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CRITICAL REVIEW

The development and use of isotope dilution mass spectrometry methods for the quantification of target proteins in certified reference materials

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Abstract

Originally developed for small molecules in the late 1970s and early 1980s, isotope dilution mass spectrometry (IDMS) also has become the "go to" method of quantification for those wishing to characterize the mass fraction or amount of substance content of proteins in matrix-based certified reference materials (CRMs). While instrumental factors can impact the accuracy of IDMS methods, the use of double IDMS, higher order approaches, and "exact matching" have done a lot to improve the accuracy of the results. This review shows the development of IDMS for the quantification of proteins from being a method only used for value assignment of primary calibrators using isotope-labelled peptides as internal standards to a method for quantification of proteins in complex biological matrices such as serum and blood using isotope-labelled recombinant intact proteins as internal standards. It will discuss the challenges and limitations of currently used organic IDMS peptide-based approaches regarding the definition of the measurands, equilibration of sample and internal standard, digestion efficiency, and stability and suitability of the internal standards. This paper reviews the steps taken to assess the accuracy of the peptide- and protein-based IDMS methods used for the characterization of CRMs and the requirement to ensure comparability in laboratory diagnostics down to the patient level. Furthermore, it will give an outlook on future challenges, such as including structural and protein activity information in the results.

Keywords Isotope dilution mass spectrometry · Internal standard · Certified reference material · Reference material characterization · Measurement comparability · Traceability

Introduction

In 1935, Rittenberg and Schoenheimer pioneered the use of stable isotopes to trace amino acid and lipid metabolism in vivo, establishing the conceptual foundation for isotope dilution (ID) [1]. Although this work predated mass spectrometry (MS), the core principle was already set: adding a known quantity of an isotope-labelled or isotope-enriched version of the analyte as an internal standard and determining the concentration of the analyte in the sample through the measurement of the analyte and labelled standard signal ratios. Radioactive ID was applied to elements like uranium and plutonium, offering the key advantage that post-equilibration losses do not affect the final result—making it ideally suited for measurements of samples with complex matrices [2].

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With the introduction of MS, IDMS first matured in inorganic analysis due to the availability of isotope-enriched elements that could be used as internal standards. Techniques such as thermal ionization mass spectrometry (TIMS), spark source mass spectrometry (SSMS), and inductively coupled plasma mass spectrometry (ICP-MS) enabled the high-precision isotope ratio measurements required for IDMS. Heumann's [3] review highlighted the rise of IDMS as the definitive method for elemental analysis [3]. TIMS-IDMS became a cornerstone in geochronology and nuclear safeguards for measuring lead, uranium, and strontium isotopes. NIST adopted IDMS for the certification of their inorganic standard reference materials (SRMs), achieving uncertainties below 0.2 % and setting a precedent for future organic and protein applications [4]. The role of IDMS as a primary reference measurement procedure (RMP) in the certification of inorganic reference materials (RMs) was summarized by Vogl and Pritzkow [5].

Deuterium- and ^{13}C-labelled internal standards became essential in pharmacokinetics [6], driving the development of synthetic methods for isotope-labelled organic molecules. The use of IDMS to certify the concentrations of small organic analytes in clinical certified reference materials (CRMs) goes back to the pioneering work at NIST (then known as the US National Bureau of Standards) with analytes such as glucose [7], cholesterol [8], and creatinine [9] in human serum. By quantifying serum cortisol and other steroids in human serum using IDMS, Siekmann and colleagues could show systematic errors in immunoassays for the first time and thus catalyzed the global movement for clinical measurement standardization [10]. Lehmann's [11] review comprehensively outlines this development [11].

Since 1993, chemical measurements were also integrated into the activities of the Metre Convention via the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) [12] in order to achieve internationally comparable results on the highest level. This led to the introduction of metrological concepts also in clinical and environmental chemistry for the above-mentioned reasons. Internationally accepted, national metrological standards in these fields started to evolve from that time on and led to the growth of metrology in clinical chemistry. This included the concept of so-called primary standards as reference points. Most often, the primary standards (or primary RMs) were certified using IDMS that uses isotope-labelled molecules as internal standards. IDMS allows for the identification of the analyte via their molar mass as well as highly accurate quantification. The early efforts by the CCQM, through its Working Group on Organic Analysis (OAWG), implemented IDMS in Key Comparison studies for clinical analytes, such as glucose [7] and cholesterol [8], as well as the measurement of environmental contaminants such as dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT), and polychlorinated biphenyls (PCBs) [13]. Stephen Wise was an important contributor to many of the OAWG's studies implementing IDMS and the use of IDMS for the certification of CRMs [14–17].

In more recent years, the importance of reliable and comparable results traceable to a common reference system for proteins has come into focus. Proteins are an essential building block of all organisms. Characterizing their behavior by measuring their concentration in relevant matrices such as bodily fluids reveals indispensable information about metabolic processes, and any deviation from their regular behavior reveals important information about health status. Furthermore, proteins can be allergens or toxins. Human health and well-being can demand measurements of the concentration of such proteins also in food and food products [18]. Proteins may occur as contaminants or residues in food or as biotoxins, for example produced by bacteria [18], and require their assessment quantitatively, e.g., because of medical decision limits or legal limits. The importance of proteins and the determination of their concentration in various biological matrices for medical applications (i.e., in clinical chemistry) was first recognized in the first half of the nineteenth century [19]. An overview of the fields in which protein analysis is important is given in Fig. 1. Although there are many other fields, this review will focus on human health including clinical diagnostics, biopharmaceuticals, and food.

Protein concentrations in bodily fluids such as blood or urine were found to be essential to characterize the health status of humans and animals alike. Modern methods for quantitative determination of protein biomarkers in clinical laboratories include chemiluminescence immunoassays, which provide high sensitivity and automation for routine testing [20]; single-molecule technology, which enables ultrasensitive detection of low-abundance proteins at femtomolar concentrations [21]; and liquid chromatography-tandem mass spectrometry (LC-MS/MS), which offers structure-specific and SI-traceable protein quantification [22]. Nowadays, more than a dozen protein biomarkers are listed in the Directive of the German Medical Association as measurands of special importance which, when quantified, require metrological quality assurance [23]. A variety of methods evolved to quantify proteins in routine laboratories for clinical chemistry. An overview of the routine methods used for protein quantification has been given [24]. However, the results of these assays are often not comparable to each other as the definition of the measurand is challenging due to the presence of different variants of the same protein and their complex structure and the measurements lack a common reference point such as a RMP or CRM. Improved reliability of diagnostic results for medical use is needed, for example, to achieve joint decision limits for clinical markers. Efforts have been made to standardize measurements in clinical chemistry on a global scale. The

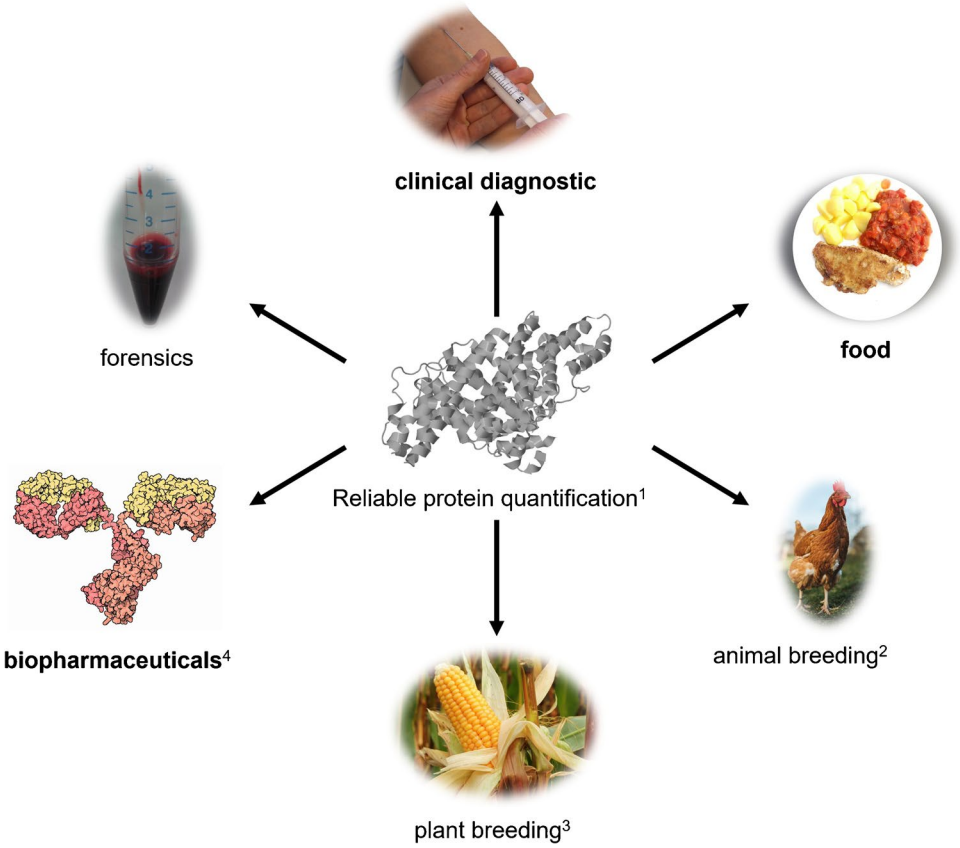

Fig. 1 Reliable protein quantification is essential for very different areas from clinical diagnostics to pharmaceutical industry and farming. RMs are currently only available in some of the areas and only for specific analytes. As these are the most advanced areas, the focus of this publication will be on clinical, biopharmaceutical, and food applications (in bold) (sources of graphics: [1]https://www.rcsb.org/3d-view/jsmol/7VR0, [2]thanks to Jan Baborák at Unsplash, [3]thanks to Marek Studzinski at Unsplash, [4]https://pdb101.rcsb.org/motm/21)

International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) was founded in 1952 for that purpose [25]. A first Directive of the German Medical Association including advice in clinical chemistry was published in 1971 [26]. Within the European Union, legal obligations for the quality of clinical chemistry were introduced by the in vitro Diagnostics Directive (IVDD 98/79/EG) in 1998 to establish quality standards for calibrators used in clinical chemistry [27]. This was reinforced in 2017 by the more stringent in vitro Diagnostics Regulation (IVDR 2017/746) [28] demanding metrological traceability.

Conventional methods such as colorimetric assays UV absorbance and fluorescence often combined with high-performance liquid chromatography (HPLC) rely on assumptions like linear detector response and require exact matrix matching of sample and calibrator to reduce the influence of potential interferences and background which might introduce systematic biases that limit true SI-traceable quantification [29]. Despite high sensitivity, immunoassays are often limited by a lack of metrological traceability, the use of non-commutable RMs, and inconsistent measurand definitions across platforms—factors that impair the global comparability of results. Furthermore, the development of immunoassays requires a prolonged and complex validation process and the definition of the measurand remains a particular challenge for heterogeneous protein analytes, where different assays targeting different epitopes may effectively measure different molecular fractions [30, 31]. In contrast, IDMS achieves SI traceable quantification through measurement of the signal ratio of the unlabelled analyte to the labelled internal standard after adding a known amount of stable isotope-labelled internal standard. This approach can compensate for recovery loss and matrix effects if the analyte and internal standard are equilibrated to achieve chemical equivalence in the samples and, thus, enable SI-traceable results. IDMS is recognized by the CCQM as a potential primary ratio method for peptide and protein quantification underpinning clinical standardization and the characterization and value assignment of CRMs [32]. Extending IDMS to peptides introduced new challenges: peptides are non-volatile, synthesis of labelled peptides was immature and biological matrices posed interference. The invention of fast atom bombardment (FAB) MS by Barber et al. in 1981 enabled direct ionization of non-volatile peptides [33]. Desiderio and colleagues later combined deuterium-labelled peptides with FAB-MS and LC to quantify neuropeptides in brain tissue demonstrating the feasibility of LC-IDMS for biological peptides [34]. Howeve,r sensitivity and background noise limit its broader application. The development of electron spray ionization (ESI) by Fenn et al. in 1989

revolutionized protein and peptide analysis [35]. ESI seamlessly integrated with LC, forming the LC-ESI-MS/MS platform, which offered unprecedented sensitivity and selectivity. In 1996, Barr et al. introduced the signature peptide approach, using stable isotope-labelled peptides as internal standards to quantify apolipoprotein A-I in serum [36]. This strategy, wherein proteotypic peptides represent the intact protein, remains the most widely used method in protein IDMS. By the early 2000s, multiple protein IDMS strategies had matured, with methods for peptide or protein purity assessment successively reported based on inorganic elements, amino acid analysis, and signature peptides. Josephs et al. comprehensively reviewed these approaches [32].

To successfully apply IDMS also for large biomolecules, ideally their isotope-labelled form is available and a clear distinction between measurands and their isotope-labelled form is ultimately required. However, if the isotope-labelled form of the protein is not available or not feasible due to high cost, isotope-labelled specific signature peptides can be a solution assuming they are released completely from the protein and are not altered during digestions. The measurement of intact protein via MS is hampered by a number of non-trivial practicalities. Their elemental composition results in the MS signal being spread across many isotopologs, which can reduce MS sensitivity. If isotop e-labelled versions of the protein are to be separated from the natural isotopic composition protein, the labelled protein requires excessive and/or total incorporation of the enriched element to be separated from the natural spread of isotopologs. This feature is further exacerbated by the multiple and often normal distribution of the added protons on the protein during the ESI process, which further reduces the sensitivity of MS for intact proteins via the spreading of the MS signal across many ions. When first developing IDMS methods for proteins, the available MS technologies lacked sufficient resolving power to separate the individual isotopologs of intact proteins and are often not routinely available even today. Furthermore, the relevant concentrations of proteins are often extremely low, for example in the case of hormones in bodily fluids but also for allergens and toxins in food, and require excellent signal-to-noise ratios. At the same time, knowledge about the structure of proteins is indispensable, and MS does not give this information a priori. Due to these challenges, the first CCQM key comparisons towards establishing internationally accepted standards for proteins in a matrix utilizing IDMS were published not before 2020 [37, 38]. New strategies, methods, and tools were required. The enormous effort to establish primary standards usable for proteins and the approaches to achieve this goal are described in this overview.

Challenges for IDMS for characterization and value assignment in matrix RMs

In IDMS, a sample with known isotopic composition (often the natural abundances as listed by IUPAC are assumed [39]) of the analyte but unknown concentration is mixed with a known amount of its isotope-labelled form, the so-called spike, used as an internal standard. This spike contains the analyte with a different isotopic composition: Ideally enriched in the rarest natural isotope and providing sufficient mass shift for complete peak separation. For organic molecules, the isotopic enrichment is usually ^{13}C, ^{15}N, or ^{2}H, with ^{13}C, ^{15}N being much preferred due to the kinetic isotope effects observed during separation and the frequent reactive exchange process of deuterium-labelled molecules. However, in the case of metal-containing molecules, such as metalloproteins, the metal can also be used for quantification. In this case, a spike material is needed with the analyte enriched in one isotope of this metal [40]. After complete mixing of sample and spike, the so-called sample-spike blend (bx) gained a new isotope ratio being between the isotope ratio of the sample and that of the spike. This blend isotope ratio directly reflects the analyte concentration in the sample [5]. This also indicates one of the most challenging steps in IDMS, the complete equilibration of sample and spike. Especially in biological matrices, this also requires that the spike behaves identical to the analyte including potential binding to other molecules present in the sample such as albumin or antibodies. This is necessary to preserve the isotopic ratio composition, present in the original sample-spike blend, in any following extraction, clean-up or measurement steps. This ensured, IDMS only requires isotope ratio determination and mass measurements. Losses of analyte after equilibration do not affect the accuracy of the analytical result, because the measurand, the blend isotope ratio, is equal in all sub-samples of the blend. This allows for extensive sample preparation schemes such as enzymatic digestion and chromatographic separation. To be used as a primary RMP, it has to be understood and clearly described in equations as well as a comprehensive uncertainty estimation has to be possible. In case of IDMS, both were clearly described by De Bièvre and Peiser [41]. This approach requires a very thorough characterization of the spike material regarding isotope abundances and purity. Due to the very low concentration of the other isotope(s) of the element enriched in one isotope, this is quite challenging. A practical solution is the use of the so-called double IDMS approach (Fig. 2). In this case, a calibrator (often a solution prepared from a pure substance or matrix (C) RM) with a known analyte content is used. By adding the spike to both the calibrator and the sample and measuring

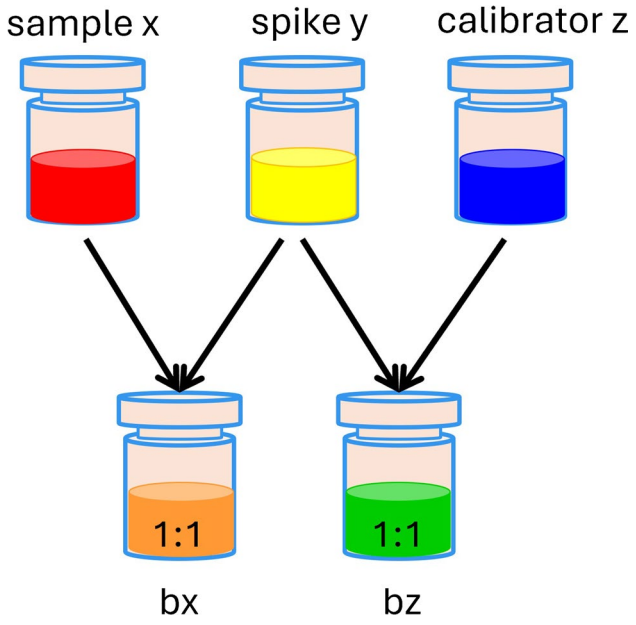

Fig. 2 For the so-called double IDMS, the isotope-labelled form of the analyte is added to both the sample and a well-characterized calibrator. By measuring the isotope ratio in both sample-spike (bx) and calibrator-spike blend (bz), the concentration of the analyte in the sample can be calculated from the known concentration of the analyte in the calibrator

the isotope ratios in both blends, the concentration of the analyte in the sample can be calculated using the known amount of analyte in the calibration blend (bz). However, this requires a well-characterized calibrator such as a (primary) RM for the target analyte.

The analyte content can then be calculated according to:

$$w_x = w_z \cdot \frac{m_{yx}}{m_x} \cdot \frac{m_z}{m_{yz}} \cdot \frac{(R_y - R_{bx})}{(R_{bx} - R_x)} \cdot \frac{(R_{bz} - R_z)}{(R_y - R_{bz})}$$

With

w_x: mass fraction of analyte in sample

w_z: mass fraction of analyte in reference

m_{yx}: mass of spike in the sample-spike blend

m_x: mass of sample in the sample-spike blend

m_z: mass of reference in the reference-spike blend

m_{yz}: mass of spike in the reference-spike blend

R_y: ratio of spike isotope to reference isotope in the spike

R_{bx}: ratio of spike isotope to reference isotope in the sample-spike blend

R_x: ratio of spike isotope to reference isotope in the sample (often the natural isotope pattern according to IUPAC [42])

R_{bz}: ratio of spike isotope to reference isotope in the reference-spike blend

R_z: ratio of spike isotope to reference isotope in the reference (often the natural isotope pattern according to IUPAC [42])

Both bx and bz are then subjected to the same sample preparation, leading to a similar matrix in both the sample and the reference. When using this so-called exact matching approach (isotope ratio in bx and bz as well as the amount of analyte in both mixtures is the same), another advantage is that a blank correction is not required; it might even be harmful in some cases, as shown by Pramann and Rienitz [43].

Also higher order IDMS such as triple IDMS are possible, having advantages like not requiring a characterization of the spike at all as both the spike content and its isotope ratios are cancelled from the equation. This is especially advantageous for ubiquitous elements such as iron or for poorly enriched spike materials and can improve the measurement uncertainty considerably. However, that comes with the price of elaborate sample preparations [44].

Assessment of primary calibrators

Element-based LC-ICP-IDMS for peptide and protein purity assessment

ICP-MS offers an orthogonal detection route for protein IDMS. Since almost all proteins contain sulfur as the same heteroatom, they yield a uniform relative molar response if the signal can be uniquely attributed to the protein of interest. LC-ICP-IDMS enables absolute quantification independent of protein sequence or structure, conferring broad applicability. Sulfur-based ICP-IDMS quantifies via cysteine and methionine residues using isotope-enriched sulfur spikes (e.g., ^{34}S-labelled methionine) [45]. Key uncertainty sources may include precise knowledge of the number of sulfur-containing residues in the target protein, polyatomic interferences (e.g., on ^{32}S from oxygen), effectively mitigated by collision-reaction cell technology, and the purity and concentration of the sulfur spike itself [46]. A disadvantage is the comparatively low sensitivity of ICP-MS for sulfur and an often high background leading to a high limit of detection. A similar approach can be used for the quantification of metal-containing proteins such as selenium or iron-containing proteins [47] using the metal for quantification. A 2024 review by Coverdale et al. systematically surveyed LC-ICP-IDMS advances for metalloprotein quantification, contrasting species-specific vs. species-unspecific IDMS and highlighting dual ESI-MS/ICP-MS platforms for simultaneous structural characterization and absolute quantification [48]. Furthermore, the simultaneous detection of metal(s) and sulfur indicates the number of metals bound to a protein, giving indications of the structure of the molecule. For

proteins with low native sulfur or selenium content and no inherent metals, artificial metal labelling may broaden the applicability. Metal chelators (e.g., DTPA, DOTA), which are labelled with naturally low-abundance rare earth elements (e.g., Lu, Eu), are covalently attached and quantified via ICP-IDMS [49]. The high sensitivity of lanthanides and their low background allows the detection of the proteins in very low concentrations.

Amino acid analysis–based LC-IDMS in value assignment of primary calibrators

Amino acid analysis (AAA) using IDMS (AAA-IDMS) is a well-established approach for the absolute quantification of peptides and proteins, providing a measure of total peptide or protein content, particularly in the context of pure primary calibrator or RM characterization. The method relies on strong acid hydrolysis (typically 6–8 mol/L HCl) to convert proteins or peptides into their constituent free amino acids, which are subsequently quantified by LC-MS/MS or gas chromatography with MS (GC–MS) using stable isotope-labelled amino acids containing heavier isotopes, e.g., ^{2}H, ^{15}N, or ^{13}C, as spike. The measured amino acid amounts are then related to the known amino acid composition of the protein or peptide to determine the amount of substance of the target protein [50–53]. The key strengths of AAA-IDMS are as follows: elimination of systematic errors from incomplete enzymatic digestion or peptide/protein loss during sample preparation as the harsh hydrolysis conditions minimizes the possibility of bias due to incomplete acid hydrolysis, establishing SI traceability via high-purity amino acid CRMs (e.g., NIST SRM 2389a), and offering broad applicability to any protein-containing pure material. Some weaknesses have to be considered when developing AAA methods. Kaiser and Brenner showed that racemization of AAs occurs in acidic hydrolysis. The degree varies between the different AAs and depends on the conditions used [54]. Furthermore, proline is prone to isomerization which is solvent dependent [55].

Nevertheless, when aware of these potential limitations and controlling them, AAA is a powerful tool for value assigning primary CRMs and calibrators. Muñoz et al. reported a complete AAA-IDMS workflow for protein calibrant quantification, demonstrating excellent repeatability and traceability across multiple protein types [52]. Li et al. applied parallel purity assessment strategies combining mass balance and AAA-IDMS to both recombinant human growth hormone and parathyroid hormone [56, 57], achieving high concordance between methods and cross-validating accuracy. Yan et al. extended this dual-route strategy to bovine lactoferrin [58]. The main limitations of AAA-IDMS arise from the harsh hydrolysis conditions, which lead to the degradation of many amino acids. Consequently, acid-stable amino acids such as proline, valine, leucine, isoleucine, and phenylalanine are the most suitable choices for quantification, as they remain stable throughout the hydrolysis process [59], although sometimes alanine and arginine are also used. Furthermore, complete hydrolysis has to be ensured, which can be challenging in certain amino acid combinations such as pairs of valine and/or isoleucine and multiple leucine in a row. However, AAA-IDMS analysis can only be applied to materials that contain only the target peptide or protein and no other source of amino acids. Otherwise, the result has to be corrected for protein or peptide impurities, which have to be identified and quantified using other methods such as LC-MS (including high-resolution MS) or quantitative nuclear magnetic resonance spectroscopy (qNMR). This approach is known as peptide impurity-corrected amino acid analysis (PICAA) [32].

Signature peptide–based LC-IDMS for peptide and protein purity assessment

The workflow of signature peptide–based LC-IDMS for protein purity assessment involves the following: (1) selection of signature peptides which result from high protease specificity, stable LC-MS behavior, and no missed cleavage or labile sites; (2) synthesis of heavy isotope-labelled (e.g., $^{13}C/^{15}N$) peptide internal standards with rigorous isotopic purity; (3) addition of the labelled standard before or after digestion; and (4) quantification by triple quadrupole or high-resolution MS in SRM/MRM or DIA mode [60–62]. Signature peptide selection is critical, and the ideal candidates must be unique to the target protein (e.g., BLAST-verified), completely and reproducibly released by the enzyme of choice, free of oxidation- or deamidation-prone residues, carry no post-translational modifications (PTMs) (unless these are the target analytes as in glycated hemoglobin) and exhibit good ionization and chromatographic performance [63]. The labelled and native peptides should also exhibit near-identical chromatographic retention and fragmentation patterns, necessitating the use of $^{13}C/^{15}N$ labelling, as deuterium (^{2}H) labelling can cause a shift in chromatographic retention time [64]. For applying the double IDMS approach, the peptides of choice have to be produced in pure form with natural isotopic composition to serve as calibrants. Different methods for verifying the purity of these calibrants have been applied. Martos et al., for example, used qNMR to characterize the calibrant peptides for SARS-CoV-2 IgG monoclonal antibody quantification, confirming a traceable purity assessment [65]. For value assignment, AAA as described in the previous section can be applied. Digestion completeness is another key factor for the signature peptide–based LC-IDMS. Arsene et al. showed that when cleavage is incomplete, the timing of internal standard addition significantly affects accuracy [62]. Thus, spike timing

is a fundamental design choice. In practice, digestion efficiency is maximized by extended digestion, high enzyme-to-substrate ratios, combined proteases (e.g., trypsin and Lys-C), and monitoring of missed cleavage peptides [66]. Another challenge are structure-related impurities present in pure protein material that might lead to the same signature peptide after digestions, but which is not related to the target form of the protein. Furthermore, IDMS quantifies total protein amount irrespective of folding state, which is appropriate for CRM mass fraction assignment but does not reflect native conformation or activity.

Other isotope dilution strategy beyond LC-IDMS

Diaz et al. developed an online carbon IDMS approach in which peptides are combusted to CO_2, and the resulting $^{13}C/^{12}C$ ratio is measured to independently verify a complete isotope labelling, providing a sequence-agnostic purity check orthogonal to LC-based methods [67]. GC-IDMS also offers a complementary route. After derivatization, analytes are resolved by GC and quantified by AAA-IDMS [53, 68]. Additionally, other ID-based methods were used as further orthogonal approaches. Vibrational spectroscopies are also sensitive to structural changes and can be useful to distinguish isotopologs of intact proteins. Frank et al. used ID Raman spectrometry to quantify hemoglobin alongside with IDMS-based approaches [69]. Song et al. proposed GC-ID infrared spectroscopy in which peptide hydrolysis is carried out and ^{13}C-labelled amino acids are used as internal standards. The quantification was performed via the amide I band shift derived by isotope-labelled amino acids, circumventing ionization discrimination effects inherent in MS and representing a candidate primary RMP [70].

Applications of IDMS for peptide and protein purity assessment

The primary application of IDMS for peptide and protein purity assessment lies in the production of CRMs. For clinically important proteins, this has become a core mandate of national metrology institutes (NMIs) worldwide. IDMS-based RMPs have been established or are under active development for an expanding range of proteins, including C-peptide [71], insulin [72], porcine insulin [73], prostate-specific antigen [74], bovine lactoferrin [58], recombinant human growth hormone [56], parathyroid hormone (PTH) [57], insulin-like growth factor-1 (IGF-1) [75], heat shock protein 90α (HSP90α) [76], interleukin-6 (IL-6) [77, 78], tumor necrosis factor alpha (TNF-α) [79], or procalcitonin [80]. During the COVID-19 pandemic, IDMS quantification methods for SARS-CoV-2-related proteins (spike, nucleocapsid) and monoclonal antibodies were also rapidly advanced [81, 82]. Collectively, these developments have established a metrologically traceable reference measurement infrastructure for protein quantification, significantly advancing the standardization of clinical laboratory measurements for these analytes.

Application of reference materials and relation to routine measurements such as immunoassay

Major challenges in IDMS for proteins

A special challenge in protein measurements is the definition of the measurand beyond the content of a specific peptide. Measurand definition is made difficult by the usually strong structural heterogeneity of proteins. Proteins in nature exist with large variations, even when the same primary amino acid sequence is present, due to PTMs such as glycosylation and phosphorylation as well as structural variations (e.g., disulfide bridge scrambling). It should be noted that our discussion of PTMs and structural heterogeneity focuses on the analytical challenges in measurand definition and protein quantification, rather than on the feasibility, cost, or yield of recombinant protein production in expression systems. Before applying IDMS for value assignment in CRMs, the measurand has to be clearly defined and that requires the determination of present variants and evaluation of their impact on protein quantification. Researchers and IVD manufacturers often identify clinically relevant biomarkers based on studies conducted relying on immunoassays that often detect a panel of isoforms that are not always completely defined. The advantage of targeted IDMS approaches of being highly selective can thus turn into a challenge as the measurand often consists of a unique, well-defined target while for immunoassays, the measurand often consists of a panel of proteins with different structures. Furthermore, while IDMS gives a result for the total amount of the protein in the sample independent of its form as long as it contains the specific peptides used for quantification, immunoassays may be blind to parts of it when bound to other proteins or in aggregates. This possible discrepancy between the measurands in IDMS and in immunoassays can compromise the success of standardization initiatives relying on an IDMS-based reference measurement system. Inconsistent measurand definition represents a critical challenge for the development of RMPs and CRMs and could explain the substantial differences that have been observed between IDMS-based results and immunoassay-based results, but are also often the cause of differences in results between immunoassays from different manufacturers nominally measuring the same analyte but including different variants (different assay selectivity) [80]. While discrepant measurand definitions can explain these differences, differences in calibration

can also play a role. While immunoassays can be calibrated with matrix-matched materials spiked or not with recombinant proteins or peptides containing the target epitope, IDMS methods also proved to be affected by the nature of the calibrators, peptides or proteins [80, 83, 84]. Peptide-based calibration has some major drawbacks as it does not account for incomplete release of the peptides from the proteins. Therefore, intact proteins are used both as calibrants and as isotope-labelled standards as far as possible [85]. A major challenge lies in the availability of isotope-labelled proteins for use as internal standards and the evaluation of whether these are fit for purpose, i.e., properly mimic the behavior of the endogenous protein. An alternative can be the use of isotope-labelled peptides, which are more affordable than labelled proteins, and a natural protein standard if processing conditions (e.g., extraction, digestion) are very well controlled (reproducible) for sample and calibration blends [86]. Despite these limitations, LC-IDMS remains the RMP of choice to determine protein concentrations in human samples [87]. Thanks to spectacular progresses in instrumentation development, top-down analysis of intact proteins becomes possible and avoids the digestion step of the bottom-up approach [88]. This represents great benefits as demonstrating the digestion completeness is not straightforward in absence of recombinant proteins that could be used as primary calibrators and/or internal standards. However, quantitative top-down approaches still have to be improved to be able to provide SI traceable results comparable with the established bottom-up methods. Another challenge in protein analysis is the stability of the measurand. Torma et al., for example, showed that brain natriuretic peptide (BNP) is not stable in plasma samples and has to be stabilized to ensure accurate quantification [89].

As immunoassays target epitopes rather than the whole protein as analyte and not all forms of a protein are really biologically active, it is also important to know not only the concentration of the target protein in the sample but also its structure variants, PTMs and even conformers. The conventional IDMS approach cannot give this information, but might be influenced by different structures, and new techniques and approaches are currently developed to answer this demand [90]. One step forward is the coupling of immune affinity techniques with MS to get the best from both worlds [91]. However, this approach also faces the challenge of the definition of the measurand, which might make traceability difficult.

Clinical requirements

Laboratory medicine results are vital for clinical decisions, but inconsistent findings across different laboratories and methods can lead to inappropriate patient care. Traceability in laboratory medicine seeks to minimize method variability, ensuring consistent results regardless of time or location. IDMS has played a pivotal role in the development of RMs traceable to the SI over the past decades with early applications for small molecule biomarkers such as creatinine or glucose. Proteins are an important class of biomarkers. Examples of widely accepted protein biomarkers include C-reactive protein for inflammation, cardiac troponin for myocardial infarction, brain natriuretic peptides for heart malfunction, prostate-specific antigen for prostate cancer, procalcitonin for sepsis, and amyloid-beta and tau protein for neurodegenerative diseases. The complexity of this class of biomarkers, including PTMs and higher order structure, poses significant challenges in the definition of the measurand of clinical relevance and the choice of the most appropriate measurand/calibrator for the development of IDMS workflows, RMPs, and CRMs. This is particularly the case when immunoassays are the routine measurements implemented in clinical settings.

In 2002, the Joint Committee for Traceability in Laboratory Medicine (JCTLM) was formed as a result of collaboration between the Bureau International des Poids des Mesures (BIPM), the IFCC, and the International Laboratory Accreditation Cooperation (ILAC) with the aim of coordinating standardization activities and implementing traceability of measurement results in clinical laboratory setting. JCTLM also offers educational support and maintains a database of RMs, reference methods, and services compliant with the relevant ISO standards including ISO 15194:2009 for RMs [92], ISO 15193:2009 for RMPs [93], and the more recent ISO 17511:2020 “In vitro diagnostic medical devices — Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples” [94]. A way to establish SI traceability of routine methods through an unbroken chain of calibration is shown in Fig. 3.

The JCTLM provides a framework for establishing metrological traceability in laboratory medicine, which is structured in a hierarchical order to ensure consistency and comparability of measurement results. The hierarchical structure aligns with ISO 17511:2020 standards, moves from highest-level fundamental references down to daily patient sample results. IDMS is considered the gold standard method for development and value assignment of primary calibrators and CRMs for clinical measurements [87]. The main challenge is, however, in the definition of the measurand where IDMS traceability for proteins is achieved through surrogates (signature peptides) of the protein itself as described above, while immunoassay-based clinical measurements rely on the binding of antibodies to certain epitopes of the protein which usually require an intact tertiary structure of the protein without in-depth chemical characterization and differentiation. Ensuring that the standards produced are fit for purpose for the intended clinical measurements

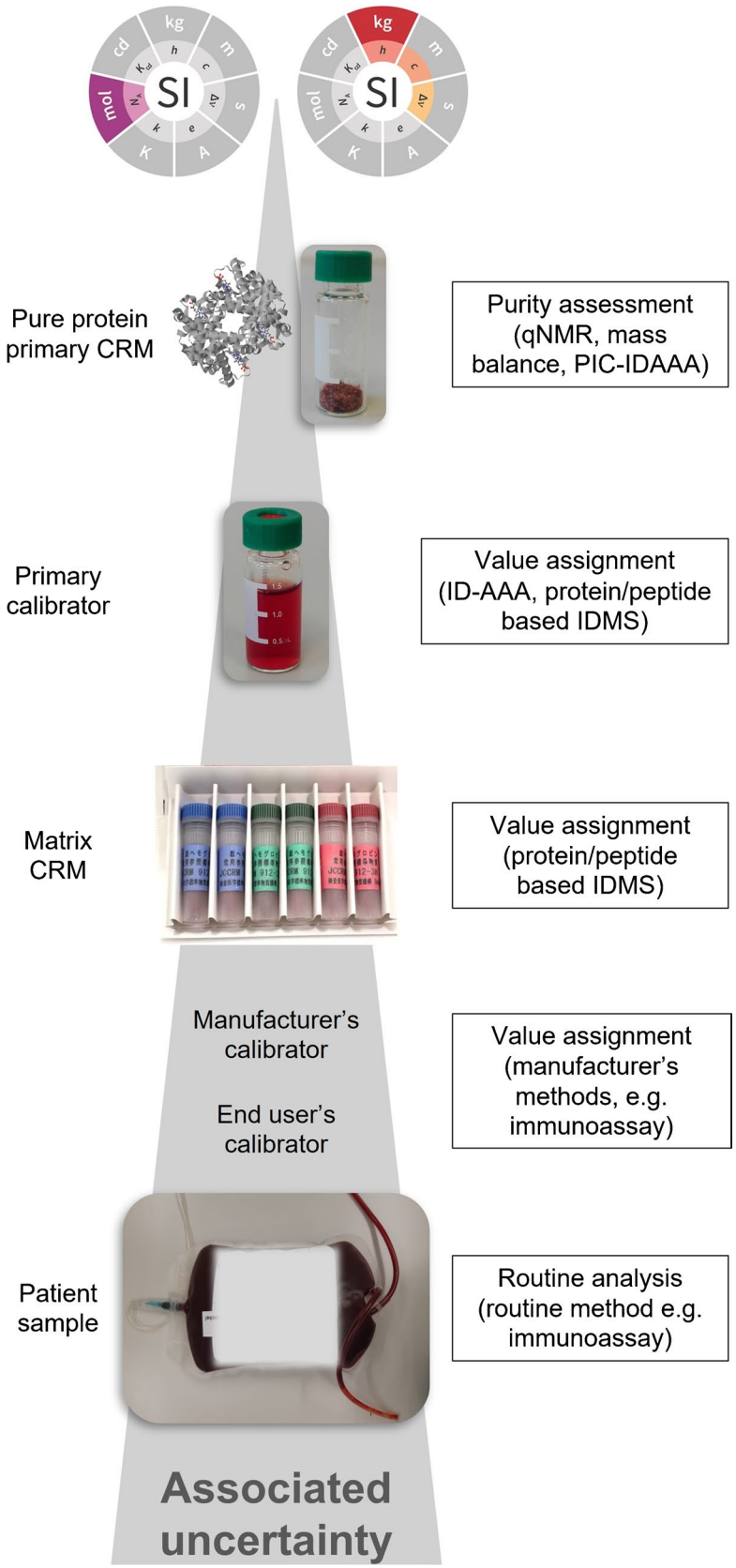

Fig. 3 The role of IDMS in establishing traceability of protein measurements in clinical diagnostics to the SI according to [94] (sources of graphics: https://www.bipm.org/en/measurement-units/si-base-units, https://www.rcsb.org/3d-view/jsmol/7VDE/1, https://www.reccs.or.jp/en/order/index.cgi?wd=JCCRM%20912)

is of paramount importance and one of the most challenging tasks of the metrological community, particularly when attempting retrospective standardization of established clinical measurements.

With the advent of LC-based methods for clinical diagnosis, however, and the broader understanding of measurement standardization requirements, the development of standards alongside the validation of emerging clinical biomarkers has significantly improved, ultimately providing benefits to patients. Examples are the amyloid peptides, mostly measured routinely by MS.

Another challenge in the development of matrix-matched RMs for proteins consists of producing materials of adequate commutability, which describes the property of a material to properly mimic the behavior of unadulterated patient samples in routine methods [87]. As the use of non-commutable CRMs will cause (i) inaccurate results for clinical samples and (ii) the inability of a common calibration material to improve harmonization of results provided by the different measurement procedures [95], commutability of calibration materials has emerged as a key requirement for establishing the metrological traceability of clinical measurements. Although commutability assessment frameworks have been established by the IFCC working group on commutability, evaluating commutability remains cumbersome, and to date, very few data are available to inform the extent to which processed and spiked materials properly mimic real patient samples [87].

Biopharmaceuticals

Biopharmaceuticals are medical drugs produced from living organisms, cells or bacteria, typically characterized by a complex structure (often large molecules such as antibodies, enzyme, or nucleic acids), high specificity, and potent activity. Examples of biopharmaceuticals are: - monoclonal antibodies such as rituximab used to treat certain lymphomas, leukemia, and autoimmune diseases like rheumatoid arthritis, adalimumab used for inflammatory conditions, pembrolizumab, an immunotherapy drug used in various cancers -hormones such as insulin, an essential drug used for regulating blood sugar and produced in recombinant form to treat diabetes or erythropoietin that stimulates red blood cell production and is used to treat anemia in kidney disease patients - vaccines that stimulate the body's immune system to protect against infectious diseases and include e.g. proteins (vaccines against hepatitis B, shingles, and some of the COVID-19 vaccines) - mRNA (COVID-19) enzymes, for example used in replacement therapy to treat genetic disorders - genetic and cell materials [96].

Biopharmaceutical standardization requirements are all about ensuring that complex biological medicines are consistently safe, effective, and high quality despite being produced in living systems and, therefore, less controlled and more variable than chemical synthesis. Biopharmaceutical product properties often depend on the cell line used and the manufacturing process, impacting for example protein PTMs. The global regulators require strict compliance with harmonized standards such as those defined by the International Council for Harmonisation (ICH) and local regulations such as those issued by the US Food and Drug Administration (FDA) and the European Medicine Agency (EMA), the National Medical Products Administration (NMPA), or the Pharmaceuticals and Medical Devices Agency (PMDA). The ICH guidelines are critical in setting the standards for the analytical requirements to measure the critical quality attributes of biopharmaceuticals (CQA) including identity, purity, quantity, potency/biological activity, and safety [97].

All biopharmaceuticals must be produced under Good Manufacturing Practice (GMP) conditions that ensure controlled manufacturing environments, validated processing and analysis, trained personnel, and full traceability of materials and qualified instruments. Because of the complexity of biopharmaceuticals, MS alone does not provide all required information. Therefore, several analytical platforms are used including biochemical and chemical platforms from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to enzyme-linked immunosorbent assay (ELISA), to capillary electrophoresis and chromatography coupled with specialized detectors such as multiangle light scattering, fluorescence detector, and MS as well as physical methods such as circular dichroism, Fourier transform infrared spectroscopy, NMR, Raman spectroscopy, dynamic light scattering, and thermogravimetric analysis, and bioassays including cell based assays for the measurements of the bio-product potency and activity [98].

The importance of standard materials to be used for comparability of measurements e.g. between different laboratories or when upgrading instrumentation is currently underestimated due to the broad range of molecules, analytics involved, and regulatory requirements. The concept of SI traceability is often perceived to be a tool for manufacturing controls more than a regulatory requirement limiting the collaboration between industry and NMIs in this space. Nevertheless, the UK National Institute for Biological Standard and Control (UK) has played a pivotal role in introducing several standards such as infliximab, rituximab, adalimumab, trastuzumab which were introduced as the World Health Organization (WHO) RMs [99]. Furthermore, the NIST collated a large amount of data on a humanized IgG1κ antibody to become the first RM (NIST RM 8671) with a large amount of published data available as reference [100, 101]. However, continuity in production is often one of the main challenges in this space.

Food and food products

The needs for CRMs to assess the accurate quantities of specific proteins in foods have been prepared to address the assessment of "gluten free" and "low gluten" containing food as defined by food regulations and the detection of food allergens for the protection of allergic consumers.

For legislative purposes, gluten has been defined as the "protein fraction from wheat, rye, barley, oats or their crossbred varieties and derivatives thereof, to which some persons are intolerant and that is insoluble in water and 0.5 M NaCl." The proteins that form gluten constitute between 70 and 80 % of total grain protein and hence find their way into many different food stuffs. However, gluten or its proteins are responsible for triggering an immune mediated disorder in individuals with celiac disease. This is a lifelong disease where the only relief is the complete removal of gluten from the diet. Due to farming practices and sharing of storage and transport infrastructure, the presence of gluten is often detected in non-gluten containing products. Therefore, the EU legislation [102, 103] requires products sold as "gluten free" and "low gluten" to contain less than 20 and 100 mg/kg of gluten, resp. The use of such thresholds requires the development of testing systems that could be used to enforce local legislation and protect consumers. For a variety of reasons, the predominant technology that was used for the detection of gluten is based on ELISA [104]. However, a lack of comparability in measurement results triggered requests for the production of reference materials with known quantities of gluten [105].

Using conventional approaches for the value assignment of an SI traceable quantity of gluten in a matrix RM provided many challenges. First, the definition of gluten results in no single protein that is present in a constant relative mass fraction to total gluten, and the difference in the gluten-containing protein solution between grain species made the conventional IDMS defunct. No single group of target proteins was agreeable to all stakeholders. This, combined with the CODEX standard [106], defining the method for determining gluten as an ELISA method using a specific antibody (R5 Mendez method), which effectively redefined gluten at the analytical level as anything detected by the R5 antibody, resulted in any higher order protein reference method based on IDMS being unnecessary. A series of harmonization materials were developed by the community, and these have been the subject of "label free" protein/peptide MS method [107].

The increase in the prevalence of food allergens, especially among children, has resulted in food labelling laws that require the indication of the intentional presence of, normally, 14 priority foods that are associated with triggering allergic reactions in the wider population. Of these 14 priority foods, 13 result from the presence of proteins or specific groups of proteins present in the offending food. Similar, to gluten, current food processing methods can result in the unintentional presence of these foods in processed foods. However, even trace amounts of these proteins can trigger a severe anaphylactic reaction in an allergic individual. This resulted in, what many consider to be, the overuse of "may contain" labelling for many of these priority foods. The impact of this on allergenic consumers has often resulted in a severe limitation in the ability to confidently consume processed foods. Unlike gluten, many of the ELISA test kits targeted the same protein or group of proteins from the offending food. However, the medical testing of thresholds, whereby the minimum eliciting dose of the offending food, is based on the mass of the total allergenic food protein. These thresholds are continuously updated and refined by an expert group under the VITAL scheme and have resulted in reference doses (based on total mass of allergenic food protein consumed per serving size) below which most allergic consumers will not have any adverse reaction [108].

As most analytical methods detect specific proteins, it is important that these are converted to "total allergenic food protein" in order to assess the real consumer risk. An assessment of the current analytical methods for use with VITAL thresholds was conducted in 2020 [109]. During this review the authors reported that one of the biggest challenges in assessing the methods was that few reported limit of detection or quantification in total allergenic food protein but instead referred to single protein analytes. It is important that any RMs to support this sector are materials that support method development and trueness controls, not only for the chosen protein analyte but for the total food allergenic protein [110]. Therefore, any IDMS methods would need to provide reference values for the individual target proteins as well as conversion of these values to "total allergenic food protein." This has focused the developments of peptide-based IDMS methods for food allergens in the late 2010 s and early 2020s. As peptides were often also used as calibrators, a complete assessment of the extraction of the individual proteins from the RM matrix was essential [111]. This was followed by an assessment of the individual uncertainty contribution of each step from protein extraction, digestion to target peptides and conversion from mole content of the target peptides to constituent protein and from each constituent protein to total food allergen protein (Fig. 4). In total, 11 peptides from five separate proteins, which represented over 95% of the total milk proteome by mass were quantified. The uncertainty for the conversions was based on reported ranges of the individual constituent proteins in total milk protein [112]. This method has been applied for the characterization of a cookie matrix RM and in providing the target reference value (for the individual mass fraction of the target analyte protein as well as total allergenic food protein) for a EU proficiency testing scheme for allergen detection laboratories

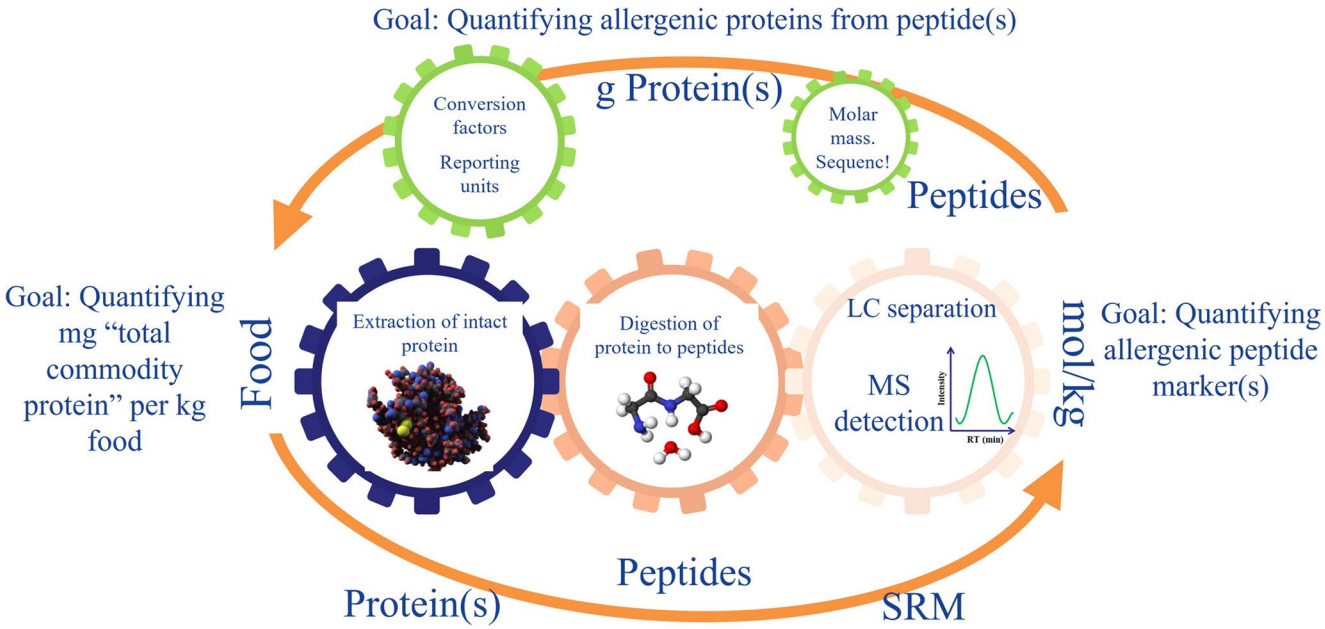

Fig. 4 Workflow for the quantification of "total food allergen protein" outlining the extraction of proteins, the determination of protein amount content from the quantification of their constituent peptides, and the conversion of individual protein content to total mass fraction of food protein (adapted from [112])

[113]. Similar peptide-based IDMS methods have been used for the characterization of a chocolate-based clinically relevant RM, whereby the same material preparation methods were used to prepare materials for clinical testing of allergenic thresholds in allergic patients as well as the detection of multiple priority allergenic food proteins via multiplexed peptide quantification methods using IDMS [114].

Outlook: future challenges

As previously underlined, CRMs are an essential part of the traceability chain, providing the basis for comparable and reliable protein measurements. Both pure materials as primary RMs and matrix-based RMs are required to allow traceability from the results of routine methods such as immunoassays via RMP to the SI. This requires a thorough characterization of the materials regarding measurand identity, purity and, in case of matrix RMs to be used in routine analysis, commutability. Although for some analytes the traceability chain could successfully be implemented, there are still analytes that show discrepancies between different measurement principles, including MS-based RMPs and immunoassays, and resist standardization mainly because the measurand seems not to be the same. This may raise doubts about measurement results and may result in improper and serious consequences, such as incorrect clinical decisions. There could be several reasons for these discrepancies: the targeted protein structure varies between methods, only proteins with certain PTMs are recognized, or proteins form aggregates in biological matrices. However, to date, most of the developed RMPs determine the protein amount based only on the protein's primary amino acid sequence without taking PTMs into account. Thus, the modifications, the whole structure of the protein, and its interaction and activity should be addressed in the future. This will require a multidisciplinary approach and the development of accurate methods for characterization of the whole protein structure (primary to quaternary) and the evaluation of protein-protein interactions in the hope of establishing traceability to the SI.

For structure analysis, classical techniques such as X-ray crystallography, NMR, small angle light scattering, or electron microscopy, including cryo-electron microscopy, can be applied to proteins [115]. However, they cannot capture solution-phase dynamics across multiple timescales or detect rare yet functionally critical conformers. In addition, these methods require large amounts of material and have limited resolution. In the last two decades, MS has emerged as a complementary technique, offering unique insights into protein structure (e.g., composition, stoichiometry, residue level proximity, solvent accessibility). Various structural MS methods, such as ion mobility MS (IM-MS), hydrogen/deuterium exchange MS (HDX-MS), chemical cross-linking MS (XL-MS), and top-down MS, have emerged. The advantage of MS is that it can be used to address PTMs, interactions, and the entire protein conformational landscape, allowing visualization of dynamic structure with high sensitivity. Recent advances in MS technologies and bioinformatics

have enhanced protein structural analyses by academic researchers [116–121].

HDX-MS, for example monitors the exchange of backbone amide hydrogens with deuterium from the solvent of D_2O [122]. In solvent-accessible regions, exchange proceeds at the intrinsic chemical rate, while residues involved in hydrogen bonding or buried in hydrophobic cores exchange far more slowly. After quenching, the protein is digested and peptide-level deuterium uptake is measured by LC-MS, generating a spatial map of structural protection. This allows the investigation of protein-ligand and protein-protein interaction, protein folding, and aggregation studies [115].

IM-MS provides useful information on protein dynamics in the gas phase and is a powerful method for the separation of conformers, especially in its high-resolution version. Collision-induced unfolding (CIU) by IM-MS can report on the stability of a protein but also provide an unfolding fingerprint that can be used as an additional means of protein characterization. By measuring the unfolding of proteins in the gas phase, it is possible to understand important features such as the preferential binding of different sub-units within a complex, subclassification of isoforms, and in-depth conformational characterization [123].

XL-MS enables the identification of residues that are close in three-dimensional (3D) space. This approach provides medium-resolution information to guidede novo structure prediction, protein interface mapping, and protein complex model building. Furthermore, XL-MS has a high potential for quantitative studies for differentiating protein conformational states, with the evaluation of the measurement uncertainty associated with the distances of linked peptides, the number of cross-linked peptides observed, the amount of cross-linked peptides, and the impact that cross-linking has on the original protein structure [124]. However, this approach is still limited by technical challenges, including the relatively low efficiency of the cross-linking reaction resulting in a low sensitivity of cross-linked peptides, the changes in cross-linked residue pairs (cross-links), and the complexity of the data analysis.

A current European project, EURAMET 23FUN06 ProMET, intends to investigate overall protein structures and their influence on measurement results and protein function and to develop a metrology framework and guidelines to better define the measurands. Further research is also needed to establish a link between the structure of a protein and its function as this might enable a better definition of the biologically active and, thus, clinically relevant form of the protein. Upcoming challenges are also the characterization of RMs for proteins in more complex systems such as viral vectors [115] or for a large set of proteins as required in (untargeted) proteomics to establish the traceability chain.

Up to now, targeted proteomic assays with results traceable to the SI have been developed for individual proteins or small multiplexed panels. However, these RMPs target only one protein or at best a handful of proteins at a time. For the comprehensive characterization of a biological sample, ideally all proteins above detection limit are quantified in a single LC-MS/MS analysis. The entire set of proteins in a biological entity is called the proteome, which varies with genetics, environment, diet, health status, and many other factors. Current approaches in research enable the identification of thousands of individual proteins, and their relative quantities can be determined with an appropriately designed experiment, with quantitative proteomics currently widely understood as ratio measurements of different samples [125]. However, an absolute quantification of the different proteoforms, which would allow results independent of the benchmark used, is currently hardly possible, because a calibration specific for each proteoform is not feasible. Thus, there is at the moment the lack of a metrological framework to support highly multiplexed measurements with absolute quantification. Such a framework will help to translate proteomics into routine use in clinical diagnostics [126], animal health [127], food assessment [128], biopharmaceuticals [129], and pharmacology [130]. This fundamental need has also been recognized by the international metrology community. A workshop on "Metrology for (multi)-omics measurements in clinical diagnostics" was organized by the CCQM and the need for RMs and methods for the standardization and calibration of omics approaches such as proteomics in personalized medicine has also been included in their 2030+ strategy. A first step in the direction of standardization of proteomics is the NIST RM 8321 "Peptide Mixture for Proteomics" meant to be used as calibration material in MS-based proteomics research. A step further has been taken by Zheng et al. who prepared the so-called Chinese quartet as multi-omics RMs, including matched DNA, RNA, protein and metabolites with results based on ratio measurements [131] intended to be used also in routine omics measurements.

Acknowledgements Steve Wise has dedicated five decades to the development of reference measurement procedures for the production of matrix reference materials for environmental and clinical monitoring. These methods, largely based on chromatographic separation coupled with mass spectrometry and isotope dilution calibration, were applied to classes of related organic molecules across a wide range of complex matrices.

The methods he developed, and many of the matrices he helped to characterize, became a learning ground for many NMIs seeking to establish their own national metrology-in-chemistry programs. They were also selected as test materials for the early international comparisons of the CCQM Organic Analysis Working Group. Steve played a central role in assessing the success of these studies and in tutoring new NMIs and Designated Institutes in the successful application of IDMS calibration methods for determining amount-of-substance content in complex matrices.

For Steve, both the measurand and the matrix mattered. Whether the material was serum, sediment, or tissue, each presented unique analytical challenges, and Steve understood these in depth. His willingness to share his experience, provide guidance, and

propose and design studies that challenged participants made his contributions invaluable to the international metrology community. This work formed part of the foundation on which IDMS methods for proteins were later developed. Before we began our own activities, we discussed with Steve the development of IDMS approaches, the assessment of interferences, and the contribution of matrix effects to the overall challenge of applying IDMS methods. His insights were invaluable, as were his enthusiasm and encouragement that we publish our findings in peer-reviewed journals as soon as possible.

The use of IDMS for characterizing matrix certified reference materials is now an accepted and viable approach for many reference material producers. Steve's support in making this possible is deeply appreciated by all involved.

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Author contributions Claudia Swart compiled all contributions to a manuscript and researched and wrote the "Introduction" to section on "Challenges for IDMS for characterization and value assignment in matrix RMs".

Gavin O'Connor researched and wrote section on "Food and food products".

Cristian Arsene contributed to the sections "Amino acid analysis–based LC-IDMS in value assignment of primary calibrators" and "Signature peptide–based LC-IDMS for peptide and protein purity assessment" as an expert for method development.

Bernd Güttler is responsible for the description of the historic development of protein analysis and metrology in this area.

Luise Luckau contributed mainly to the section "Major challenges in IDMS for proteins".

Milena Quaglia wrote the section on "Biopharmaceuticals".

David Bunk and Karen Phinney are responsible for the history of IDMS used for metrology in CCQM in the "Introduction" and Steve Wise's contributions as close former colleagues.

Liesbet Deprez and Guy Auclair contributed as experts in the production of CRMs to the section "Challenges for IDMS for characterization and value assignment in matrix RMs" from the RM producer side.

Qinde Lui contributed to the section "Major challenges in IDMS for proteins".

Liqing Wu is responsible for the sections "Other isotope dilution strategy beyond LC-IDMS" and "Applications of IDMS for peptide and protein purity assessment" and contributed to the outlook.

Vincent Delatour researched and wrote the section "Clinical requirements".

Amandine Boeuf is mainly responsible for the section "Outlook: future challenges", especially for the protein structure as coordinator of the project 23FUN06 ProMET.

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Declarations

Conflict of interest The authors declare no competing interests.

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