolute quantification; SEA, staphyloccocal enterotoxin A; SEB, staphyloccocal enterotoxin B; TSST-1, toxic shock syndrome toxin-1.
enterotoxin B (SEB) as a potential warfare contaminant of food and water supplies. To date, 19 staphylococcal enterotoxins have been identified (variant forms excluded) and are associated with different clinical profiles (15–17). Extensive sequence and structure similarities between staphylococcal superantigenic toxins have so far precluded the development of specific and comprehensive immunological tools. As a result, no diagnostic test is presently available for the staphylococcal toxic shock syndrome. Furthermore, no assay is referenced for the identification and quantification of these toxins in accidentally or deliberately contaminated food or water supplies. In this context, MS, which circumvents the need for antibodies, offers great potential for staphylococcal superantigenic toxin-specific detection and quantification.

In this work, we present an MS-based methodology to detect and quantify the staphylococcal superantigenic toxins SEA and TSST-1, which are the most frequently involved toxins in staphylococcal toxic shock syndrome (15, 16). Drinking water and human urine were selected as preferred matrices for warfare agent surveillance and clinical diagnosis. Comparisons with the current state-of-the-art methodologies demonstrate the great potential of isotope-labeled full-length proteins as standards for MS-based absolute quantification.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—AQUA \[^{13}C_6,^{15}N\] L-leucine-labeled peptides were synthesized by Sigma-Genosys (Saint Quentin Fallavier, France). These peptides were quantified by amino acid analysis (AAA) by the provider. Recombinant staphylococcal enterotoxins SEA and TSST-1 were purchased from Toxin Technology (Sarasota, FL). The dilutions of quantification standards and commercial toxins were systematically performed in low adsorption tubes (Dutscher, Bru-math, France).

**Synthesis, Purification, and Quantification of the Isotope-labeled QconCAT Concatemer**—The QconCAT protein was designed as shown in Fig. 1. Briefly, tryptic peptides from eight staphylococcal superantigenic toxins (SEA, SEB, TSST-1, staphylococcal enterotoxin G, staphylococcal enterotoxin I, staphylococcal enterotoxin M, staph...
ylococcal enterotoxin N, and staphylococcal enterotoxin O) were selected according to their uniqueness of sequence among the staphylococcal superantigens and their detectability in nano-LC-MS analysis. These peptide sequences were concatenated into an artificial QconCAT protein and retro-translated to design the corresponding artificial QconCAT gene (see references 9 and 11 for more details). The QconCAT gene was synthesized from 53 5’ phosphorylated oligonucleotides (Sigma-Genosys) covering the forward and reverse strands (Supplemental Fig. 1). The synthetic QconCAT gene was assembled by ligase chain reaction with TaqDNA ligase (New England Biolabs, Frankfurt, Germany) and amplified with the Expand High Fidelity polymerase (Roche, Meylan, France) using the primers mentioned in Supplemental Table I. The amplified QconCAT gene was purified, digested with Ncol (Roche) and SmaI (New England Biolabs), and inserted into the pIVEX 2.3d vector (Roche) as providing a C-terminal hexahistidine purification tag. Ligation was achieved using the Rapid DNA Ligation Kit (Roche). The resulting plasmid was cloned into strain XL1-Blue (Stratagene, Amsterdam, Netherlands) and was purified using QIAprep Spin Miniprep Kit (Qiagen, Courtaboeuf, France). Finally, we checked the QconCAT construct sequence before its use for recombinant protein synthesis (Genome Express, Meylan, France). QconCAT protein production was performed in vitro using the RTS 500 ProteoMaster Escherichia coli HY Kit (Roche) according to the manufacturer’s instructions with the following modifications: we used the RTS Amino Acid Sampler Kit (Roche) instead of the amino acid mix provided, and we replaced L-lysine and L-arginine by isotope-labeled [13C5, 15N3]-l-lysine and [13C8, 15N4]-l-arginine (Cambridge Isotope Laboratories, Andover, MA). The isotope enrichment of [13C6, 15N4]-l-lysine and [13C6, 15N4]-l-arginine was 98% 13C and 98% 15N. QconCAT protein was efficiently produced in a precipitated form and was solubilized in guanidine 6N. QconCAT purification was performed on a nickel affinity column (Ni Sepharose 6 Fast Flow, Amersham Biosciences, Freiburg, Germany) using a 20 mM-250 mM imidazole gradient in guanidine 6N. After purification, QconCAT was sequentially dialyzed against pure water and 1% SDS, Tris HCl 50 mM, pH 7.5. QconCAT quantification was performed by AAS on a Biochrom 30 Amino Acid Analyzer (Biochrom, Cambridge, UK). QconCAT primary structure and isotope labeling was further assessed by nano-LC-MS/MS and nano-LC-MS analysis (Supplemental Fig. 2).

Synthesis, Purification, and Quantification of Isotope-labeled SEA and TSST-1 PSAQ Standards—Two S. aureus strains carrying SEA or TSST-1 genes were selected from the strain collection of the French National Staphylococci Reference Center. Isotope-labeled SEB standard was not synthesized as its production is officially restricted. Genomic DNA was prepared using the QIAamp DNA Stool Mini Kit (Qiagen). The primers used for PCR amplification are described in Supplemental Table I. SEA and TSST-1 PCR fragments were purified, digested with KspI (Roche) and SmaI (New England Biolabs), and cloned into the pIVEX 2.4d expression vector providing an N-terminal hexahistidine hexahistidine purification tag (Roche). Our PSAQ strategy relies on biochemical equivalence between each toxin and its PSAQ standard. We thus privileged an N terminus cleavable tag to allow a polishing of the limited N terminus heterogeneity reported for proteins produced by cell-free synthesis (18). These constructs were cloned in XL1 blue, purified, sequenced, and used for in vitro protein synthesis in the presence of [13C6, 15N3]-l-lysine and [13C6, 15N3]-l-arginine as described above for QconCAT. Isotope-labeled SEA and TSST-1 were readily produced in a soluble form and were purified on a nickel affinity column (Ni Sepharose 6 Fast Flow, Amersham Biosciences) using an imidazole gradient. The N-terminal hexahistidin tag of each isotope-labeled protein was cleaved by biotinylated Factor Xa (Factor Xa Removal Kit, Roche) according to the manufacturer’s instructions. Both the resulting hexahistidin tag peptide and the biotinylated Factor Xa were removed in a single step using a mix of streptavidin coated beads and Ni Sepharose 6 Fast Flow resin. These isotope-labeled SEA and TSST-1 were quantified by AAA. Primary structure of the proteins and labeling efficiency were verified by nano-LC-MS/MS and nano-LC-MS analysis. The incorporation yield of [13C6, 15N3]-lysine and [13C6, 15N4]-arginine in these cell-free expressed standards was greater than 98% (data not shown).

SDS-PAGE Quality Control—Home-produced PSAQ proteins and purchased SEA and TSST-1 were all checked for purity on SDS-PAGE using both Imperial Protein Stain and SYPRO Ruby staining (Bio-Rad, Marnes-la-Coquette, France). The quantities of commercial toxins were too limited for AAA analysis, and commercial TSST-1 toxin displayed a contamination by a higher molecular weight protein precluding an accurate AAA quantification. Thus, commercial toxin concentrations were evaluated by comparison with our AAA calibrated PSAQ standards on SDS-PAGE using SYPRO Ruby staining (19). SYPRO Ruby fluorescence was scanned (Laser 532 nm, filter 610BP30) on a Typhoon 9400 (Amersham Biosciences). When compared with our AAA calibrated PSAQ standards, the announced concentrations of commercial SEA and TSST toxins turned out to be overestimated (Supplemental Fig. 3). Therefore, the commercial toxin concentrations were corrected, and the corrected values were used in all subsequent calculations.

Water Sample Preparation and Trypsin Digestion for MS Analyses—Drinking water samples were contaminated with five different quantities of SEA and TSST-1 commercial toxins. Each sample was divided into nine aliquots of 120 μl each. Three aliquots (analytical replicates) of each sample were spiked with either QconCAT or PSAQ toxins standards in defined quantities. Trypsin digestion was performed in solution using sequencing grade modified trypsin (Promega, Madison, WI) at a 1:2 protease to toxins ratio in 25 mM NH4HCO3 overnight at 37 °C. Samples were dried by vacuum centrifugation and resolubilized in 5% ACN, 0.2% formic acid. Before nano-LC-MS analysis, AQUA peptides were added in defined quantities into the aliquots that contained neither QconCAT nor PSAQ standards.

Urine Sample Preparation and Trypsin Digestion for MS Analyses—Urine from a 30-year-old healthy woman was collected and contaminated with four quantities of SEA and TSST-1 commercial toxins. Each sample was divided into nine aliquots of 100 μl each. Three aliquots (analytical replicates) of each sample were contaminated with PSAQ toxin standards in defined amounts. Each 100 μl aliquot was adsorbed on 5 μl of Stratscreen resin (Stratagene) according to the manufacturer’s instructions. Following elimination of supernatant, proteins adsorbed onto the resin were directly eluted in 10 μl of a depolymerization buffer containing 2% SDS and 5% β-mercaptoethanol. At this stage, QconCAT standard was added in controlled quantities into half of the samples devoid of PSAQ standards. After a thermal denaturation step at 95 °C for 5 min, samples were loaded on precoat Novex NuPAGE Bis–Tris gels (4–12% acrylamide gradient) purchased from Invitrogen (Cergy Pontoise, France). Gels were run for 30 min under 200 V, fixed for 30 min in 30% ethanol-7.5% acetic acid, and stained with Biosafe Coomassie blue (Bio-Rad). In the 25 kDa region of the gel encompassing toxins and QconCAT, protein bands were excised and were destained by repeated cycles of incubation in 25 mM NH4HCO3 for 15 min and then with 50% (v/v) ACN in the same buffer (25 mM NH4HCO3) for 15 min. After drying by vacuum centrifugation, the gel pieces were incubated with an oxidizing solution (7% H2O2) for 15 min (20). Gel pieces were then washed in HPLC grade water (Sigma-Aldrich) for 15 min before being dehydrated with 100% ACN. nano-LC-MS analysis.
TABLE I
Marker peptides used for the detection and quantification of SEA and TSST-1 staphylococcal superantigenic toxins.

| Staphylococcal superantigenic toxin | Swiss-Prot accession no. | Sequence of the specific tryptic peptides used for detection and quantification analyses | Monoisotopic mass of the precursor (observed) | m/z (observed) | z (observed) | Retention time min |
|------------------------------------|--------------------------|---------------------------------------------------------------------------------|------------------------------------------|----------------|--------------|-------------------|
| SEA                                | P0402                   | NVTVQELDLQAR<sup>a</sup>, QNTPPLETVK, YNLNSDVFDFGK<sup>a</sup>, HQLTIQHGLYR, LPTPIELPLK<sup>a</sup>, NTDGSILIFFSPYSPAFTK, QLAISTLDEIR | 1384.7, 1127.6, 1433.7, 1364.7, 1119.7, 2417.2, 1404.9 | 693.4, 564.8, 717.9, 455.9, 560.9, 806.8, 703.4 | 2+, 2+, 2+, 3+, 2+, 3+, 2+ | 27.6, 17.7, 32.6, 15.8, 36.3, 29.8, 43.0 |
| TSST-1                             | P06886                  |                                                                                   |                                            |                |              |                   |

<sup>a</sup> Synthesized as AQUA peptide standard.

RESULTS

Selection of Staphylococcal Superantigenic Toxin Marker Peptides—SEA and TSST-1 recombinant staphylococcal toxins were submitted to SDS-PAGE and in-gel digestion with trypsin. The peptide digests were analyzed by nano-LC-MS/MS (see Supplemental data files) and nano-LC-MS. Specific tryptic peptides (marker peptides) were selected for each of the two toxins (Table I). Marker peptides were chosen according to their sequence uniqueness among staphylococcal superantigens and their optimal detectability in MS analysis. Three of these marker peptides were made synthesized as AQUA peptides with one <sup>13</sup>C<sub>6</sub>, <sup>15</sup>N-l-leucine (mass increase 7 Da).

Quantification of Staphylococcal Superantigenic Toxins in Drinking Water Using Isotope-labeled AQUA Peptides—Commercial SEA and TSST-1 staphylococcal toxins were added in defined amounts into drinking water. Trypsin digestion was performed in-solution. Known amounts of AQUA peptides were added to the peptide digests before nano-LC-MS analysis. For the three unlabeled/labeled peptide pairs selected (Table I), peak doublets were integrated to determine the total ion signal of the natural peptide (mass m) and its corresponding labeled AQUA standard (mass m + Δm). The ratio of these signals allowed a direct calculation of the estimated natural toxin amount which was plotted against the added amount (Fig. 2). Ideally, 100% recovery should have been observed. The SEA and TSST-1 titration curves obtained were linear for added amounts of toxins ranging from 50 to 750 pg. Deviation from linearity above these values resulted from ion signal saturation. The measurements of accuracy and precision were evaluated through the slope values of the titration curves and the standard mean errors (S.E.) values of the data, respectively. AQUA peptide standardization is a highly precise quantification strategy (Fig. 2). However, regarding accuracy, an important discrepancy between the two AQUA peptides targeting SEA was observed (slope value = 1.37 for YNLNSDVFDFGK peptide and slope value = 0.44 for NVTVQELDLQAR peptide) (Fig. 2A). These potential biases may account for such results: 1) AQUA peptides were quantified by the provider using AAA, obtained lyophilized, and re-solubilized according to their own guidelines. Nevertheless, quantitative re-solubilization should not be taken for granted, and a subsequent evaluation of the re-solubilized peptide by AAA would require much more material than provided. 2) AQUA standards have to be highly diluted prior to their addition into the samples. Depending on their physicochemical properties, dilution of pure peptides can lead to important losses of peptide by adsorption onto vials. 3) Standardization with AQUA peptides does not take into account the yield of the protease digestion step. This step introduces variability due to the intrinsic susceptibility of each protein or protein domain to proteolysis. In addition, variability between samples can also originate from the digestion con-
ditions (e.g., composition of the sample buffer, amount of trypsin, temperature during incubation). Standard peptide adsorption onto vials or incomplete solubilization both lead to an overestimation of the true standard concentration and consequently of the targeted protein. Accordingly, the 37% overestimation of SEA abundance given by the AQUA peptide YNL*VYNSDVFSGK (slope value $y = 1.37$) likely resulted from incomplete re-solubilization and/or partial adsorption of this standard peptide onto vials. Conversely, the peptides NVTVQELDQAR and LPTPIELPLK may not have been efficiently generated by trypsin digestion and therefore led to an important underestimation of SEA and TSST-1 abundances (slope values $y = 0.44$ and $0.54$, respectively). This stresses a major limitation of the AQUA peptide strategy: it does not take into account the actual digestion yield of the different peptides from the native protein. Actually, this drawback is especially problematic for proteins such as staphylococcal superantigenic toxins, which are known to be poorly protease sensitive (15).

Quantification of Staphylococcal Superantigenic Toxins in Drinking Water Using Isotope-labeled QconCAT Protein—We designed and produced an isotope-labeled concatemer of staphylococcal superantigenic toxins marker peptides (QconCAT) (Fig. 1A and B). In this construction, we included all the peptides previously chosen as AQUA standards. The presence of multiple peptide standards for each toxin aimed at improving the quantification robustness. When QconCAT was digested and analyzed, among the 14 marker peptides theoretically generated, 11 were detected by MALDI-TOF analysis (Fig. 1C) and 13 were observed in nano-LC-MS analysis (data not shown). The remaining peptide (peptide P11, Fig. 1A) was not observed in nano-LC-MS analyses possibly due to its high hydrophobicity.

In agreement with previous reports (21), the cell-free expression of QconCAT in the presence of [13C6, 15N2]-lysine and [13C6, 15N4]-arginine yielded a high rate of isotope-label incorporation. A nano-LC-MS analysis of the pure isotope-labeled QconCAT indicated an isotopic purity greater than 98% (Supplemental Fig. 2). Moreover, [13C6, 15N2]-lysine and [13C6, 15N4]-arginine-labeling leads to constant mass increments of arginyl and lysyl peptides after trypsin cleavage, which greatly simplifies the processing of LC-MS data required for quantification. Therefore, for each of the marker peptides selected (Table I), peak doublets separated by a mass of 8 Da ([13C6, 15N2]-lysine-labeled peptides) or 10 Da ([13C6, 15N4]-arginine-labeled peptides) were investigated. The ratios of unlabeled over labeled peptide signals allowed a direct calculation of the estimated natural toxin amount that was plotted against the added amount (Figs. 3 and 4).

We showed from the previous AQUA titrations that the tryptic digestion efficiency influenced the quantification accuracy. The intrinsic susceptibility of proteins to proteolysis and the experimental conditions of the digestion may constitute major influencing parameters. In an attempt to underline the role of proteolysis conditions, two experiments were designed with the QconCAT chimeric protein. In the first experiment, QconCAT was added in known amounts into drinking water samples and was codigested with the toxins. In the second experiment, QconCAT was digested separately, and the resulting digest was used as a mixture of standard peptides to calibrate the toxin digests. As shown in Fig. 3, poor linearity and high S.E. were obtained when the QconCAT standard...
was digested separately. These results emphasize the benefits of codigesting the standard with the target proteins for linear and reproducible quantitations. Therefore, in subsequent experiments aimed at quantifying SEA and TSST-1 in drinking water, the QconCAT standard was codigested with the artificially contaminated samples. Although the six marker peptides could be observed in nano-LC-MS analysis, they exhibited quite different MS detectability. To enhance quantification sensitivity, we focused on the four marker peptides that gave the best nano-LC-MS signals (NVTVQELDLQAR, QNTVPLETVK, YNLYNSDVFDGK for SEA, and LPTPIELPLK for TSST-1). In contrast with SEA titrations obtained with the two AQUA peptides YNLYNSDVFDGK and NVTVQELDLQAR, the three QconCAT-generated SEA peptide standards displayed only limited variance (compare Fig. 2A and Fig. 4A). This higher consistency is attributed to the equi-stoichiometric representation of peptide standards in the QconCAT construct. However, QconCAT quantifications led to an underestimation of SEA and TSST-1 by a factor over 2 (Fig. 4, A and B). As staphylococcal enterotoxins are reputed as poorly protease sensitive (15) and QconCAT concatemers are reported to be highly susceptible to trypsin digestion (9, 12), the straightforward rational for this underestimation is to postulate different digestion rates between the toxins and the QconCAT protein.

Quantification of Staphylococcal Superantigenic Toxins in Drinking Water Using Isotope-labeled PSAQ Standards—Commercial SEA and TSST-1 toxins were added in defined amounts into drinking water samples. These samples were spiked with known quantities of SEA and TSST-1 PSAQ standards. The water samples were digested in solution, and nano-LC-MS data were analyzed as described for QconCAT. SEA and TSST-1 PSAQ standards generated 6 and 7 tryptic peptides, respectively. However, due to amino acid sequence similarity between staphylococcal superantigens and marked differences in nano-LC-MS detectability at low concentrations, only three and two labeled marker peptides were considered as highly specific standards for sensitive quantification of SEA and TSST-1 in drinking water, respectively (Fig. 5). Calibration with PSAQ standards was highly precise and improved the accuracy of measurements. Regarding SEA, the results obtained using the three standard peptides were highly consistent but slightly overestimated (slope values ranging from 1.26 to 1.42). This likely originated from the partial adsorption of SEA PSAQ standard onto vials during the

Fig. 3. Comparative use of predigested or codigested QconCAT standard. Variable amounts of SEA and TSST-1 were mixed into drinking water samples, and QconCAT concatemer was used as calibration standard. The contaminated water samples were digested in solution with trypsin either separately (○) or concomitantly (◆) with QconCAT. We show here the titration curves obtained with the marker peptides YNLYNSDVFDGK (A), NVTVQELDLQAR (B) and QNTVPLETVK (C) addressing SEA and LPTPIELPLK (D) addressing TSST-1. Each data point is the mean value ± S.E. of three analytical replicates.
dilution process. In the case of TSST-1, the two standard peptides LPTPIELPLK and QLAISTLDFEIR both allowed a recovery of 81% (slope value = 0.81).

Quantification of Staphylococcal Superantigenic Toxins in Prefractionated Urine—In the final set of experiments, we contaminated human urine samples with SEA and TSST-1, which are the most frequently involved toxins in staphylococcal toxic shock syndrome (15, 16). Contaminated samples were prefractionated on Strataclean resin, decomplexified by SDS-PAGE, and digested in-gel with trypsin. These samples were spiked with either PSAQ toxins, or QconCAT concatemer, or AQUA peptides (Fig. 6A). The PSAQ standards were directly added in known quantities into the urine samples. Regarding QconCAT standard, we improved the previously described QconCAT concept (9) by adjusting its molecular weight to that of the target toxins (24 kDa), so that: 1) it comigrates with the toxin targets in an electrophoresis gel, 2) it is codigested in-gel with the toxins, and 3) the peptides generated by QconCAT and toxin proteolysis are concomitantly extracted from the gel. The adjustment of QconCAT to 24 kDa was accomplished by incorporation of additional peptides potentially useful as quantification standards for other staphylococcal superantigenic toxins (Fig. 1, other toxins not quantified in the present study). As the QconCAT construct was solubilized in 1% SDS, it could not be captured on Strataclean resin. Consequently, it was introduced into the samples just before SDS-PAGE. The AQUA peptides standards, the size of which imposes their addition after SDS-PAGE

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**Fig. 4.** Quantification of SEA and TSST-1 in drinking water samples using QconCAT. Drinking water samples were contaminated with different amounts of SEA and TSST-1. QconCAT concatemer was added to the samples and codigested in solution with the toxins. The peptides generated by QconCAT digestion were used as calibration standards in nano-LC-MS analysis. Three marker peptides (mentioned) enabled the titration of SEA (A), and one peptide allowed that of TSST-1 (B). Each data point is the mean value ± S.E. of three analytical replicates.

**Fig. 5.** Quantification of SEA and TSST-1 in drinking water samples using PSAQ standards. Variable amounts of SEA and TSST-1 were mixed into drinking water samples. PSAQ toxin standards were added as calibration references. The contaminated water samples were digested in solution with trypsin. The peptides generated by PSAQ toxins digestion were used as quantification references in nano-LC-MS analysis. SEA (A) and TSST-1 (B) could be titrated with three and two marker peptides (mentioned), respectively. Each data point is the mean value ± S.E. of three analytical replicates.
fractionation step, were added to the samples just before nano-LC-MS analysis as previously described (6).

In comparison with the detection and quantification of SEA and TSST-1 in drinking water samples, lower sequence coverage was obtained in urine samples even when the PSAQ standards were used. Indeed, a high background generated by urinary proteins prevented the detection of marker peptides QNTVPLETVK and YNLYNSDVFDGK. In drinking water, toxins were quantitated down to 7.7 pM (signal-to-noise ratio 75:1) for SEA and 3.8 pM (signal-to-noise 15:1) for TSST-1, respectively. In contrast, the high protein complexity limited the quantitation sensitivity in urine to 0.4 nM (signal-to-noise ratio 35:1) for SEA and to 1.3 nM (signal-to-noise ratio 20:1) for TSST-1. Fig. 6 shows the estimates of SEA and TSST-1 amounts obtained with the different standards compared with the added amounts. Both AQUA and QconCAT standardizations severely underestimated the toxin amounts in urine. This is due to addition of these standards at late stages of the analytical process (Fig. 6A). This emphasizes a major limitation of these strategies: their incompatibility with sample pre-fractionation. In contrast, PSAQ was the only quantitation strategy that allowed an accurate estimation of the toxins in this complex matrix (slope values = 1.05 and 1.08 for SEA marker peptide NVTVQELDLQAR and TSST-1 marker peptide LPTPIELPLK, respectively) (Fig. 6, B and C).

**DISCUSSION**

In various fields ranging from fundamental biology to clinical diagnostic and public health surveillance, the specific and accurate quantification of proteins in complex biological samples remains a recurrent and challenging problem. For many protein biomarkers, this problem has been solved by immunological techniques. However, the success of immunological approaches relies on the heavy duty production and validation of high specificity and high affinity antibodies. Although recent efforts are being made to design antibody arrays (22), the adaptation of immunological methods to multiplexed analyses remains limited. Indeed, the simultaneous optimization of several protein assays is hardly ever possible (23). Alternatively, the power of MS-based proteomics can be harnessed to allow proteome-wide quantifications. Label-free spectral counting approaches have been especially designed for this purpose (2, 3). However, so far, the low cost and high potential of these approaches for large scale studies is counterbalanced by their rather poor accuracy. To gain in measurement accuracy, absolute quantitative methodologies still require an internal standardization via stable isotope dilution strategies (6, 24).

The commercial availability of highly pure synthetic isotope-labeled peptides renders the AQUA peptide strategy very...
attractive. This methodology has been successfully used to quantify neuropeptides (25) or protein phosphorylations with phosphopeptide standards (5–7). However, individual chemical synthesis, purification, and quantification of isotope-labeled precursors may result in a reduced incorporation yield and a dispersion of the label over different amino acids of the protein (label scrambling) (28) that potentially introduce important biases in MS quantification. Thus, we favored cell-free protein expression and labeling, which allow a high isotope incorporation yield (greater than 95%) with negligible scrambling (21, 29). (Supplemental Fig. 2). This technique is particularly suited to toxic proteins and allows an experimental confinement that is critical for biological and health hazard control.

In this study, we present an in depth comparison of the existing AQUA (5–8) and QconCAT strategies (9–12), to our original PSAQ methodology for biomarker quantification. The choice of staphylococcal superantigenic toxins SEA and TSST-1 as typical biomarkers that could be added to water or biological samples in defined amounts allowed a precise assessment of the accuracy of the three methods. With that goal in mind, we designed a set of AQUA peptides, a QconCAT concatemer and full-length isotope-labeled recombinant toxins (PSAQ standards) to quantify SEA and TSST-1 in drinking water and urine samples. In contaminated drinking water, two AQUA peptides addressing SEA gave diverging estimations (Fig. 2A). Moreover, two AQUA peptides markedly underestimated SEA and TSST-1 amounts (Fig. 2). Interestingly, similar observations have been reported for the quantification of C-reactive protein in serum using AQUA peptides (30). These biases mostly result from variabilities in trypsin digestion efficiency, which are not accounted for when AQUA peptides are used. When codigested with the targets, QconCAT standards gave more consistent results between the different marker peptides of a same protein. However, essentially due to differential susceptibility to proteolysis, QconCAT standard nonetheless led to an underestimation of the toxins (Fig. 4). Finally, the PSAQ strategy demonstrated a marked superiority over AQUA and QconCAT approaches for toxin quantification in drinking water both in terms of interpeptide consistency and accuracy (Fig. 5). The 26–42% overestimation of SEA abundance may originate from the adsorption of SEA PSAQ standard onto vials during the dilution process (final concentration before spiking: 10 nM). Accordingly, an excellent accuracy was observed for both SEA and TSST-1 titrations in urine samples (Fig. 6, B and C). In comparison with drinking water samples, the PSAQ standard solutions that were used for spiking urine samples were much more concentrated (200 nM), which may have prevented protein adsorption on vials.

For both QconCAT and AQUA strategies, the choice of the best peptide(s) to use for the quantification of any given protein is frequently based on an educated guess. Depending on biological matrix and prefractionation strategy, the choice of a single standard peptide can be inadequate (e.g. when the standard peptide is suppressed by other dominant peptides). The PSAQ strategy, which allows maximal protein coverage, circumvents these potential problems and provides a more robust quantification of the targets. As suggested by Anderson and Hunter (10) for small proteins, the difficulty to find a
good peptide reporter can impose to swap trypsin for a distinct protease for peptide digestion. In a long term study, QconCAT or AQUA standards freeze the choice of quantification standards, whereas PSAQ strategy opens the way to alternative peptide standards.

The possibility to integrate the prefractionation and digestion yields renders PSAQ strategy exquisitely attractive for quantitative analysis of biomarkers in biological fluids. This improvement was demonstrated by the comparative quantification of SEA and TSST-1 toxins in a complex sample (e.g., urine) after Stratalclean resin capture and SDS-PAGE prefractionation. Compared with drinking water samples, the protein complexity of urine samples generated high background and ionization competition that prevented MS detection of several toxin marker peptides. With the remaining markers, both AQUA and QconCAT severely underestimated the toxin amounts. The tendency of the AQUA strategy to underestimate protein targets was seriously aggravated after the SDS-PAGE prefractionation. This was also true for QconCAT despite our SDS-PAGE-compatible design. Finally, only PSAQ standards gave reliable quantification after this prefractionation protocol. Even with the most powerful MS technologies, decomplexification often appears as a mandatory step before quantification of medium to low abundance proteins (10, 13). Actually, AQUA quantifications are often realized on prefractionated protein samples (6, 23). As illustrated in the present study, uncertainty on the yield of the target protein recovery due to these prefractionation steps can introduce important quantification biases that are efficiently corrected using PSAQ standards. This constitutes the major advantage of the PSAQ strategy over AQUA and QconCAT approaches.

Improvement of detection sensitivity was beyond the scope of the present study. Nevertheless, the staphylococcal superantigenic toxins could be detected at the picomolar level in water samples using our LC-QTOF instrumentation. A reliable quantification of toxins could be achieved down to 3.8 pm (i.e., 0.5 fmol equivalent to 10 pg of TSST-1 in the 120 μl sample analyzed). This limit was comparable regardless of the standardization procedure used, e.g., AQUA, QconCAT, or PSAQ, and is inherent to the QTOF performances. For comparison, a recently developed real-time immuno-PCR technique detected SEA toxin down to 6 pg/100 μl (i.e., 2 pm) that is reportedly 100 times more sensitive than previous commercial toxin detection ELISA (31). In urine, due to the complexity of the matrix, QconCAT and PSAQ standards allowed quantification of SEA and TSST-1 down to the nanomolar range (i.e., 44 fmol equivalent to 1.2 ng in the actual 100 μl sample analyzed). This compares favorably with a previous report on staphylococcal SEB enterotoxin MS-based quantification (32), in which the limit of quantification of SEB spiked in apple juice was estimated to be 50 ng/ml. Thus, despite a much higher protein complexity of human urine compared with apple juice, we demonstrated a lower quantification limit in this complex matrix. This can be partly attributed to the difference of sensitivity of the LC-MS instrumentation used. In the near future, we intend to improve the sensitivity of our quantification procedure through the use of multiple reaction monitoring (MRM). The discriminating power of MRM assays renders them especially suited to address the protein complexity and high dynamic range of biological fluids such as urine (10, 33, 34). The lower density of species detected in nano-LC-MRM runs should enhance the robustness of the PSAQ quantification method. On this respect, the opportunity to monitor different peptide standards offered by the PSAQ strategy can be a compelling advantage compared with single peptide strategies. Along the same line, when using single peptide standards, any sequence variation or post-translational modification in a marker peptide will dismiss quantification of the corresponding protein. The larger protein coverage allowed by the PSAQ strategy can also favor the detection and differential quantification of variant species of any protein of interest. It is a valuable feature when addressing the survey of potentially variable proteins such as staphylococcal superantigenic toxins (35).

Conclusion—We have demonstrated the advantages of our PSAQ strategy over existing approaches for biomarker absolute quantification in complex samples, such as biological fluids. Considering the high sensitivity of MS analyses, a single medium-scale, cell-free protein expression experiment provides sufficient amounts of a given PSAQ standard for thousands of quantification analyses. Moreover, simple quality controls, such as SDS-PAGE, coupled to quantitative fluorescent detection can be performed whenever required. In this regard, as it was realized for nuclear magnetic resonance structural studies (36), we propose to initiate a community-based effort for the construction of large reference PSAQ libraries for protein biomarkers. This could extend the actual standardization initiatives of the proteomic community to the developing fields of biomarkers discovery and validation by enabling truly accurate quantitative proteomics (37).

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