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# Should LC-MS/MS Be the Reference Measurement Procedure to Determine Protein Concentrations in Human Samples?

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## Introduction

The harmonization of laboratory results is one of the most important ongoing efforts to improve the practice of laboratory medicine and clinical chemistry. While agreement of results between laboratories is a laudable goal, the extra step of standardization can help investigators and practitioners build systems to achieve unbiased truth in measurements, which is particularly important for assays that have specific cut-points that dictate patient care. But to get there, there is a need for reference measurement procedures (RMP) and reference materials (RM) that can be used by the in vitro diagnostic industry and the creators of laboratory-developed tests to better define and understand their targets.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been the cornerstone of standardization for small molecules for many years, but it is now becoming more prominent for proteins. Examples of established RMPs for proteins include hemoglobin A1c, C-peptide, and Aβ1–42. However, issues around the consistency and completeness of proteolysis in traditional clinical proteomic assays, as well as the complexities surrounding calibration, have hampered many efforts to develop solutions for proteins. In addition, given the specificity of LC-MS/MS over immunoassay, the fundamental conundrum that has plagued biochemistry for decades has become even more relevant: what is a protein? Is it a specific isoform with a uniform amino acid sequence and consistent post-translational modifications (PTM)? Or is it a family of isoforms that contain polymorphisms and variable PTMs?

Here, 5 experts in proteomics and metrology consider whether LC-MS/MS has advantages over

immunoassay in serving as RMPs for proteins and discuss the issues that still face a field interested in providing unbiased results with LC-MS/MS assays for proteins.

## *Are there advantages of using targeted mass spectrometry over immunoassay in the development of reference measurement procedures for proteins?*



**Christa Cobbaert:** Yes. In contrast to immunoassay-based RMPs used for protein quantification, targeted mass spectrometry does not necessarily require specific antibody reagents and is characterized by high selectivity of the analyte, large capacity for multiplexing (multiple peptides per analysis) and

rapid, cost-effective transition from assay development to deployment. The concept of Selected Reaction Monitoring/Multiple Reaction Monitoring utilizing triple quadrupole mass analyzers provides inherent reproducibility and unparalleled sensitivity and selectivity to efficiently differentiate isoforms, PTMs, and mutated forms of proteins. Several RMPs based on targeted mass spectrometry have been developed, or are in the process of being developed, as RMPs to enable standardization of routinely used medical tests (e.g., hemoglobin A1c, hemoglobin A2, C-peptide, insulin, and human growth hormone).

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**Mark Lowenthal:** I agree, targeted mass spectrometry (MS) maintains several advantages over immunoassays for developing protein RMPs: specificity, the ability to use isotope dilution, multiplexing, and traceability. An RMP must be able to assess and validate measurement trueness and analytical bias, while also being unvarying over

time. Immunoassays often suffer from nonspecific antibody binding, decreasing our confidence in measurement trueness. It is well-known that immunoassays may undergo autoantibody interference to varying degrees between subsets of patient populations (e.g., thyroglobulin), leading to quantitative bias (i.e., false negatives). In some cases, immunoassays may not be available if the proper antibodies haven't been or can't be developed. Lastly, immunoassay traceability is challenged by matrix effects and a lack of internal normalization of the measurement system. In contrast, direct detection of analyte mass-to-charge by targeted MS, is unvarying (assuming proper instrument calibration). The high specificity of MS permits for proteoforms to become distinguishable within the RMP and targeted MS assays can claim direct traceability to the International System of Units (SI) through an appropriate double isotope dilution calibration system (with internal normalization and external calibration).



**Neil Kelleher:** I also absolutely agree. Lower propensity for bias or interference along with higher quantitative agreement across laboratories are just a few reasons. Many ELISAs are highly refined, yet for some applications they can give erroneous results at unacceptable rates. Of course, we can view the re-

search assays differently that do not need to perform 100% robustly, but one wants extremely high reliability for RMs, their standard operating procedures, and assays!



**Vincent Delatour:** I also agree, but achieving the lower limit of quantification needed to meet medical needs can be challenging for MS-based methods. Additionally, traceability to SI units is sometimes not possible (e.g., when it is not possible to formulate or assess a pure standard). In those cases, harmonization

through immunoassays may be the only alternative, and given the number of analytes for which traceability chains are urgently needed, it could be considered more pragmatic, or even necessary, to make some compromises and resort to harmonization protocols that rely on consensus target values from immunoassays. However, to be clear, in my opinion, standardization with full traceability to the SI units should always be preferred and pursued to the greatest extent feasible.

**For reference measurement procedures, is it appropriate to consider using proteolysis-aided approaches to protein quantification or should we instead focus only on the quantification of intact proteins?**

**Neil Kelleher:** Given the current landscape of the field, yes—and further—we should be doing both. One of the big sources of analytical variation is the digestion step itself, and for RMPs, controlling for that in the laboratory is important; intact proteoform quantification could really help us understand digestion variability in routine assays.



**Chris Shuford:** Yes. For the majority of protein measurements currently used in clinical medicine, proteolysis-aided workflows would likely be the preferred methodology for an RMP. Given that the proteoform or collection of proteoforms being measured is often not well defined in the immunoassay serving as the standard-of-

care, it is presumed that a “total” analyte measurement is most likely being made in those measurement

procedures. Except in cases of small proteins with minimal well-characterized posttranslational processing, “total” analyte measurements would be exceedingly challenging to achieve using an intact workflow, because each of the several proteoforms would need to be discretely quantified and then summed.

**Vincent Delatour:** Although recent developments in instrumentation now make it possible to perform absolute quantification of intact proteins (top-down strategy), proteolysis-based protocols (bottom-up strategy) remain the most frequent approach and these are generally fit for purpose, under conditions in which metrologically sound protocols are employed. If peptide-based calibrators are used, the main limitation of proteolysis-aided approaches is that these require the demonstration that digestion consistently proceeds to completeness or that the digestion yield and its reproducibility are very well characterized. Using protein-based calibrators as primary standards can help overcome this issue, in the instance that recombinant proteins and their labeled homologues really behave like the endogenous protein.

**Christa Cobbaert:** The practice of quantifying proteins by the peptide fragments that are derived from their enzymatic proteolysis (digestion) using LC-MS/MS and calibration with peptide standards is appropriate for protein quantification in the case that 3 important criteria are met: (a) the selected peptides and labeled internal standard must be stable throughout digestion, (b) the selected peptide standards must be demonstrably pure, and (c) the proof of equimolar release of the selected peptides. Of course, the accuracy of protein quantification by proteotypic fragments has to be evaluated thoroughly by comparison of peptide results to those obtained for the same intact protein sample by amino acid analysis. The rate of cleavage (and thus the digestion protocol used) turns out to be crucial to the quality and accuracy of results in protein quantification using enzymatic fragments.

**When developing a reference measurement procedure, the quantity intended to be measured, or the measurand, must be identified. If we accept that the specification of a measurand in laboratory medicine requires knowledge of the kind of quantity (e.g., mass concentration), a description of the matrix carrying the quantity (e.g., human plasma), and the chemical entity involved (i.e., the analyte), then what is the measurand in a proteolysis-aided assay?**

**Christa Cobbaert:** In a proteolysis-aided MS assay, the measurand is the selected quantifying proteotypic peptide, in molar units. Of course, the quantifying peptide

should be proteotypic and well-chosen so that these are representative of the molar concentration of the protein intended to be measured, and not confounded by, e.g., (size) polymorphisms, genetic variation, or PTMs. Confirmation of the protein concentration derived from the quantifying peptides, is needed through cross-checking and calculation from one or more qualifying peptides.

**Chris Shuford:** I would answer this question a little differently. It seems to me that the measurand defined by any proteolysis-aided workflow is the total concentration of all proteoforms in the matrix of interest that contain the indicated surrogate peptide(s). This is easily conceptualized when using a single surrogate peptide; however, when using multiple surrogate peptides to achieve a more precise definition of the measurand, as may be desirable for an RMP, the quantity yielded works under the assumption that all surrogate peptides have equivalent stoichiometry among all the collection of proteoforms, such that all surrogate peptides are expected to represent the same collection of proteoforms and produce the same “total” quantity.

As such, careful consideration should be taken when selecting surrogate peptides—particularly for an RMP. If one surrogate peptide, “peptide A,” is found in 4 out of 5 proteoforms, while the other surrogate peptides are found in 5 out of 5 proteoforms, then peptide A would be expected to yield a lower quantity than the other surrogate peptides because peptide A does not define the same collection of proteoforms. This should be straightforward to determine empirically, by comparing the quantities derived from each individual surrogate peptide and observing which ones are in agreement. To eliminate ambiguity in what collection of proteoforms are comprising the “total” measurement, the definition of a measurand for a proteolysis-aided workflow should also include the sequence of the surrogate peptides monitored.

**Neil Kelleher:** I agree, when using a peptide-based assay, the measurand is the set of digested proteins (e.g., from plasma) that contain the targeted peptide(s). One must accept all the ripple effects of digestion when doing peptide-based assays, which includes the inference step back to what endogenous proteoforms might be present in the original sample of intact proteins.

**Vincent Delatour:** It should be kept in mind that the measurand is what is intended to be measured, not what is actually measured. So, in some way, the measurand is actually the protein and what is measured is one or more peptide (potentially from various isoforms).

***What is the most appropriate calibration system for a reference measurement procedure that uses targeted mass spectrometry to quantify a protein?***

**Mark Lowenthal:** Double isotope dilution (ID) techniques. ID requires that stable isotopically labeled analogs (SIL, commonly  $^{15}\text{N}$  or  $^{13}\text{C}$ ) for each targeted analyte be used as an internal quantitative control. Double ID techniques spike SIL into external calibration standards in addition to the testable samples—the SIL analogs act as normalization factors for sample preparation and instrumental drift. The quantitative trueness of the RMP hinges on the quality of the calibration standards that are available. Only traceable, commutable, higher-order standards with estimated imprecision should be considered, preferably a Certified or Standard Reference Material from a metrological organization or institute. The quantitative precision of the analytical portion of a double ID MS workflow can be <1%, but only for an ideal case.

For quantifying a protein, the calibrators should be in a matrix as similar as possible to the testable samples, and SIL should be spiked as early as possible within the sample preparation procedure. While in a top-down MS analysis, the analyte is the measurand [i.e., what is detected is the proteoform(s) of clinical significance], in a proteolysis-aided analysis the analyte(s) are peptide surrogate(s) of the measurand(s). For immunoenrichment schemes, internal standards must be SIL analogs of the measurand (intact proteoforms). However, for minimally processed (but proteolyzed) samples, SIL peptide surrogates suffice.

**Christa Cobbaert:** According to ISO 17511, metrological traceability to SI is the most complete calibration hierarchy that enables the highest order of metrological traceability of test results. To standardize protein measurements, we will have to choose between pure value-assigned peptides or intact proteins, which could both be used as the primary RMs in LC-MS/MS assays. In my opinion, peptide-based primary RMs are preferred when the intact proteins are complex and large, whereas intact protein-based primary RMs may be preferred in the case of small well-defined proteins. In both cases, purity has to be assessed and amino acid analysis composition has to be determined with internationally recognized procedures for amino acid value assignment.

**Chris Shuford:** If the ultimate goal of a RMP is to produce matrix-based, secondary RMs that are likely to be commutable with patient samples across multiple laboratory measurement procedures (so as to enable/foster interlaboratory agreement), then the calibration system for a RMP is one that should be highly reproducible above all other things. As such, the calibrant for a RMP

should ideally be a primary reference material that is available in sufficient quantities to last in near perpetuity and is stable for the duration of use. To provide the greatest chance of the RMP calibration system being commutable with patient samples, it would also be desirable if the calibration system used a calibrant comprising all proteoforms defined by the measurand and, if the calibration system was prepared in the same matrix defined by the measurand. However, use of such a “prototypic calibration system” would almost necessarily require the use of patient specimens (or pools thereof) to create the calibration system, which may be in opposition to the paramount requirement for availability and stability.

**Vincent Delatour:** Both peptide-based and protein-based calibrations are suitable calibration strategies, as long as the purity of primary calibrators can be accurately and consistently determined. The current state-of-the-art is mature enough to properly determine purity of peptide calibrators of reasonable size. Although protein-based calibrators are preferable, purity assessment is more difficult because impurities will generally be larger and thus more challenging to be identified and accurately quantified. The most frequent approach is therefore to further purify protein-based calibrators by several orthogonal purification strategies. It should be kept in mind that primary calibrators are only intended to calibrate the MS RMP; the MS RMP is then used to value assign panels of clinical specimens or secondary calibrators with which routine assays will be calibrated.

***How will we ensure the accuracy of reference measurement procedures that use mass spectrometry for the quantification of proteins? Are there specific experiments that should be included during development and validation?***

**Vincent Delatour:** The most frequent approach used to demonstrate accuracy of RMPs that use MS is to conduct recovery experiments. Typically, a blank sample (matrix material) is spiked with a known amount of a protein-based primary calibrator and the concentration measured with the RMP is then compared with the theoretical concentration determined by gravimetry. When blank samples are not available, the standard addition approach can be used: the matrix is spiked with increasing amounts of a protein-based primary calibrator and the concentration determined by linear regression analysis is then compared with the concentration measured with the RMP.

When these approaches are not applicable (e.g., in the absence of a well-characterized primary calibrator), accuracy can be validated thorough interlaboratory comparisons between laboratories that have developed

candidate reference methods by mass spectrometry. Although demonstrating close agreement between results does not rule out that results of the different laboratories could all be biased, it remains the best indication that the results are accurate. What is clear is that accuracy of a mass spectrometry RMP cannot be demonstrated through a comparison with routine clinical assays.

**Mark Lowenthal:** The RMP must contain a clear definition of the measurand. The biggest challenge is then ensuring availability of proper RMs—often the limiting factor. Next, the RMP must ensure that the appropriate calibration system is implemented. The RMP should be thoroughly assessed for all potential sources of bias (known and unknown), and it should be validated to measure the measurand. The calibrators must be deemed fit for purpose and have a clearly established traceability chain. Comparability to orthogonal techniques is informing, but not sufficient. Interlaboratory comparability studies are essential, with agreement over time, between instrumentation, and experts, and through the use of commutable RMs.

**Chris Shuford:** Accuracy of RMPs should be verified in the context of the defined measurand. For RMPs using intact workflows to quantify a discrete proteoform, validation studies would have the same expectations as any RMP for small molecules. However, for RMPs using proteolysis-aided workflows, it would be ideal if each surrogate peptide used in defining the measurand was independently validated (i.e., for interference, precision, linearity, parallelism, etc.). Further, the quantities derived for each surrogate peptide should be compared within patient samples (from the expected testing population). Results for each surrogate peptide used to define the same measurand should be in close agreement (i.e., demonstrate 1:1 stoichiometry) to infer each surrogate peptide is describing/quantifying the same collection of proteoforms. Discordance from one surrogate peptide would indicate either: (a) posttranslational processing in patient samples that uniquely affects the surrogate peptide stoichiometry among the collection of proteoforms intended to be the measurand, (b) posttranslational processing in the primary reference material that uniquely affects the surrogate peptide stoichiometry of the RMP calibrant (which may also be a collection of proteoforms), or (c) posttranslational processing that effects digestion of the collection of proteoforms comprising the native measurand and/or RMP calibrant.

#### **Will we always need liquid chromatography?**

**Mark Lowenthal:** For the foreseeable future, yes. But assuming instrumentation continues to improve as in

recent years (i.e., dynamic range, sensitivity, and resolution), it's possible that targeted MS in the clinical laboratory could become LC-free in some unique situations. We are not there yet.

**Neil Kelleher:** No, for 2 reasons: (a) we are seeing very good performance with capillary electrophoresis, and vendors are spending more time on product and market development, despite the relatively small market at present and (b) we have seen good performance for proteoform-specific assays using ion mobility spectrometry with direct infusion of moderately complicated mixtures (e.g., provided by immuno-capture) and cleaned up on-line with size-selection technologies. It will take some time for approaches like these to mature, but lower cost per assay and less training for those at the bench are drivers for the future.

**Chris Shuford:** For RMPs, it is probably prudent to always use liquid chromatography given the added selectivity it is likely to provide. That said, liquid chromatography is simply a tool used to overcome any shortcomings in selectivity of the mass analyzers and there are already examples in which selectivity of the mass analysis is sufficient without LC separations, particularly for intact analyses of large proteins by high resolution MS in which isobaric interference is less common after some other enrichment step (e.g., immunoaffinity purification). Indeed, there is likely to be some movement away from LC interfaces in laboratory measurement procedures given they significantly limit the throughput of analyses.

**Vincent Delatour:** Not necessarily. Liquid chromatography is obviously a wonderful tool to reduce sample complexity and decrease ion suppression in the mass spectrometer but other approaches could perfectly be suitable. For example, capillary electrophoresis is increasingly used and asymmetric flow field-flow fractionation is another example of a separation technique that could potentially be employed prior to mass spectrometric analysis.

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**Nonstandard Abbreviations:** RMP, reference measurement procedure; RM, reference material; MS, mass spectrometry; PTM, posttranslational modification; SIL, stable isotopically labeled.

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