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REVIEW ARTICLE

Quantification of snake venom proteomes by mass spectrometry-considerations and perspectives

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Abstract

The advent of soft ionization mass spectrometry-based proteomics in the 1990s led to the development of a new dimension in biology that conceptually allows for the integral analysis of whole proteomes. This transition from a reductionist to a global-integrative approach is conditioned to the capability of proteomic platforms to generate and analyze complete qualitative and quantitative proteomics data. Paradoxically, the underlying analytical technique, molecular mass spectrometry, is inherently nonquantitative. The turn of the century witnessed the development of analytical strategies to endow proteomics with the ability to quantify proteomes of model organisms in the sense of "an organism for which comprehensive molecular (genomic and/or transcriptomic) resources are available." This essay presents an overview of the strategies and the lights and shadows of the most popular quantification methods highlighting the common misuse of label-free approaches developed for model species' when applied to quantify the individual components of proteomes of nonmodel species (In this essay we use the term "non-model" organisms for species lacking comprehensive molecular (genomic and/or transcriptomic) resources, a circumstance that, as we detail in this review-essay, conditions the quantification of their proteomes.). We also point out the opportunity of combining elemental and molecular mass spectrometry systems into a hybrid instrumental configuration for the parallel identification and absolute quantification of venom proteomes. The successful application of this novel mass spectrometry configuration in snake venomics represents a proof-of-concept for a broader and more routine application of hybrid elemental/molecular mass spectrometry setups in other areas of the proteomics field, such as phosphoproteomics, metallomics, and in general in any biological process where a heteroatom (i.e., any atom other than C, H, O, N) forms integral part of its mechanism.

This manuscript is dedicated to Dr. Emilio Gelpì, pioneer of implementating mass spectrometry in Spain. His long and fruitful scientific career inspired next generations of Analytical Chemists and Protein Chemists converted into Proteomists, who now show the master that the conjunction of both disciplines offers novel perspectives for biological mass spectrometry.

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KEYWORDS
absolute quantification, elemental mass spectrometry, hybrid MS configuration, ICP-MS, molecular mass spectrometry, proteome quantification, shotgun proteomics

1 | INTRODUCTION

Rooted in ancient Greece's Peripatetic School founded by Aristotle (384–322 BC), the study of natural phenomena (*Natural Philosophy*) involved for much of its history qualitative observations and reasoning about the natural world. Aristotle's *Natural Philosophy* of nature prevailed throughout the Middle Ages which lasted between the collapse of Roman civilization in the 5th century and the flowering of the Renaissance from the 14th to the 17th centuries (Manchester, 1992). On the night of the 7th of January of 1610, the Italian astronomer, physicist, and engineer Galileo Galilei was the first to use a telescope for scientific observations of celestial objects. Galileo pioneered the experimental scientific method whose founding principles are still valid today (Croy, 2021). The laws of nature are mathematical, and associating numbers with physical quantities and phenomena to establish causal relationships among variables is at the core of the scientific revolution. Quantitative research plays an important role in mind-ordering nature. Paraphrasing the 1965 Nobel Prize winner in physics Richard Feynman (Gribbin & Gribbin, 2018), “people who wish to analyze nature without using mathematics must settle for a reduced understanding.” Comprehending biological processes revolves around the quantitative correlation of intrinsic and extrinsic attributes of the molecular entities involved, that is, structure-derived biological activities and their concentration in the solution in which they are found, respectively. Our field of interest, snake venomics, to which this review/essay is especially addressed, represents an example to illustrate this assertion. Venom is an ecological trait used by snakes primarily for the purpose of subjugating prey but also for defending themselves from potential adversaries, including humans (Calvete, 2013; Gutiérrez et al., 2017; Kazandjian et al., 2021). Snake venoms are proteomes of relatively low complexity, comprised of tens to hundred peptides and proteins derived from a limited number ($2 < n < 20$) of gene families. Venom's toxins acting alone or synergistically wreak havoc on the vital systems of the animal prey or human victim. The individual toxin abundances and their pharmacological profile are conjugated parameters, that should be analyzed into an appropriate, ecological or clinical, model (Calvete et al., 2019), to disclose the pathophysiology of envenoming.

Since Galileo facilitated the transition from the middle age to modern science, and throughout most of the twentieth century, the study of nature has been reductionistic. The advent of omics technologies at the turn of the 21st century has revolutionized biology. Data integration across multiple omics layers has expanded the scope of biological research from description to mechanistic understanding of complex phenotypes (Munsky et al., 2018). In this context, molecular mass spectrometry (MS)-based proteomics developed in the 1990s (Müller et al., 2020; Tamara et al., 2022) represented a breakthrough by enabling both within and between proteome-wide measurements in single separate experiments (Aebersold & Mann, 2016; Aebersold et al., 2018; Cristea & Lilley, 2019; Robey et al., 2021). Paradoxically, and in striking contrast to its contribution to describing the proteome with unprecedented resolution, molecular MS is inherently a not quantitative technique. Due to numerous confounding factors involved in the detection of peptide ions, there is no physical law or empirical equation that relates the intensity of the ion current recorded by the mass spectrometer detector to the amount of ionized matter. Most notably, the distinct physicochemical properties of different bioanalytes unpredictably affect their ionization and detection efficiency (Eyers & Gaskell, 2014; Urban, 2016). Consequently, at its birth and initial implementation in biology during late 1990, proteomic workflows were essentially qualitative. Coinciding with the turn of the century (Gygi et al., 1999), and over the first decade of the XXI century, novel technologies for the systematic quantitative analysis of proteins represented a milestone in the field (Eyers & Gaskell, 2014; Glish & Burinsky, 2008; Urban, 2016). The implementation of quantitative approaches has proven essential for proteomics to move beyond mere protein identification. In the following, we critically review the field, pinpointing some lights and shadows in the proteomic analyses of non-model versus model organisms.

The term “model organism” was coined to indicate accessible and convenient systems to study a particular area of biology. Labeling an organism as a model led to the development of databases and a wealth of toolkits and methods specifically for these organisms. Rapid progress on the selected organism makes it obvious why early genome projects focused on model organisms. In this essay, we refer generically as “nonmodel organisms” to those species for which comprehensive genomic or

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transcriptomic databases are not available (Armengaud et al., 2014; Calvete, 2014a; Dickinson et al., 2020; Franz-Odendaal & Hockman, 2019; Gulia-Nuss, 2019; Kwon, 2017; Heck & Neely, 2020; Russell et al., 2017). Strategies for analyzing proteomics data from nonmodel organisms have been reviewed (Armengaud et al., 2014; Carpentier et al., 2008; Fox & Serrano, 2008; Heck & Neely, 2020; Modahl et al., 2021; von Reumont et al., 2022; Tan, 2022) and will not be repeated here. Instead, here we will discuss an issue that has not been addressed in any previous review: why and how the common misuse of label-free shotgun approaches developed for model organisms imposes a serious hurdle to the quantification of the individual proteins within the same proteome of nonmodel species. The conceptual basis of this circumstance will be discussed and illustrated using examples from our field of research, snake venomics.

2 | GENERAL PROTEOMICS APPROACHES TO UNRAVEL A PROTEOME'S COMPOSITION

Two main analytical workflow configurations, bottom-up and top-down, co-exist in the proteomics arena. In top-down proteomics, the intact disulphide-bond-reduced sample proteins are fractionated via a variety of off-line or hyphenated orthogonal pre-MS decomplexation steps, including reverse-phase high-performance liquid chromatography (RP-HPLC), capillary electrophoresis or size-based separations, to achieve deep proteoform characterization (Cupp-Sutton & Wu, 2020). Intact protein ions generated by electrospray ionization (ESI) are trapped in a high-resolution, most commonly Orbitrap (Eliuk & Makarov, 2015; Zubarev & Makarov, 2013) and Fourier transform ion cyclotron resonance (FT-ICR) (Park et al., 2021) mass spectrometers to resolve the proteins' isotope patterns. Subsequently, the monoisotopic topoisomers are sequentially trapped and submitted to gas-phase fragmentation. Protein assignments are then achieved by matching the daughter ion production spectra against a species-specific transcriptomic or genomic database (Fornelli et al., 2018; Schaffer et al., 2019; Skinner et al., 2018).

Conversely, all variants of bottom-up proteomics involve the site-specific proteolytic cleavage of the crude proteome (shotgun proteomics approach) or of its front-end fractionated proteins (protein-centric bottom-up approach) before the analysis of the daughter peptide ions by tandem mass spectrometry (MS/MS) (Eng et al., 1994). Most commonly, before entering the mass spectrometer the mixtures of proteolytic peptides generated via shotgun or protein-centric approaches are fractionated by one-dimensional RP-HPLC or through multidimensional configurations in which two or more separation methods are coupled (Rathore et al., 2020). Within the mass spectrometry system there are currently two broad approaches toward generating the MS/MS data: data-dependent acquisition (DDA) and data-independent acquisition (DIA). In DDA mode, also known as information-dependent acquisition mode (IDA), individual precursor peptides ions are sequentially selected from the first MS cycle, the MS1 precursor ion survey scan, for fragmentation (Defossez et al., 2021). Amino acid sequence information from the parent proteins is then reconstructed by matching the DDA-recorded product ion spectra against a reference database using search algorithms (Han et al., 2008). Additionally, manual de novo peptide ion sequencing through tandem MS, although a low-throughput method that also requires a high degree of skill from the user, should not be ruled out (Marina & Calvete, 2014; Seidler et al., 2010).

With the DIA approach, MS/MS spectra are acquired either fragmenting simultaneously all the ions that enter the mass spectrometer at a given time (broadband DIA). MS^E is a broadband DIA technique that uses alternating low- and high-energy collision-induced dissociation to acquire, respectively, precursor ion mass spectra and product ion information by tandem mass spectrometry (Plumb et al., 2006). SWATH-MS (Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra) consists of the simultaneous fragmentation of all the peptide ions within 32 consecutive, slightly overlapping precursor isolation windows with defined increments of 25 m/z, stepping through the whole mass range (Gillet et al., 2012). A current drawback regarding the scoring of SWATH-MS data is the need of considerable upfront effort to generate a priori experimental or in silico knowledge about the chromatographic and mass spectrometric behavior of peptides of interest in the form of spectral libraries and specific peptide query parameters. Data are acquired using a mass spectrometer in DIA mode, but analysis requires a spectral library acquired using a mass spectrometer in DDA mode. The spectral library is necessary for protein identification while the spectra acquired in SWATH-MS mode are used for quantification (reviewed by Ludwig et al., 2018).

Although top-down mass spectrometry approaches are gaining momentum (Fornelli et al., 2018; Garcia, 2010; Toby et al., 2016), bottom-up approaches still represent the most used protocols for characterizing complex proteomes in large-scale proteomic studies. However, this strategy generates peptide sets far more complex than their parent protein mixtures, only a small

part of which can be mapped to their proteins of origin. The reason for this seemingly counter-intuitive strategy is that proteolytic (most commonly tryptic) peptides, are easier to separate by liquid chromatography (LC) than whole proteins, and are also more effectively fragmented and sequenced in a tandem mass spectrometer at substantially lower concentration than the parent proteins (Duncan et al., 2010). Concomitant with its high throughput nature, bottom-up proteomic approaches bear the intrinsic limitation of the so-called "protein inference problem" (Duncan et al., 2010; Huang et al., 2012; Li & Radivojac, 2012): the same peptide sequence can be present in different proteins or proteoforms (Smith & Kelleher, 2018) and thus the loss of connectivity between the precursor protein and its fragment ions complicates the computational analysis and biological interpretation of bottom-up proteomics data. Thus, the inherent drawback of loss of intact proteoform information entails the inability to decipher the combinatorial aspects of protein modifications in enzymatically digested unfractionated proteomes submitted to automated LC-MS/MS analysis.

3 | LABEL-BASED AND LABEL-FREE PROTEOME QUANTIFICATION

Proteome quantification in both bottom-up and top-down approaches fall into two main categories, label-free methods and those involving the use of stable isotope labels. Quantitative bottom-up peptide-centric approaches are based on analysis of peptides derived from parent proteins, whereas top-down workflows provide quantitative estimates of intact proteins. Since the development of selectively reacting functional groups for specific protein amino acid residues, such as cysteine in the ICAT (Isotope-Coded Affinity Tag) protocol in 1999 (Gygi et al., 1999), amine (Hsu et al., 2003; Ross et al., 2004), and phosphate groups (Ficarro et al., 2002; Oda et al., 2001; Zhou et al., 2001), numerous labeling strategies found their way into the field of quantitative proteomics (Figure 1). These early methods of quantification analysis using tandem mass spectrometry (MS/MS) included stable "heavy/light" isotope-coded protein labeling such as TMT (Tandem-Mass Tag) (Liu et al., 2021; Thompson et al., 2003), stable-isotope dimethyl labeling (Hsu et al., 2003), iTRAQ (Isobaric Tag for Relative and Absolute Quantitation) (Ross et al., 2004) or ICPL (Isotope-Coded Protein Label) (Schmidt et al., 2005); metabolic (SILAC, Stable Isotope Labeling by Amino acids in Cell culture) (Krijgsveld et al., 2003; Ong et al., 2002) and chemical (^{18}O) (Shevchenko et al., 1997; Yao et al., 2001) labeling; and a number of isobaric tags (Rauniyar & Yates, 2014). However, because of the many shortcomings of labeling strategies, such as high cost of reagents, extensive sample preparation, risk of incomplete labeling, the increased complexity in the interpretation of MS/MS spectra, and the need of advanced MS instrumentation and data analysis software (Nahnsen et al., 2013), untargeted label-free protein quantification, based either on the integrated peptide ion signal intensities of extracted ion chromatograms (XIC), or on spectral counting (SpC), of the identified protein-specific peptide ions increasingly gained the interest of proteomics researchers (Geis-Asteggiante et al., 2016; Millán-Oropeza et al., 2022; Old et al., 2007; Trudgian et al., 2011). Label-free bottom-up and top-down workflows yield, respectively, relative quantifications of peptide or protein levels (Ankney et al., 2018; Rozanova et al., 2021). However, since neither the number of spectra nor the amplitude of XIC areas are strictly correlated to peptide concentrations but are context- and peptide-dependent, quantitative information based on ion intensity or spectral count conceptually cannot be applied to compare abundances of different peptides within a sample, but only to compare relative abundances of a same peptide/protein between samples. In this sense, a major purpose of peptide-centric, bottom-up mass spectrometry-based relative label-free quantification of a proteome is to identify and compare differences in the expression of the components of the same system in different biological situations, that is, for discovering diagnostic or prognostic protein markers; for detecting new therapeutic targets; or for understanding basic biological processes. Differential protein expressions gathered from comparative proteomics yield unitless "fold change" values. The fold change of protein "*i*" in condition *X* versus condition *Y* can be computed as $(y_i–x_i)/x_i$, where x_i and y_i are the relative abundances of protein "*i*" in condition *X* and *Y*, respectively.

Three relative quantitative approaches have been applied in quantitative top-down proteomics analysis: Label-free quantitation, metabolic labeling, and chemical labeling (Cupp-Sutton & Wu, 2020; Schulze & Usadel, 2010). Label-free quantification methods directly compare normalized combined MS1 signal intensity data for each charge state of a proteoform between two or more LC-MS runs. Metabolic labeling techniques supplement cell cultures with isotopically labeled compounds so that they express isotopically labeled proteins, while chemical labeling methods rely on incorporating site-specific isotopically labeled chemical tags on proteins. In a typical comparative proteomics workflow, equimolar amounts of samples labeled with two or more different

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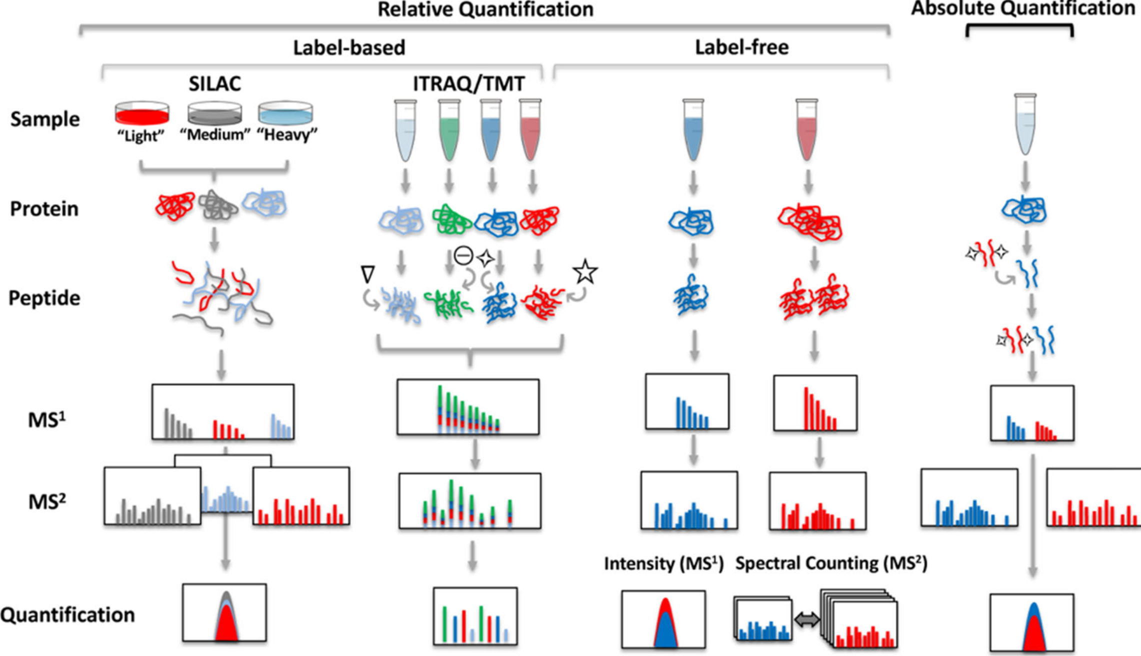

FIGURE 1 Generalized comparison of different quantitative proteomic strategies. Absolute quantification is achieved by spiking known concentrations of internal standards, often heavy labeled synthetic peptides, and comparing the acquired mass spectrometric signal to that of the endogenous peptide in the mixture. Label-based relative quantification can be achieved by metabolically incorporating stable isotopes into proteins during cell growth using approaches such as Stable Isotope Labeling by Amino acids in Cell culture (SILAC) or ^{15}N labeling, or chemically labeling digested peptides with iTRAQ or TMT reagents. In both labeling strategies, samples are pooled before mass spectrometry which reduces sample manipulation and chances of error. With SILAC, quantitation is achieved by directly comparing the differences in MS^1 intensity of the isotope-labeled peaks, whereas relative abundances are measured from the acquired MS^2 spectra in iTRAQ/TMT experiments. Label-free quantification (LFQ) is based on the strategy of using either the intensity of a peptide precursor ion in MS^1 or the number of acquired MS^2 spectra associated with a given protein, as relative values. Relative quantification methods are designed to compare abundance changes of the same protein in different samples. Due to the lack of absolute correlation between the intensity and the amount of biomolecular ions, fold changes of different ions cannot be ranked in quantitative terms within the same proteome or between proteomes. [Color figure can be viewed at wileyonlinelibrary.com]

isotopic variants of the same molecule are mixed, and the ratio of the MS-detected labels is used to quantify the relative abundances of proteoforms in the different samples. In bottom-up proteomics, label-free shotgun MS methods provide relative estimates of protein levels by counting the number of identified MS2 spectra matched against each parent protein ("spectral counting") (Arike & Peil, 2014; Neilson et al., 2013; Vogel & Marcotte, 2012) or by summing up the peak areas of the MS1 peptide ions matched by MS/MS to a given protein ("ion intensity" or "XIC, Extracted Ion Chromatogram") (Gerster et al., 2014; Wu et al., 2012). These label-free quantification strategies are based on (i) the principle that the likelihood of data-dependent precursor ion selection is higher for abundant precursor ions and (ii) on the assumption that the number of successful peptide identifications is directly correlated with the abundance of the parent proteins. However, due to the lack of absolute correlation between the intensity and the amount of biomolecular ions, the relative abundances of different ions can not be quantitatively compared within the same proteome or between proteomes.

4 | MASS SPECTROMETRIC LABEL-FREE QUANTIFICATION IN MODEL VERSUS NON-MODEL ORGANISMS

Label-free proteome quantification was developed for model organisms, that is, species for which comprehensive genomic or transcriptomic databases are available, and thus inferring protein abundance based on the frequency of identification of surrogate peptides does not represent a limiting factor (Bantscheff et al., 2007, 2012; Choi et al., 2008; Liu et al., 2004; Neilson et al., 2011; Old et al., 2007; Tang et al., 2006). The frequency with which spectra remain unassigned in proteomics analysis of nonmodel organisms points to a serious limitation of proteome quantification with label-free methods

(Ntai et al., 2014). In other words, whereas the ultimate goal of protein quantification is to determine the abundance of each protein present in the sample, in the case of nonmodel species the abundance values of an unpredictable set of missing proteins, present in the sample but not identified in the proteomic analysis, are neglected in the computation. The protein inference problem can be thus regarded as a special protein quantification problem (He et al., 2016) that bias the distribution of relative protein abundances toward the successful peptide identifications (Figure 2). This assertion is deduced from the empirical mathematical formulae underlying the methods proposed for estimating protein abundances from peptide counts and ion intensities in single shotgun LC-MS/MS experiments, such as the representative emPAI and NSAF (spectral counting), and the T3PQ (ion intensity-based) methods.

The Exponentially Modified Protein Abundance Index (emPAI) (Ishihama et al., 2005; Shinoda et al., 2010), implemented in the popular Matrix Science's Mascot server (https://www.matrixscience.com/search_form_select.html), offers label-free relative quantification of the proteins in a mixture based on protein coverage by the peptide matches in a database search result. The formula used to calculate the emPAI for protein "k" is $(\mathrm{emPAI})_k = (10^{\mathrm{PAI}} - 1)_k = (10^{[N\mathrm{observed}/N\mathrm{observable}]} - 1)_k$, where N_{observed} and $N_{\mathrm{observable}}$ represent, respectively, the number of experimentally observed peptides and the calculated number of theoretically observable peptides for protein "k." The count of observable peptides corresponds to the sum of all possible nominal fragments (no missed cleavages allowed) generated in a complete in silico-specific proteolytic digestion of the protein which falls within the MS scan mass range. The relationship between PAI and molar protein concentration has been empirically modeled to an exponential function $a^{\mathrm{PAI}} - 1$ (Ishihama et al., 2005). However, there is no absolute consensus on the value of the exponent base. In their original papers, Ishihama et al. (2005) Shinoda et al. (2010) proposed to use $a = 10$ but more recently Kudlicki (2012) claimed that a generalized exponentially modified PAI (gemPAI) with $a = 6.5$ ($\mathrm{gemPAI65} = 6.5^{\log10(\mathrm{emPAI}+1)} - 1$) performed significantly better. The denominator of the Protein Abundance Index (PAI) is a figure that ultimately depends on the amino acid sequence of the protein. It includes the number of peptide matches with scores at or above an identity threshold, and hence the count of N_{observed} is strongly influenced by the degree of sequence coverage of the search database. $N_{\mathrm{observable}}$ also depends on, among other factors, the degree of completeness of the database. Thus, although the information required for emPAI is always present in a search result, the applicability of this label-free quantification method is conceptually limited to model organisms.

The Normalized Spectral Abundance Factor (NSAF) (Florens et al., 2006; Zybailov et al., 2005, 2006) was introduced to account for the fact that trypsinolysis of

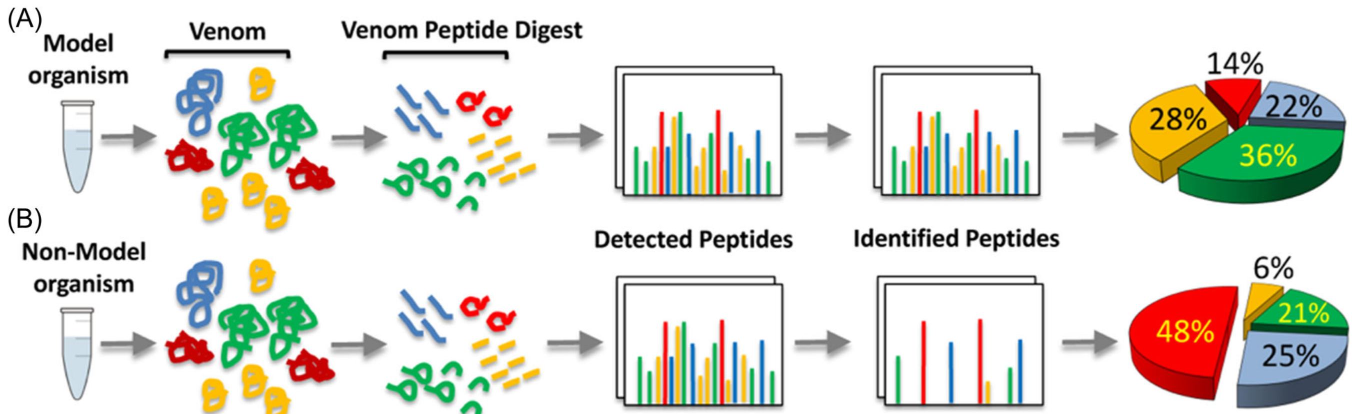

FIGURE 2 Different proteomics outcomes for shotgun analysis of model versus nonmodel organisms. The cartoon displays a theoretical simulation of the different outcomes of the label-free (ion intensity-based or spectral counting-based) quantification (here expressed as % of the same color-coded four protein families in the pie charts) when the same shotgun-generated tryptic venom peptidome was matched to a species-specific (genomic/transcriptomic) reference database (A, model species) or when the genomic/transcriptomic resources were not available (B, nonmodel species). Please note that the biased relative abundance of the proteome of the nonmodel versus the model organism toward the successful peptide identifications was introduced by the mismatch between the same peptides present in the tryptic digests and the different identification levels due to the distinct species-specific protein coverages of the search databases used in the computation of the relative abundances of the venom toxin families of the model versus the nonmodel species. [Color figure can be viewed at wileyonlinelibrary.com]

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higher molecular mass proteins tend to produce larger number of peptides, thereby contributing more peptide-spectrum matches (PSM) than lower molecular mass proteins in a typical shotgun experiment (Zhang et al., 2015). NSAF for protein "k" is calculated as the number of spectral counts identifying the protein divided by the protein's length $(\text{SpC}/L)_k$ divided by the sum of SpC/L for all proteins in the experiment $\sum_i^N (SpC/L)i$: $(\text{NSAF})_k = (\text{SpC}/L)_k / \sum_i^N (SpC/L)i$. As with the emPAI method, the absence of a comprehensive database determines that the calculated NSAF values for proteins not represented in the search database result in their underestimation in the quantified proteome.

Deriving relative abundances from PSMs alone, without differentiating between MS spectra with different ion intensities may lead to systematic errors of quantification, especially for the low-abundant peptides. Based on the empirical observation that the average MS signal response for the three most intense tryptic peptides per mole of protein appeared to be constant within a coefficient of variation of ±10% for 11 common serum proteins, led Silva and coworkers to propose in 2006 the T3PQ (Top 3 Protein Quantification) method as an alternative label-free absolute quantification strategy (Grossmann et al., 2010; Silva et al., 2006). In this method, the average MS signal response gathered from internal standard proteins is used to determine a universal signal response factor (counts/mol of protein), which is then applied to the other identified proteins in the mixture to determine their corresponding absolute concentration. This basic premise of the Top3 method has been contested by Krey et al. (2014), who instead proposed a normalized relative (r) "top-three" abundance factor, $r\text{Top3}_k = \text{Top3}_k / \sum Top3$, where Top3_k is the average intensity for the three most abundant peptides of an individual protein "k" divided by the sum of all Top3 values in the experiment. Krey et al.'s rTop3 abundance factor is conceptually identical to riBAQ (relative intensity Based Abundance Quantification), which converts intensities to a value proportional to molar abundance by dividing the summed protein's MS1 intensities by the homologous number of theoretically observable peptides (Krey et al., 2014). As discussed above for spectral counting strategies, the validity of the application of ion-intensity methods in shotgun bottom-up proteomics is also conceptually limited to model species.

Clearly, label-free methods can conveniently provide comparative estimates of peptide levels in multiplex differential proteomics experiments (Bakalarski & Kirkpatrick, 2016; Pappireddi et al., 2019). However, regardless of the label-free method used, accurate relative quantification of the components of a proteome in a single experiment is only consistent if the identification of the peptides is carried out against a complete transcriptome/genome database. When this is not the case, deriving reliable quantitative information from peptide-centric MS/MS data should be based on a procedure that does not depend on sequence coverage of the search database. In our experience with snake venom proteomics, pre-MS decomplexation represents an opportunity to accurately quantitate the relative abundances of the components of venom proteomes (Calvete, 2014b; Eichberg et al., 2015). The following sections develop the authors' view that snake venomics, apart from its contributions to molecular and translational toxinology, is also playing a role as a proof-of-concept and proteomics technology development field at the frontier between molecular and elemental MS.

5 | LEVERAGING PRE-MS DECOMPLEXATION TO UNCOVER AND QUANTIFY SNAKE VENOM PROTEOMES

For the proteomic analysis and relative quantification of the venom arsenal of nonmodel venomous snakes we have developed since 2004 a bottom-up analytical pipeline termed "snake venomics" (Calvete, Lomonte, et al., 2021; Juárez et al., 2004; Lomonte & Calvete, 2017). As occurs in the proteomic analysis of any nonmodel species, the paucity of genomic and venom gland transcriptomic sequence information for venomous snakes in public domain databases represents a major limitation of current snake venomics. Among the 236+ nominal snake species that have their venom proteomes characterized, only 114 taxa have the venom gland transcriptome been reported (Calvete, Lomonte, et al., 2021). Although demanding a high level of user skill, manual de novo sequencing of high-quality fragmentation spectra followed by BLAST analysis of the deduced sequence, remains a valid option for low-throughput identification of proteins from venoms, which usually contain <100 toxins belonging to a limited number, $2 < n < 20$, of protein families (Calvete, 2013). Venom proteomes assembled from such low-resolution data provide nonetheless sufficient information to unveil and quantitate the relative composition of toxin families that make up the venom proteome notwithstanding the lack of a species-specific database.

To quantify venom proteomes, snake venomics leverages three hierarchical quantification levels, two pre-MS and one post-MS decomplexation steps (Figure 3). The first pre-MS level of quantification (a) consists of an initial estimate of the percentage representation of the different toxin families in the venom from the ratio of the summed areas of the

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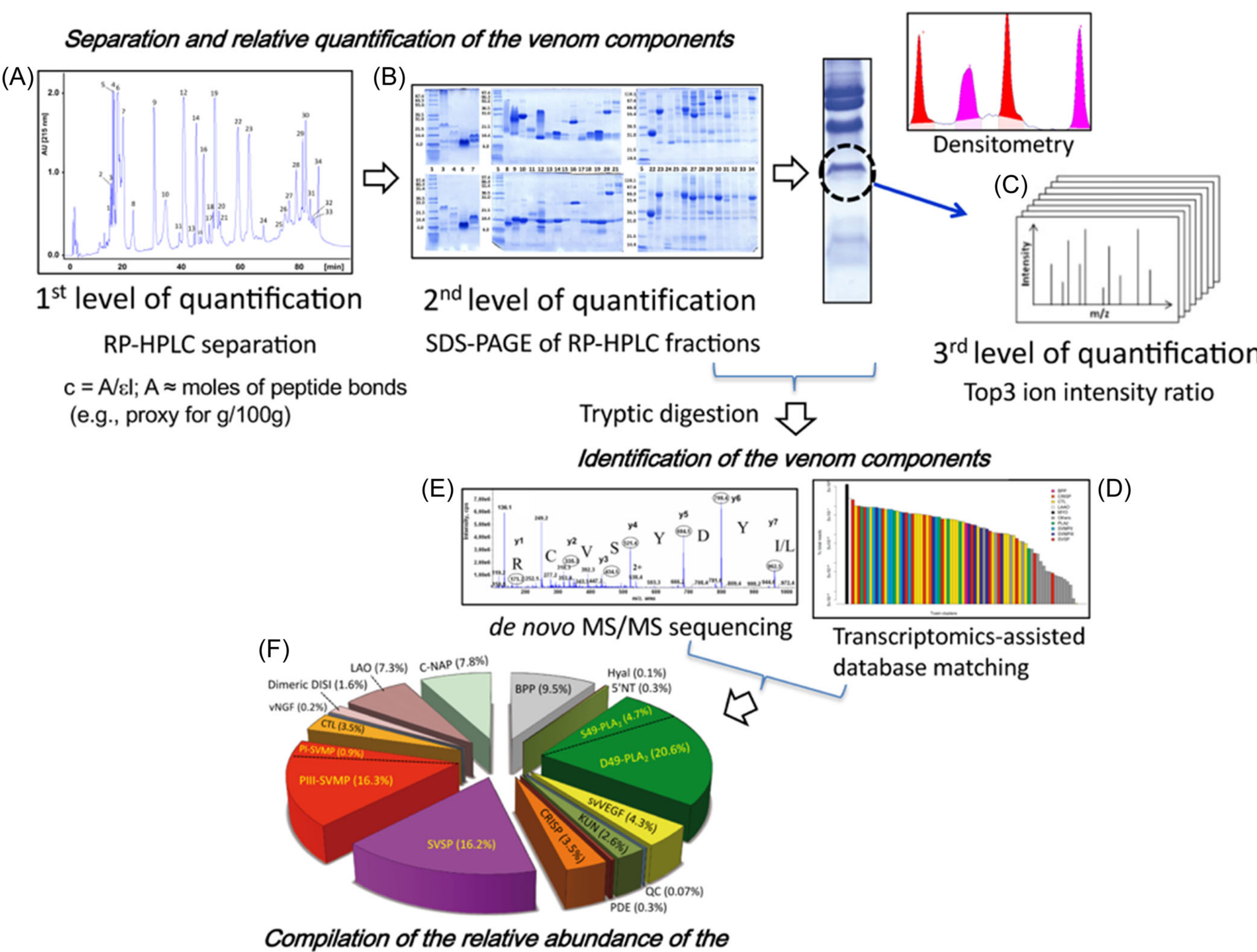

FIGURE 3 Snake venomics workflow. Scheme of analytical steps of a typical bottom-up snake venomics strategy: decomplexation and quantification of the venom proteome through a hierarchical three-step (RP-HPLC [A], SDS-PAGE [B], and Top3 ion intensity ratio [C]) workflow, tryptic digestion of electrophoretic bands (D) and toxin identification by combination of specific (e.g., venom gland transcriptome) matching and de novo sequencing (E), and compilation of the relative abundance of the venom components (F). RP-HPLC, reverse-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis. [Color figure can be viewed at wileyonlinelibrary.com]

reverse-phase (RP) chromatographic peaks containing proteins from the same family to the total area of venom protein peaks in the RP chromatogram. Recording the RP-HPLC run at the wavelength of absorbance for the peptide bond [190–230 nm], and according to the Lambert–Beer law ($A = \varepsilon cl$, where A = absorbance; ε is the molar absorption coefficient, also called molar absorptivity per molar concentration [$M^{-1}\,cm^{-1}$]; c = concentration [M]; and l = path length [cm] of the solution that the light passes through), these percentages correspond to the "% of total peptide bond concentration in the peak." This figure can be transformed into percentage of molecules dividing by the number of peptide bonds per mature venom molecule (=n residues per molecule −1). For chromatographic peaks containing single components (as judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE] and/or MS), this figure is a good estimate of the relative abundances of the chromatographic fraction as % by weight (g/100 g), or dividing by molecular mass to %mol, of the total venom proteins (Calderón-Celis et al., 2017; Calvete, Lomonte, et al., 2021). When more than one protein is present in a reverse-phase fraction, their proportions are estimated in the second level of pre-MS quantification (b), where the relative abundances of the proteins coeluting in the same RP fraction are assigned to the different toxin bands by densitometry of Coomassie-stained SDS-polyacrylamide gels. Finally, in the third level of quantification (c) the relative abundances of different proteins comigrating in the same SDS-PAGE band are estimated based on the relative ion intensities of the three more abundant peptide ions assigned by MS/MS

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analysis to the corresponding comigrating proteins. Figure 3 illustrates how dissociating the venom decomplexing (a–c) from the protein identification (d, e) steps represents an opportunity to achieve parallel relative quantification and locus-resolved insight into the venom proteome. The major current limitation to reach this goal is the lack of chromatographic baseline separation of all the different proteome components. This drawback can be partly minimized by deconvoluting the quantitative contribution of the different coeluents using hierarchical levels of orthogonal techniques, for example, SDS-PAGE and ion intensity. In contrast to most multidimensional separation approaches, the hierarchical relationship between the quantification levels guarantees that each level refines the value obtained in the previous level and that the sequential venom subfractionation steps do not alter the relative proportions of the components in the original venom. Furthermore, the relative quantification of the proteome toxins in g/100 g of the total venom proteins provides a way to transform the compositional data into absolute figures knowing the amount of venom injected in the RP-HPLC column (Eichberg et al., 2015). Conversely, the compositional distortion introduced by shotgun bottom-up proteomics in the relative quantification of the venom proteome, derived from the combination of the protein inference problem and the incompleteness of a specific database, is illustrated by the two case studies displayed in Figure 4. Panels A and B show, respectively, comparisons of protein-centric (snake venomics) versus peptide-centric (shotgun) bottom-up proteomics relative quantifications by ion-intensity (A) or spectral counting (B) of the venom proteome of the nonmodel species white-lipped Sunda Island pitviper (*Trimeresurus insularis*) (Jones et al., 2019) and the two-striped Amazonian palm pitviper (*Bothrops bilineatus smaragdinus*) (Sanz et al., 2020).

6 | THE CHALLENGE OF ABSOLUTE QUANTIFICATION

Percentage compositions gathered from single shotgun experiments that neglect pre-MS proteome decomplexation provide relative compositional values subject to the constant-sum constraint, a mathematical property embedded in any compositional close data set, where all variables in a sample always add up to the same constant figure (1% or 100%) (Aitchison, 1986; Aitchison & Egozcue, 2005). Because of this "constant sum constraint," individual variables of compositional data are not allowed to vary independently. Analyzing adimensional relative compositional data derived from shotgun MS as if they were absolute figures can yield erroneous results. For example, correlation analyses based on statistical models that assume independence between compositional features are flawed because of the mutual dependency between components of closed data. There is thus a need in all the fields of natural sciences, but particularly in biology, to conduct absolute quantification studies, that is, to accurately correlate molar changes with biological responses. Absolute quantification through targeted proteomic approaches (Borràs & Sabidó, 2017; Calderón-Celis, Ruiz Encinar, et al., 2018; Keerthikumar & Mathivanan, 2017; Manes & Nita-Lazar, 2018; Vidova & Spacil, 2017), for example, Selected Reaction Monitoring (SRM), also known as Multiple Reaction Monitoring (MRM), and Parallel Reaction Monitoring (PRM) (Rauniyar, 2015), requires for each targeted quantification of a specific protein spiking the experimental sample with defined amounts of isotope-labeled analogues of proteotypic peptide(s), which uniquely represent target proteins or a protein isoform, using AQUA or QconCAT strategies (Brun et al., 2009; Kirkpatrick et al., 2005) or a PSAQ ("Protein Standard for Absolute Quantification") (Adrait et al., 2012; Brun et al., 2007, 2009). Targeted proteomic techniques are gaining importance in Systems Biology (Manes & Nita-Lazar, 2018) and in the field of biomarker validation (Keerthikumar & Mathivanan, 2017). PSAQs are whole synthetic isotopically labeled analogues of the proteins to be quantified of certified concentration and similar ionization efficiency as the target analyte (Calderón-Celis, Ruiz Encinar, et al., 2018; Picard et al., 2012). Though state-of-the-art targeted assays, can measure simultaneously in the unfractionated digest of biological fluids up to 100 peptides representing 100 medium-to-high-abundance proteins (Bailey et al., 2014), and the recombinant or synthetic production of PSAQs for each and every one of the proteoforms of a relatively simple proteome may be technically possible, proteome-wide targeted proteomics is currently not a feasible option in practice.

7 | ELEMENTAL MASS SPECTROMETRY FOR THE ABSOLUTE QUANTIFICATION OF PROTEINS

7.1 | Inductively coupled plasma (ICP) mass spectrometry: Operational principles and its emerging role in quantitative proteomics

The popular saying "no one-size-fits-all" applies perfectly to scientific instrumentation, including mass spectrometers. The trend toward hybrid configurations of mass analyzers has dominated recent advances in biological

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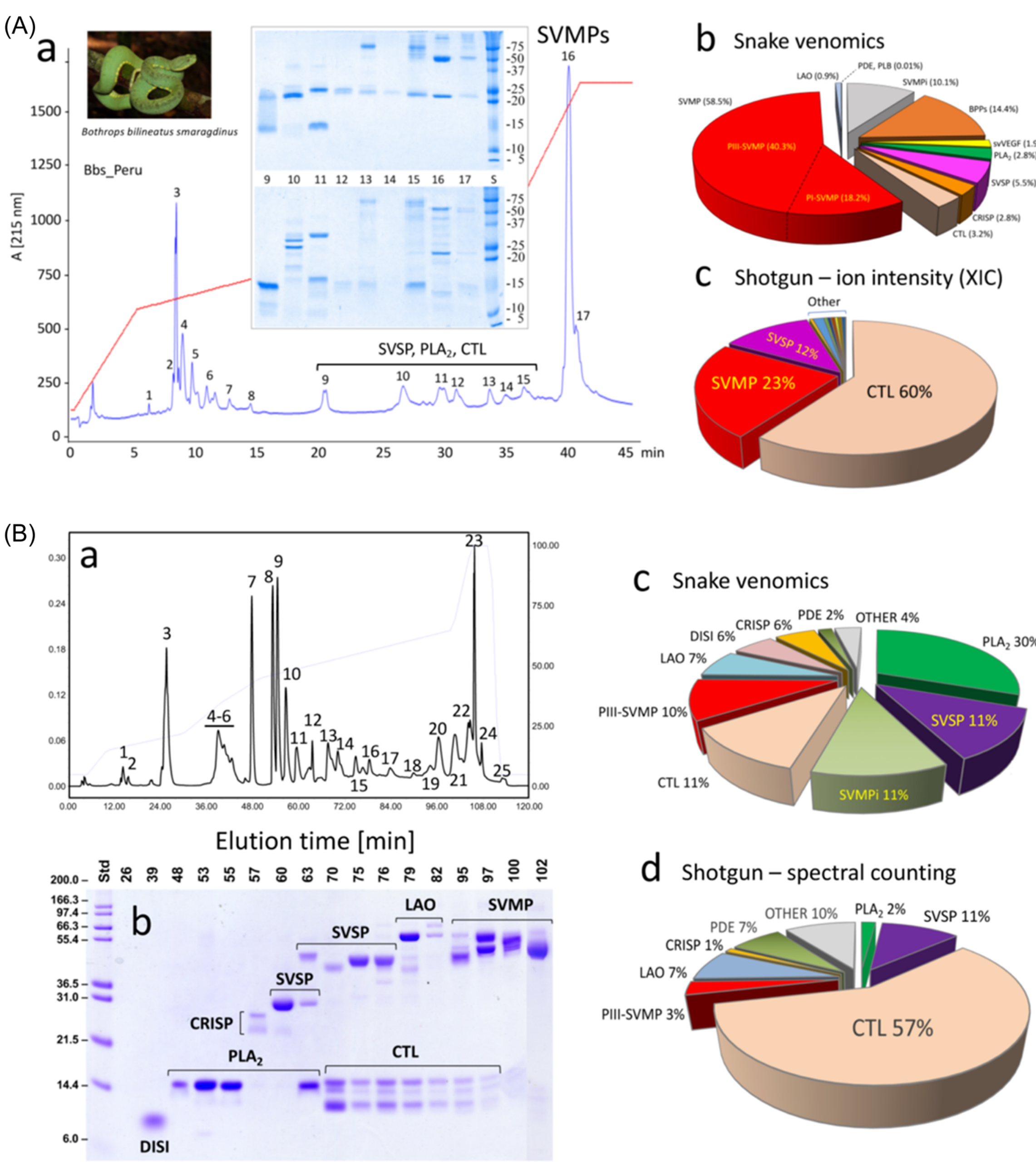

FIGURE 4 Comparison of venom protein family abundances gathered by snake venomics versus shotgun bottom-up proteomics workflows. (A) RP-HPLC separation of the venom proteins of the South American palm pitviper *Bothrops bilinatus smaragdinus*. Chromatographic fractions were collected manually and analyzed by SDS-PAGE (inset) under nonreduced (upper panel) and reduced (lower panel) conditions as described and processed using our standard snake venomics workflow (Lomonte et al., 2020). (B) Displays the computed venom protein family abundances. The same whole venom of *B. b. smaragdinus* was analyzed through a shotgun bottom-up proteomics approach as described (Sanz et al., 2020) using PEAKS X (Bioinformatics Solutions) for spectral processing against the UniProt "*Serpentes*" database and assignments to known protein families by similarity. The analysis resulted in the identification of a minimum of 82 distinct proteins, whose relative abundances were estimated from the sum of the extracted ion peak area intensity as % of spectral area (C) of the MS1 precursor ion chromatograms recorded by the PEAKS software. (B) panels a and b show, respectively, RP-HPLC separation and SDS-PAGE analysis of the chromatographic peaks of the venom of the nonmodel species white-lipped Sunda Island pitviper (*Trimeresurus insularis*) (Jones et al., 2019). Panel c shows the relative composition of the venom toxin families gathered through the hierarchical three-step snake venomics workflow. Panel d displays the protein family relative abundances of *T. insularis* venom characterized and quantified by shotgun proteomics and the normalized spectral abundance factor, NSAF, approach. The shotgun strategies illustrated in (A) and (B) show distorted venom compositions, strikingly biased towards an over-representation of CTL and SP (A), and over-representation of CTL and under-representation of PLA_2s (B) compared to the same venom quantified through the snake venomics workflow schematized in Figure 3. BPP, bradykinin-potentiating peptide; CRISP, cysteine-rich secretory protein; CTL, C-type lectin-like protein, DISI, disintegrin; LAO, L-amino acid oxidase; NGF, nerve growth factor; PLA_2, phospholipase A_2; PI- and PIII-SVMP, SVMPs of class PI and PIII, respectively; PDE, phosphodiesterase; PLB, phospholipase B; SVMPi, tripeptide inhibitors of snake venom metalloproteinase; SVSP, snake venom serine protease. [Color figure can be viewed at wileyonlinelibrary.com]

mass spectrometry. Current hybrid mass spectrometry systems use various designs of beam-type and ion-trapping spectrometers to combine the different performance characteristics offered by the individual in-space and in-time analyzers into one system (Calvete, 2014c; Senko et al., 2013). Incorporating novel capabilities into current configurations represents an instrumental complication, which entails an additional economic cost. On the other hand, the possibility of determining in the same experimental system the identity and the absolute quantification of the components of a complex proteome, would however compensate for all the extras. In recent years we have explored novel molecular and elemental MS hybrid configurations for the absolute quantification of proteomes using snake venomics as proof of concept (Calderón-Celis et al., 2016, 2017; Calvete et al., 2017; Calvete, Pla, et al., 2021). ICP-MS, a type of elemental MS introduced in 1980 (Houk et al., 1980) and commercialized in 1983 (Becker & Spectrometry, 2007) came out as a realistic alternative to overcome the need of spiking a Stable Isotope-Labeled (SIL) standard for each target protein (Calderón-Celis, Ruiz Encinar, et al., 2018).

ICP-MS is a particularly powerful spectrometric technique traditionally engaged with the detection of metallic elements (Ammann, 2007). Liquid samples are first nebulised in the sample introduction system and the fine aerosol is subsequently transferred to the ionization source. A high-temperature energized Ar plasma, created by inductively heating the gas with an electromagnetic coil, atomizes the sample generating positive atomic and small polyatomic ions derived from the constituent (analytes and matrix) elements. With the aid of electrostatic focusing devices, ions produced in the ICP are guided through the quadrupole mass analyzer to the detector [Houk et al., 1980; Wilschefski & Baxter, 2019). In contrast to other forms of inorganic mass spectrometry, such as Glow Discharge Mass Spectrometry (GDMS) (Quarles et al., 2017) and Thermal Ionization Mass Spectrometry (TIMS) (Lassiter, 2019), ICP-MS has the unique ability to sample continuously the analyte introduced at atmospheric pressure. This is an essential feature for hyphenating it to identical RP-HPLC pre-MS proteome decomplexation conditions applied in molecular MS-based bottom-up and top-down proteomic workflows.

The main virtue of ICP-MS in proteomics is undoubtedly its quantitative nature. ICP-MS ionization process is virtually unaffected by the analyte structure and the signal detected is directly proportional to the amount of the detected element. However, atmospheric pressure ionization source makes ICP-MS unsuitable for detection and quantification of biomolecules through their main elements, C, H, N, and O. The background signal of these elements along with their high ionization potentials are major constraints preventing the sensitive detection of biological analytes. On the other hand, another significant feature of ICP-MS is its multielement capability, which allows multiple elements to be measured simultaneously in a single analysis, including all metal and metalloid, semimetals and several nonmetals (and their different isotopes) at concentration levels as low as one part in 10^{15} (part per quadrillion, ppq) using adequate noninterfered low-background isotopes (Calvete et al., 2017; Pröfrock & Prange, 2012). The only elements that ICP-MS can not measure are H and He (which are below the mass range of the mass spectrometer), and Ar, N, and O (which are present at high level from the ICP argon plasma used to atomize and ionize the sample, as well as in the ambient air). The concept of heteroelement-tagged proteomics has been proposed for the complementary application of elemental and molecular mass spectrometry for the screening and quantification of heteroatom-containing biomolecules (Bettmer et al., 2009; Sanz-Medel et al., 2008; Szpunar, 2005). This unique capability results in the possibility to carry out the quantification of biologicals (e.g., peptides or proteins) using as generic quantification standard any compound that shares with the bioanalyte an ICP-detectable element (Calderón-Celis, Ruiz Encinar, et al., 2018). The lack of requirements of specific standards opens up a wide range of generic standards of known and traceable concentration that fit the instrumental and methodological criteria.

7.2 | Absolute protein quantification via ICP-MS sulfur detection

The applicability of ICP-MS in protein analysis has been exploited for more than 30 years. The first applications focused on the speciation of metalloproteins through the monitorization of different metals (Cu, Zn, Fe, Cd, etc.) noncovalently complexed to the proteins of interest (High et al., 1995). These studies led to the development of alternative quantification methods using chemical labeling with element-containing tags for multiplexed absolute quantification (Esteban-Fernández et al., 2012). Developments in immunoassays and nanotechnology using probes tagged with nanoparticles carrying hundreds to thousands ICP-detectable elements resulted also in a significant amplification of ICP-MS signal (Cid-Barrio et al., 2018). Further, Collision-Reaction Cell (CRC) technology in conjunction with ICP-MS (Tanner et al., 2002) enabled the sensitive detection of the nonmetallic elements phosphorous and selenium in proteomics studies, thereby widening the range of biological application of ICP-MS, notably protein

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phosphorylation (Krüger et al., 2009; Maes et al., 2016). These approaches are conditioned by the presence of ICP-detectable elements incorporated in the target protein post-translationally in vivo or through in vitro labeling strategies and are led astray from the purpose of generic, universal, label-free protein quantification though. Universal label-free protein quantification demands that all proteins in the sample be quantified in identical conditions with the same protocol and generic standard. In the case of ICP-MS-based quantification, it requires that all proteins carry one naturally present ICP-detectable element. Sulfur present in cysteine and methionine, amino acids that are statistically present in 98% of all proteins, was recognized as the key element for the absolute quantification of individual proteins but also proteomes via elemental MS (Wind et al., 2003). Yet, due to its high ionization potential and the occurrence of a great polyatomic interference ($^{16}O_2$) at the m/z of its most abundant isotope (^{32}S, 95%), sulfur detection was hampered to ICP-MS. To overcome the $^{16}O_2$ interference problem Bandura and coworkers (Bandura et al., 2002) applied quadrupole ICP-MS with a dynamic reaction cell to oxidize sulfur to $^{32}S^{16}O$ allowing the detection of sulfur at the less interference m/z 48. More recently, the introduction in 2012 of a tandem mass spectrometry configuration (ICPQQQ) for efficient polyatomic interference removal (Diez Fernández et al., 2012) enabled the sensitive (11 fmol) detection of sulfur (and other nonmetallic elements, i.e., P), boosting enormously the applicability of ICP-MS in biological research. In parallel, the use of miniaturized total consumption nebulization systems was found to be essential to transfer native proteins from the liquid sample solution to the ICP plasma with the same efficiency as the low molecular weight compounds used as generic standards (e.g., methionine) (Cid-Barrio et al., 2020). Notably, the operating flows demanded by such miniaturized systems (few $\mu L\ min^{-1}$) make LC-ICP-MS approaches more compatible with most of the LC-ESI-MS-based proteomics platforms. This compatibility allows running parallel detection of the same LC separation analysis with elemental and molecular MS. MS^n top-down proteomics analysis provides protein identity and sequencing in each LC peak, which can be correlated with the mass of sulfur corresponding to that same peak with ICP-MS. Sulfur-based protein quantification does require the use of just one sulfur-containing compound (organic or inorganic, e.g., sulfate salt) of known (certified) concentration for each and all of the chromatographically separated species, measured prior (external standardization) or spiked (internal standardization) to the LC-ICP-MS analysis. Protein sequence knowledge or characterization by proteomics approaches makes it possible to directly translate sulfur mass into protein absolute quantities. Therefore, the inclusion of this element MS step in the integrated MS platform does not bring about any significant impact on the global throughput of the approach.

Due to issues concerning the chromatographic separation of native proteins, most early studies of absolute quantification of biomolecules via sulfur determination involved isolated proteins or samples of low protein complexity (Pröfrock & Prange, 2012). However, since 2016 we have incorporated ICP-MS absolute quantification of S in our snake venomics platform (Calderón-Celis et al., 2016, 2017). Our contributions to this emerging aspect of venomics have recently been reviewed (Calvete, 2018) and the most significant advances to date are briefly discussed in the next section.

8 | HYBRID MOLECULAR AND ELEMENTAL MASS SPECTROMETRY CONFIGURATIONS FOR THE ABSOLUTE QUANTIFICATION OF VENOM PROTEOMES

Venom emerged as a key evolutionary innovation that underpinned the explosive radiation of caenophidian snakes in the wake of the Cretaceous-Paleogene Mass Extinction that swept away the reign of the dinosaurs. Extant snake venoms have evolved for predatory and defensive purposes. They comprise mixtures of toxins of varying complexity, which act individually or as an integrated phenotype to wreak havoc on internal organs of the prey. The study of snake venoms ("snake venomics") is of great interest in the field of evolutionary ecology, but also in biotechnology, as the principles that have weaponized ordinary proteins to act on the vital systems of the prey suggests the existence of novel mechanisms of biotechnological significance (Calvete et al., 2017; Calvete, Lomonte, et al., 2021). On the other hand, snakebite envenomings inflicted on humans are an occupational hazard and a disease in many tropical and subtropical regions, especially in Africa and Asia (Calvete, Lomonte, et al., 2021; Harrison et al., 2009). Literature reports estimate that 400,000–1,200,000 snakebite envenomings occur annually, causing 81,000–138,000 deaths and many more injuries, such as physical sequelae (stigmatizing disfigurements and amputations) and chronic mental morbidity (Gutiérrez et al., 2017). Snakebite envenoming affects not only the victims but often their entire families, which may enter a cycle of generational poverty that is difficult to break (Calvete, Lomonte, et al., 2021; Longbottom et al., 2018).

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The abundance and specific toxicity of the individual toxins are important features for inferring composition-activity correlations that provide us with a deeper and more integrative understanding of the evolutionary and translational biology of snake venoms. Research on venoms has continuously enhanced by technological advances (Slaagboom et al., 2022). Besides its contributions to molecular and translational toxinology (Calvete, 2013; Calvete, Lomonte, et al., 2021; Lomonte & Calvete, 2017), it should be mentioned that venomics is also probing to represent an excellent testing ground to check the applicability of nonconventional technologies in the field of proteomics. In this regard, Calderón-Celis et al. (2016, 2017) have demonstrated a proof-of-principle use of a novel hybrid molecular and elemental mass spectrometry configuration for the MS1 identification of the snake venom toxins and their absolute quantification through parallel RP-μHPLC-ICP-QQQ MS and on-line Isotope Dilution Analysis (IDA). The signal provided by the continuously added isotopic tracer (^{34}S) is used to compensate for the significant variation of the protein (natural sulfur, ^{32}S) response factor obtained along the whole RP-HPLC chromatogram. Significantly, these studies also showed quantitative ($99 \pm 1\%$) chromatographic protein recovery calculated by comparing the sulfur mass of sample eluting from the chromatographic column with respect to the sulfur mass directly recorded by flow injection analysis (FIA). Complete chromatographic column protein recovery is a strictly necessary condition to achieve accurate ICP-MS-based generic absolute protein quantification. The ICP quantification approach using online IDA was subsequently simplified and improved in its sulfur quantification performance through control change of the chemical composition of the ICP-MS plasma (Calderón-Celis, Sanz-Medel, et al., 2018; Calderón-Celis et al., 2019). In particular, the continuous addition of carbon dioxide/argon gas mixture (CO_2/Ar, 10:90) to the plasma provided excellent signal variation corrections along the whole chromatographic separation for all elements simultaneously (<6 RSD%) while maintaining a sensitivity enhancement of twofold to ninefold. The approach abolished the need for correcting sulfur response factor variation using complex ^{34}S-isotope dilution procedures, thereby simplifying the mathematical treatment of the data (Calderón-Celis, Sanz-Medel, et al., 2018; Calderón-Celis et al., 2019). We have recently applied this methodology to the absolute quantification of the venom proteomes of the two species of desert black cobras, *Walterinnesia aegyptia* and *W. morgani* using the combined molecular and elemental mass spectrometry configuration (Calvete, Pla, et al., 2021) schematically depicted in Figure 5. Whereas the combination of elemental and molecular MS has proved the quantitative characterization of "toxinomes" of snake venoms, its early yet for its application into the absolute quantification of more complex organisms' proteomes (hundreds to thousands of proteins). The nature of the elemental signal in ICP-MS lacks the capacity to discriminate the species from which the detected element comes from when proteins coelute in LC chromatography. To apply this hybrid strategy in proteome-level quantification, pre-MS separation resolution must improve substantially. Potential combination with intact protein multidimensional separations or novel developments in the field of miniaturized separation processes and instruments, seem to be some of the most feasible ways to go to break this barrier.

9 | CONCLUDING REMARKS AND PERSPECTIVES

This review provides a conceptual and technical view of current methods for quantification of a proteome's components. The drafting of this essay was motivated by the widespread misuse in the literature of methodologies designed for model organisms in the quantification of proteomes of nonmodel organisms, particularly in the field of snake venomics (addressed in Calvete, Lomonte, et al., 2021; Tan, 2022). Additionally, the fact that molecular mass spectrometry is not an inherently absolute quantitative technique, and relative quantification has not standard units of measurement, represent a serious drawback for comparing the outcomes of independent molecular MS-based experiments. This circumstance makes it necessary to quantify the proteome through a truly quantitative orthogonal method. The strategy that we have been proposing for more than a decade takes advantage of pre-MS decomplexation of the proteome by RP-HPLC monitoring the eluate at the absorption wavelength of the peptide bond, a procedure that provides a frame of reference for the relative quantification in units of % of peptide bonds (a proxy for % by weight or mol%) of the RP-HPLC-separated proteome components. Biological MS has been continuously enhanced by technological advances. Hybrid configurations combining the complementary performances offered by in-space beam-type and in-time ion-trapping mass analyzers have given a great boost to biological MS applications, notably the development of stable isotopic labeling and label-free methods to quantify the individual components of a proteome. However, the performance of these approaches is contingent upon the species proteome coverage in the reference search database. Recently we have demonstrated the feasibility of incorporating ICP-MS into hybrid elemental and molecular snake venomics workflows. The compatibilty of the duty cycles of molecular

FIGURE 5 Cartoon of the more recent hybrid molecular and elemental MS configuration developed for the absolute quantification of snake venom proteomes. The workflow comprises RP-HPLC venom protein separations hyphenated to bottom-up and top-down venomics workflows for relative quantification and locus-resolved identifications (Calvete, Pla, et al., 2021). Continuous absolute quantification of sulfur along a reverse-phase capillary HPLC run with parallel ESI-QToF molecular mass profiling served to assign the parent venom toxins gathered from homologous transcriptome-assisted bottom-up and top-down venomics. Molar ratios sulfur/protein computed throughout the chromatogram were translated into the corresponding absolute protein amounts using the equation [μmol $S/\Sigma(C+M)] \times MT_i = \mu g\ T_i$, where $\Sigma(C+M)$ is the number of sulfur-containing amino acids cysteine and methionine residues in the amino acid sequence of toxin "i" (T_i) and MT_i is the ESI-MS determined monoisotopic molecular mass of toxin "i." The hybrid molecular and elemental MS configuration combines the relative (%mol) venom proteome quantification, the unparalleled molecular resolution of top-down MS, and the absolute proteome quantification of ICP-MS. ICP-MS, inductively coupled plasma mass spectrometry; MS, mass spectrometry; RP-HPLC, reversed-phase high-performance liquid chromatography. [Color figure can be viewed at wileyonlinelibrary.com]

mass spectrometers (≥ scans/s) and elemental mass analyzers (able to read a single element in about 0.3 s) allows to combine their unique performances (e.g., the unparalleled molecular resolution of top-down MS and the absolute quantification of ICP-MS) into a single instrumental setting. Our proof-of-concept on snake venomics via a hybrid elemental and molecular MS configuration paves the way for broader and more routine applications in other areas of the proteomics field, notably, but not limited to, protein phosphorylation, quantification of S-containing proteins, and metallomics (Diez Fernández et al., 2012; Mounicou et al., 2009; Singh & Verma, 2018; Wang et al., 2019; Zhou et al., 2022). Further, it is estimated that our planet hosts approximately 10 million species of plants and animals (Scheffers et al., 2012). However, our knowledge of biological processes comes from studies of a few model organisms. This situation barely reflects the true diversity of life on Earth. The breakthrough of the hybrid elemental and molecular MS configuration makes feasible the study of life processes where the underlying chemistry involves a heteroatom playing a crucial role in its mechanism. In this sense, metallomics, "the systematic approach to the study of metal content, speciation, localization and use within organisms and ecosystems" (Thiele & Gitlin, 2008) is becoming increasingly important. Hence, taking advantage that biological systems utilize metal ions in fundamental processes like folding, stability and assembly of proteins, signaling, catalysis, cellular homeostasis, detoxification responses, including bioremediation, gene expression, as well as processes involved in how living creatures sense, and dynamically adapt to ecosystems (Chasapis et al., 2022; reviewed by Singh & Verma, 2018), proteomic studies on ample aspects of nonmodel organisms' biology would benefit from the application of a hybrid elemental and molecular MS configuration.

The integration of top-down molecular MS and elemental ICP-MS into a compact and automated

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platform bears the potential to revolutionize the proteomics arena in general and snake venomics in particular. Of the around 3970 extant snake species, 600 are venomous, and about 200 are able to kill a human. However, as of 2022 only a handful of sequenced genomes (~24) and venom gland transcriptomes (~80) of venomous snakes have been reported (Calvete, Lomonte, et al., 2021; Rao et al., 2022; Tan, 2022). Although we have not reached the ultimate goal of characterizing and quantifying all unique proteins in a venom proteome, current bottom-up or top-down approaches in conjunction with database-assisted and/or de novo MS/MS sequencing, have provided insights into the relative composition of 240+ venoms at different resolution levels (individual toxins or toxin family) (Calvete, Lomonte, et al., 2021; Damm et al., 2021; Tasoulis et al., 2022). Implementing top-down and absolute quantification approaches into next-generation proteomics workflows will represent a quantum leap in the study of venom proteomes. A challenge that remains to be solved to achieve this goal includes efficient, ideally baseline separation, of all the venom proteome components before their parallel proteoform-resolved identification and absolute quantification.

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