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Quantifying Proteins

Quantifying Proteins by Mass Spectrometry
Mark W. Duncan, Alfred L. Yergey, and P. Jane Gale

Quantifying Small Molecules

Quantifying Small Molecules by Mass Spectrometry
P. Jane Gale, Alfred L. Yergey, and Mark W. Duncan

Quantifying Biopharmaceuticals

In Biological Samples

Advances in Liquid Chromatography–Tandem Mass Spectrometry (LC–MS–MS)–Based Quantitation of Biopharmaceuticals in Biological Samples
Nico C. van de Merbel
The quantification of proteins in a complex biological sample is an important and challenging task. Mass spectrometry (MS) is increasingly used for this purpose, not only to give a global survey of the components and their amounts, but also to precisely and accurately quantify specific target proteins. Here, we review the essential elements of MS approaches to protein quantification and critically compare the available options.

Proteins are the most abundant macromolecules in biological systems. Together with their smaller relatives, peptides, they are polymers comprising amino-acid building blocks joined through amide bonds. In contrast to the repeating units of other biopolymers (for example, polysaccharides and polynucleotides), the constituent amino acids are diverse in their chemical and physical properties. Consequently, the polymers derived from them are also a complex, chemically and physically diverse ensemble.

This structural diversity lends itself to extensive functional diversity. Proteins serve as antibodies, enzymes, messengers, structural components, and transport or storage molecules. For that reason, the majority of drug targets are proteins.
Significantly, the genetic machinery of the cell is tasked with synthesizing proteins. Accordingly, one might argue that much of each cell, and therefore any organism in toto — its structure, function, reproduction, repair, and regulation — relies on proteins. Understanding biology — function or dysfunction, health or disease — is therefore about defining and understanding proteins.

Protein identification and quantification are thereby the two central objectives of many biological and biomedical studies. Historically, these tasks were performed on purified proteins that were exhaustively sequenced (such as Edman) or quantified by the immuno-based western blot or enzyme-linked immunosorbent assays (ELISAs). Today, however, because of advances in mass spectrometry (MS) and the development of a set of global, protein-analysis tools that some call the “proteomics toolbox,” improved analytical strategies have evolved, and the objectives of researchers have changed. Typically, investigators now aim to study biological entities at the “systems” level; that is, they seek to resolve and identify a multitude of proteins simultaneously in a single sample and to quantify each in relative or absolute terms. (See definitions in Table I.)

Quantification is an important component of most studies. Defining differences or changes in protein abundances (or, more appropriately, the abundance of specific protein species including isoforms and post-translational variants) between two or more groups or states (such as control and test) is often at the heart of understanding function and regulation.

As the proteomics toolbox evolves, new approaches to protein quantification by MS are continually reported. These methods can be categorized into several major classes, all of which share features and performance characteristics. Furthermore, though some additional considerations are specific to protein quantification, it is also important to state that the process of quantification remains essentially the same, regardless of the nature of the analyte (that is, small molecules versus biopolymers). Consequently, the principles and practices that have guided the development and evaluation of quantitative methods (for example, replicate measurements to characterize the variance of a method) are no less applicable in this setting.

Non-MS Approaches to Protein Quantification
This article focuses on MS methods for protein quantification because of their growing importance. Yet it is important to acknowledge the existence of other strategies and that, moreover, in certain settings those alternatives may be the methods of choice. For example, the mainstay for targeted protein quantification for almost 50 years has been the western blot immunoassay, in which antibodies are used to detect
Quantifying Small Molecules

Proteins transferred from polyacrylamide gels to nitrocellulose or polyvinylidene fluoride membranes. Refinements of the basic protocols yield detection limits in the attomolar range (1). Evolution of the principles underlying the western blot led to the development of the radioimmunoassay (RIA) and ELISA (2,3). In a clinical setting, specific proteins are commonly quantified by ELISA. Similarly, for several decades, quantification of multiple protein components in complex biological samples has relied on two-dimensional (2D) gel electrophoresis (discussed in some detail later). Additionally, a growing array of multiplexed, selective capture methods, including aptamer and antibody arrays, are increasingly used for protein quantification. Numerous reviews discuss these and related strategies (4–9).

### MS Approaches to Protein Quantification

#### An Overview of the Approaches

We categorize protein quantification by MS into several groups. These are outlined below, represented in Figure 1, and discussed in more detail in the sections that follow.

**Global, System-Wide (Multicomponent) Strategies**

Here, hundreds or thousands of proteins are compared in two or more samples and quantification is typically relative. Nevertheless, with modification to the basic method, absolute quantification is sometimes possible.

**Protein-Centric or Top-Down Strategies**

These approaches involve resolution of a complex mixture of proteins (for example, by 2D gel electrophoresis) and quantification in their intact form. The approach often involves differential radio- or chemical-labeling of proteins in distinct samples (such as, difference gel electrophoresis [DIGE]). After tagging, the samples are combined, the proteins are resolved in two dimensions, and the relative amounts of the tagged proteins are measured. Protein spots are excised from the gel, digested, and identified by MS by means of peptide mass maps (that is, peptide masses), peptide sequences

### Table I: Some essential definitions

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Relative quantification</td>
<td>Relative quantification strategies compare the levels of individual proteins in a sample to those in another sample. Results are typically expressed as a relative fold change, or percent change, of protein abundance.</td>
</tr>
<tr>
<td>Absolute quantification</td>
<td>Absolute quantification is the determination of the amount, in units of mass or concentration, of a protein in a sample. For example, protein X expressed in units of nanograms per milliliter of plasma.</td>
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(that is, tandem mass spectrometry [MS-MS]), or a combination of both techniques.

**Peptide-Centric, Bottom-Up, or Shotgun Strategies**
Here, a complex mixture of proteins, an extracted proteome, is digested to peptides. The peptides, which serve as surrogates of the original intact proteins, are then separated, quantified, and identified. These strategies are further subdivided into label-free strategies and labeled strategies, depending on whether a label is incorporated:

**Label-Free Strategies:**
- Isolate proteins in a sample → proteolysis → separate peptides → sequence peptides → identify protein → repeat procedure for additional samples. We then compare either the number of peptides recovered for each protein (that is, spectral counts) or relative abundances of specific peptide ions (that is, peptide peak intensities by liquid chromatography–mass spectrometry [LC–MS]) to quantify proteins.

**Labeled Strategies:**
- This involves differential metabolic labeling with stable isotopes of proteins in two or more samples → combine samples → isolate total proteins → proteolysis → separate peptides → quantify differentially labeled peptides → sequence peptides and identify proteins (for example, stable isotope labeling by amino acids in cell culture [SILAC]).
- Differential chemical labeling with stable isotopes of proteins in two or more samples → combine samples → isolate total proteins → proteolysis → separate peptides → quantify differentially labeled peptides → sequence peptides and identify proteins (for example, isobaric tags for relative and absolute quantitation [iTRAC]). (Note the similarities of this approach to DIGE. The primary difference is that separation and quantification are performed on surrogate peptides, not intact proteins.)

**Targeted (Single-Component or Several-Component) Quantification Strategies**
In these approaches, one or a few components are selectively isolated from a sample and quantified in relative or absolute terms. Approaches fall into two categories, top-down and bottom-up.

**Top-Down Approach**
Direct quantification by matrix-assisted laser desorption-ionization (MALDI) or protein isolation–concentration by an approach such as mass spectrometric immunoassay (MSIA, Thermo Fisher Scientific): Selective isolation of one or more proteins → determine protein abundances based on ion current. Protein identification or selectivity is derived from antibody and mass of target protein;
amount is based on ratio of peak heights/areas for analyte and an internal standard. Absolute concentrations are determined referring to a calibration curve containing a fixed amount of internal standard (IS) and varying amounts of the intact target protein.

**Bottom-Up Approach**

Multiple-reaction monitoring (MRM) methods including stable isotope standard capture with anti-peptide antibodies (SISCAPA). Approaches selectively isolate target protein or proteins → digest proteins → quantify one or several peptides according to parent-ion (MS) or product-ion chromatograms (MS-MS).

**General Considerations in Quantitative Proteomics by MS**

An abundance of reviews discuss protein quantification by mass spectrometry, but most focus on instrumental considerations (10–13). Furthermore, most authors have almost exclusively focused on electrospray ionization (ESI)-based approaches and have neglected the findings of precise and sensitive intact-protein quantification by MALDI-based methods. Our focus is on the overarching steps in system-wide protein quantification.

**Sources of Inaccuracy and Imprecision**

Multiple sample-manipulation steps are common before instrumental analysis (for example, protein precipitation or isolation, fractionation, selective depletion and enrichment, proteolysis, and tagging and labeling reactions). Each step is a source of pre-analytical sample variability that can compromise both precision and accuracy. For example, high-abundance proteins like albumin are sometimes removed from plasma samples by means of immunodepletion before analysis. However, that removal process introduces a risk of codepleting other components of interest because of nonspecific binding to both the antibodies used and to the albumin itself (by other sample components). Similarly, other steps, such as protein precipitation...
and enzymatic digestion, can introduce significant imprecision and inaccuracy because proteins are not recovered or digested quantitatively. Irreproducibility in other sample-handling steps, including chemical labeling, together with instrument perturbations (such as pressure and temperature fluctuations and tuning); the laboratory environment (for example, temperature and humidity); reagent variability; the presence of coeluted species or their levels; and analyte concentration can contribute to imprecision by altering the ionization process and thereby the measured signal intensity. Intensity comparisons are therefore compromised at a fundamental level. Studies that compare results across different analytical runs are most susceptible to these factors. Minimizing or carefully controlling key variables is critical. Yet even so, given the number of steps in the analysis and the number of species being measured, many potential sources of imprecision remain.

Validation of Proteomic Methods
It is important to remain mindful that the primary aim of any quantitative proteomics study is to provide timely, accurate, and reliable data that are fit for an intended purpose. Nevertheless, depending on the specific approach and the rigor with which the analysis is performed, the quality of the data will be variable and undefined. Quantifying thousands of components in a sample is a formidable challenge, to say the least. Defining specificity, linearity, accuracy, precision, range, detection limit, upper and lower limits of quantification, and robustness — all central considerations in the validation of a conventional quantitative analysis — is, given the scope of the task, empirically impossible for every protein species. Similarly, recovery and stability studies are not possible on each of the components. Consequently, in global proteomic studies, validation of the assay is typically perfunctory, and the resultant data are of uncertain and ill-defined reliability.

Although conventional validation is not practical, measures of precision and accuracy remain essential so that experimental findings can be put into context. Therefore, we must be confident that the measured differences are real and not merely an artifact of the method itself.

A method’s assessed precision for a subset of analytes measured, at various concentrations, in one or more test samples and the derived data, can be used to determine the method’s suitability. These data can also help validate subsequent findings derived from the method. (14). Similarly, technical replicates (that is, repeat analyses of each of the samples in the study) provide additional support that a change is real, not an artifact of the analytical method itself. In the same vein, the issue of specificity must also be considered. Accurate quantification cannot be assumed on the basis of one — or even a
few — peptides simply because a single peptide defines only a single segment of any protein, and modifications elsewhere in the molecule are missed (14,15). Quantification based on a peptide common to multiple, related forms will always lead to an overestimate of the amount of any single variant whereas quantification based on a unique peptide fails to “recognize” and quantify closely related variants of that protein, even if they are significantly more abundant. Precise and accurate quantification of a specific protein variant is therefore achievable only when the targeted peptide or peptides are derived from a single precursor protein or, in the case of protein-centric methods, in instances in which we can resolve and quantify the specific (intact) protein species without interferences.

The situation, however, is not as bad as it might seem at first. Because a common objective in proteomics is to compare groups — for example, disease versus control or control versus test — absolute levels are not (necessarily) important. Defining percent change (or difference) is the overarching objective. Therefore, it is possible to take advantage of differential (isotopic) tags and the exquisite selectivity of mass detection to compare two (or more) samples worked up and assessed in the same experimental run under identical conditions. For example, in a typical experiment all proteins in a sample (such as control) are labeled with a chemical tag; separately, all of the proteins in a second sample (such as disease) are labeled with an isotopic variant of the same tag. The samples are then mixed and treated as one. Thereafter, each tagged protein and its isotopic variant behave in an identical manner during sample handling. Yet because of their difference in mass, they can be specifically detected and quantified by MS. While this detection and quantification strategy markedly reduces variance in one sense, such an approach is limited to “A versus B” comparisons. Numerous modifications of the basic strategy have been developed and will be discussed in more detail later in this installment.

Global or Proteome-Wide Protein-Centric Quantitative Tools

2D Gel-Based Methods

Using 2D gel-based methods is the most frequently adopted top-down strategy, and it is based on quantitative analyses of intact proteins resolved via 2D gel electrophoresis. Typically, the first step is protein separation by isoelectric focusing (IEF; first dimension [1D]) and then orthogonal separation of the proteins distributed on the 1D strip by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; second dimension). Protein spots (that is, discrete species) are visualized by dyes, fluorophores, or radioactive labels tagged. (These can be visible or fluorescent post-electrophoretic dyes, or fluorophores or radioactive labels that
are tagged to proteins before resolution. Examples include silver stain, Pro-Q Diamond, 2,4-dinitrophenylhydrazine, or Coomassie blue.) The quantity of each protein is assessed by measuring the spot’s density using a customized software package. Two-dimensional gels provide excellent sensitivity, precision, and linearity over a wide dynamic range. Nevertheless, subsequent identification of proteins requires resecting, by hand or robot, the individual spots from the gel followed by MS (that is, a peptide mass map with or without additional MS-MS sequence information).

To minimize the influence of gel-to-gel variations, and to reduce the total number of gels required, variants of this general strategy involving differential labeling (or tagging) of the proteins in two or more samples have been developed (for example, with fluorophores or radioactivity labels). The objective of these approaches is to retain the physical properties of the proteins so that their mobility in each of the two dimensions of separation remains unchanged. Therefore, both can be run as a mixture on one gel, and each can be independently quantified, because of the tag.

The most common manifestation of this approach is DIGE (16). Here, three (or more) different protein-containing samples can be labeled with size-matched, charge-matched, spectrally resolvable fluorescent dyes (for example, Cy3, Cy5, and Cy2) before 2D gel electrophoresis. In this way, the number of gels to be resolved is reduced, precision is increased, and the time and cost of the comparison is also reduced. For example, running two samples on a single gel significantly increases reproducibility. Nevertheless, because three distinct “tags” are available, comparisons can also be made between multiple samples run across multiple gels by using a pooled internal standard (17). Composed of a mixture of all of the samples constituting the study, this standard is tagged and run on each gel. The pooled sample acts as an internal standard for every protein spot on each of the gels. As such, it is used to normalize all spots and to optimize inter-gel precision. Typically, reciprocal labeling (a dye-swap experiment) is performed to ensure the observed changes are not associated with dye-dependent interactions. Commercial software is used to detect differences and assign statistical confidence to them.

**Critical Evaluation**

With high precision, 2D gel electrophoresis and its variant, DIGE, allow relative abundance comparisons that detect modest changes of one to several thousand proteins in multiple samples. Provided they can be resolved, variant forms of the same protein (that is, protein variants or proteoforms) can also be independently quantified. Because each separation is visually represented, only proteins that differ in abundance need be resected from
the gel and identified. Two-dimensional gel electrophoresis and DIGE have been successfully adopted by many investigators. Both methods have been thoroughly reviewed and have stood the test of time (18,19). However, the approach is cumbersome, labor-intensive, and difficult to fully automate. These limitations, especially the last, have dampened the enthusiasm of many to adopt 2D gel electrophoresis (and DIGE). While it is frequently suggested that the resolution of a 2D gel is limited, it is important to stress that no other approach rivals 2D gel electrophoresis for practical, intact protein separation. Comigration of multiple proteins to the same location on the gel is also often cited as a problem, but it is rarely an issue (20). Similarly, although large (>150 kDa) or small (<5 kDa) proteins are difficult to separate on gels, this difficulty is not a significant limitation because work-arounds are available, at least for peptides. DIGE is subject to variations in the extent of labels incorporated into the proteins, a potential source of variance in the measurements. Identifying proteins is sometimes problematic, especially with DIGE. Alignment problems can occur because identification requires running a separate (preparative) gel and then matching it to images for the set of analytical gels.

Other Top-Down Strategies
In this review, we forego discussion of instrument-intensive, top-down approaches simply because they are not yet practical for routine use. As alternatives to 2D gel, top-down proteomic approaches are, however, being developed by several groups. These alternatives involve introducing intact proteins into a mass spectrometer and fragmenting them directly. By working with the intact protein, complete sequence coverage is possible, and post-translational modifications (PTMs) are preserved. Until recently, however, top-down proteomic strategies were restricted to the analysis of purified proteins or simple mixtures. Over the last few years the approach has been extended to complex mixtures of proteins (21), but quantification remains a challenge nonetheless.

Global or Proteome-Wide Peptide-Centric Approaches

General Comments
Peptide-centric (bottom-up or shotgun) quantitative strategies are dominant because of their purported ability to quantify multiple components simultaneously in an automated or semiautomated manner. These strategies involve a common step: that is, site-specific cleavage of a mixture of isolated proteins (a proteome) to generate a substantially more complex mixture of peptides. Typically, peptide-centric applications utilize trypsin and quantify based on tryptic peptides. The selective cleavage of proteins to peptides is undertaken for these reasons: Peptides
can be separated by LC better than proteins; most proteins generate one or more soluble peptides even if the antecedent protein is poorly soluble; peptides fragment better in a tandem mass spectrometer, giving spectra that can be sequenced; and peptides can be detected at much lower levels than their protein precursors. The peptides are then fractionated by LC and analyzed by MS-MS (14).

It is important to acknowledge, however, that all peptide-centric approaches to quantification are based on the assumption that when a protein is cleaved by a specific reagent, the reaction will go to completion, or at the very least, that the cleavage will be reproducible and predictable. Further, it is assumed that the target peptide or peptides are sufficient to define and selectively quantify the antecedent protein (that is, the target peptide is solely derived from a single antecedent protein). In practice, however, a target peptide or peptides may be degenerate and shared by multiple proteins. Through digestion, connectivity between the peptides and their antecedent protein is lost, a phenomenon referred to as the protein inference problem (22). In fact, multiple variants of a protein (variant protein species or proteoforms) are common, and unless peptides incorporating the specific, modified residue or residues (for example, the oxidized, reduced, nitrated, phosphorylated, glycosylated, or differentially “altered” amino acid) are targeted, quantification will be inaccurate. As a specific example, a single-point amino acid mutation may exist in a target protein, but if quantification is based on any tryptic peptide other than the one incorporating the modification, the variant will not be detected. Similarly, other variants of the precursor protein including truncated or alternatively spliced forms are often misidentified (15). On the other hand, if the focus is on identifying specific modifications, and the correct peptide is targeted, the peptide-centric approach offers advantages. The influence of a modification on mass is more evident at the level of the peptides than it is at the protein level because the percent change in mass is greater.

Relative quantification by peptide-centric methods can involve the separate analysis of multiple samples by MS and their subsequent comparison (for example, label-free methods). Alternatively, tags (such as isotopic tags or stable isotopes) can be incorporated into proteins or their proteolytic peptides. These cause a shift in mass of the labeled protein or peptides in the mass spectrum. Differentially labeled samples can be combined and analyzed together, determining differences in the peak intensities of the isotope pairs. These intensity differences correlate with differences in the abundance of their antecedent proteins.

With these overarching issues in mind, the approach can be implemented in many, disparate ways. This review
does not aim to be an encyclopedia of all available methods. Instead, it is a description of the basic divergent strategies and their strengths and weaknesses.

**Label-Free Approaches**
Label-free quantification is fast, cost-effective, and easy to implement. It is frequently used when stable isotope incorporation is impractical or cost-prohibitive. In these approaches, samples are analyzed separately and results from multiple runs are compared. The two main, label-free approaches each rely on proteolytic digestion of a sample followed by analysis by LC–MS or LC–MS-MS. Both strategies are used to make comparisons between two or more samples and to determine relative change in protein abundance (with the caveats noted above).

**Spectral Counting**
This is a practical, semiquantitative measure of protein abundance in proteomic studies. Relative quantification by spectral counting compares the number of identified spectra associated with the same protein between different samples — that is, the total number of tandem mass spectra that match peptides to a particular protein as a measure of protein abundance within a complex mixture. The approach is based on the finding that increasing protein abundance results in an increase in protein-sequence coverage. Therefore, abundant proteins produce more MS-MS spectra than less-abundant proteins, and their antecedent peptides are sampled more often in fragment-ion scans than those derived from low-abundance proteins. However, important caveats are associated with this approach. Low-mass proteins (that is, those generating fewer fragments on proteolysis) are problematic; the dynamic range of the approach is limited; precision is poor and, consequently, small changes in protein abundances are difficult to determine. Several modifications of spectral counting, including the normalized spectral abundance factor (NSAF) approach (23), have also been reported. NSAF corrects for the fact that larger proteins yield more peptides on digestion than shorter proteins and also accounts for sample-to-sample variations associated with replicate analysis. A modified spectral counting strategy, absolute protein expression (APEX) profiling, has been used to measure the absolute protein concentration per cell after the application of several correction factors (24). Further refinements have also been made and the approach was recently reviewed (25).

**Quantification Based on Peptide Peak Intensities as Determined by LC–MS**
This approach to quantification is based on the observation that for a specific peptide separated and detected by LC–MS, the measured ion current increases with increasing concentration. Typically, ion chromatograms for each peptide of interest are extracted from
Quantifying proteinS

an LC–MS run, and their peak areas are integrated over time. Peak areas for the same ion are then compared between different samples, to give relative quantification; absolute amounts can also be calculated by reference to a calibration curve. Most often, ion currents derived from the intact, protonated, peptide ions are monitored, but product ions generated by MS-MS can also be used for quantification. (Product-ion detection increases selectivity, but at the expense of sensitivity.) The approach and computational strategies to manage the data have been reviewed (26).

While the relationship between the actual amount of protein and generated ion current holds true for standard samples of limited complexity, in practice, the analysis of digests of complex biological samples is far more problematic. For example, variations in temperature, pressure, sample preparation, injection volume, retention time, and the presence of coeluted species can significantly compromise precision. (Studies often extend over weeks — or even months — and changes in column, mobile phase, instrument condition, and calibration begin to manifest themselves.)

Critical Evaluation of Label-Free Approaches

Label-free approaches are inexpensive and simple to implement, but the old aphorism, “You get what you pay for” may apply. They allow “semiquantitative” comparisons between samples, but precision and reliability is low, in large part because without an internal standard the measured ion current is susceptible to many factors when it is measured in many separate runs. In a recent study by The Association of Biomolecular Resource Facilities (ABRF), data generated from digests of parallel lanes of gel-separated proteins were supplied to several groups. The task was to “identify” the proteins in the sample and determine which were elevated or reduced in intensity relative to the adjacent lane. Not surprisingly, participants failed to agree, and there was no evidence that either approach — spectral counting or intensity-based quantification — could reliably address this question (27).

Labeled Approaches to Global Protein Quantification

General Comments

These approaches uniquely tag the proteins in two or more samples with a stable-isotope tag. The tagging can be done metabolically (that is, by adding enriched amino acids into cell culture medium) or chemically (that is, by covalently binding a labeled moiety to the proteins). The samples are then combined and analyzed in a single run. Precision is markedly improved because two or more samples are compared within one run, but at the expense of the time, cost, and complexity of the overall analysis. The earlier in the analytical process the label is incorporated into the proteins, the better,
but its (global) incorporation is far from straightforward.

**Metabolic Labeling**

In this approach to relative quantification, the proteins in two or more samples are labeled with isotopically distinct forms of amino acids by growing cells in enriched culture medium (for example, SILAC). The first report of this approach was by Ong and colleagues in 2002 (28). Typically, two populations of cells are grown in separate cultures, one in standard medium and the other in medium containing stable-isotope-labeled amino acids. After the samples are combined, the mass difference between proteins and their proteolytic peptides in the two populations can be detected by MS. The ratio of peak intensities in the mass spectrum for the labeled versus unlabeled forms reflect the relative protein abundances in the two samples. This approach delivers the highest precision because the label is incorporated before any analytical steps are undertaken, and it therefore accounts for sample handling biases through the whole analytical process. These advantages are in part offset by the cost of the strategy and the fact that the metabolic labeling approach is far from widely applicable. For example, it cannot be applied to the assessment of protein differences in biological fluids collected from human subjects.

**Chemical Labeling**

Because metabolic labeling is often not feasible, if a stable-isotope label is to be used, it must be introduced later in the workflow by chemically tagging peptides or proteins. Two basic strategies are commonly adopted, as discussed below.

*Isotopic Labeling*

The many variants of this general strategy all aim to add isotopic atoms or isotope-coded tags to peptides or proteins. Some are simple in concept whereas others combine multiple elements to react with differentially tagged and selectively recovered peptides. Once again, two separate samples are differentially tagged with isotopic labels, mixed, and analyzed. Labeling strategies include enzymatic labeling with 18O at the C-terminus of proteolytic peptides (29); global internal standard technology (GIST), in which deuterated acylating agents (for example, N-acetoxy succinimide [NAS]) are used to label primary amino groups on digested peptides (30); and chemical labeling with formaldehyde in deuterated water, to label primary amines with deuterated methyl groups (31).

Commercial isotopic labeling reagents are also available. The best known commercial option is, perhaps, the isotope-coded affinity tag (ICAT) method (32). Several iterations of ICAT tags have emerged. The first generation of the reagent comprises three separate parts: a sulfhydryl-reactive chemical crosslinking group, a linker, and a biotin entity. The reagent’s two versions are an unlabeled form and a heavy form.
incorporating eight deuterium atoms. The sulfhydryl-reactive group reacts with free thiols (that is, on cysteine residues); the biotin tag is used to selectively recover the tagged peptides (that is, through binding with avidin); and the linker provides the opportunity to differentially (mass) label two samples. Since not all proteins contain a cysteine residue, this approach is limited in that about 20% of the proteome may be missed. Furthermore, the incorporation of deuterium as the label is suboptimal because of a discernible isotope effect, which manifests itself as differences in retention time. A variant ICAT reagent incorporating 13C was reported several years later (33). A further refinement of the same basic strategy, isotope-coded protein labeling (ICPL), which tags lysine residues and the N-terminus on the intact proteins has also been reported (34). Importantly, ICPL allows the simultaneous comparison of three groups in a single experiment (that is, [2H7], [2H3], and [2H0] forms).

Isobaric Labeling
These are the most commonly used isotope tags. Isobaric labels are a set of matched reagents designed to react with peptides to give products of identical masses and chemical properties. Significantly, these products can incorporate carefully selected combinations of heavy and light isotopes. Although many different manifestations of isobaric labels exist, they all comprise the same basic components. Those components are a reactive moiety that functionalizes groups such as primary amines or cysteines, a mass reporter with a unique number of isotopic substitutions, and a mass normalizer with a unique mass that balances or equalizes the mass of the tag. Each different tag is designed to be of equal mass when bound to a peptide, but to cleave on collision-induced dissociation (CID) at a specific linker location, thereby delivering different-sized tags (reporters) that can be quantified independently. In a typical workflow, the proteins in various samples are isolated, enzymatically digested to peptides, and labeled with different isobaric tags. The separately labeled samples are then mixed and analyzed as one. On LC–MS analysis, the peptides are separated, fragmented to produce sequence-specific product ions, to determine sequence, and the abundances of the reporter tags are used to determine the relative amounts of the peptides in the original samples. Commercially available isobaric mass tags (for example, TMT and iTRAQ) allow the simultaneous analysis of multiple samples in one run (such as 4, 6, or 8 mass-unit differences).

Critical Evaluation
Labeled approaches to global protein quantification offer relatively high precision and multiplexing capability, and they suit many sample types. Nevertheless, they are based on the assumption that analytes will be
quantitatively — or at least uniformly — labeled in all samples. Because these strategies are based on measuring proteolytic peptides as surrogates of proteins, the general considerations raised previously (that is, the assumption of complete digestion and selection of diagnostic peptides) apply to all of these methods.

**Targeted Protein Quantification**
Approaches to targeted protein quantification similarly can be divided into two distinct groups: those that detect and quantify intact proteins (typically by MALDI) and those that quantify one or more surrogate peptides derived from each protein (typically by LC–MS-MS).

**Intact Protein Quantification**
Although numerous investigators have demonstrated the ability of MALDI as a precise and accurate approach to protein quantification (35–37), the most powerful and widely adopted manifestation of MALDI protein quantification is the mass spectrometric immunoassay. Developed by Nelson and colleagues (38), this assay combines immunoaffinity column capture with MALDI detection and quantification to reduce the number of components in the sample. In contrast to a conventional ELISA, selectivity is achieved through both the antibody and mass-specific detection. In practice, the sample is passed through an immunoaffinity column; the column is washed, to remove other components; and the bound antigen is eluted directly onto a MALDI target, ready for MS.

For quantification by mass spectrometric immunoassay, fixed amounts of a modified form of the antigen, or a similar protein, are typically added to the sample early in the process, as an internal standard. Absolute quantification is possible by reference to a calibration curve prepared and run in concert with the samples. Mass spectrometric immunoassay offers high-throughput protein quantification. It is important to note that it can also provide details about PTMs and genetic variants. In fact, not only is it possible to identify protein heterogeneity, but the variant forms of the same protein can be independently quantified. Several different antibodies can be combined in a single column, to allow multiplexed antigen quantification. Although mass spectrometric immunoassay is most commonly combined with MALDI, ESI-based methods have also been developed (39).

**Multiple Reaction Monitoring Approaches**
Targeted quantification of proteins following their proteolysis to constituent peptides has increasingly become a routine task (40). With a few significant modifications, the process follows the same strategy, essentially, as that described earlier for “label-free methods.” First, the target peptides are monitored in MRM mode. Then stable,
isotope-labeled versions of the target proteolytic peptides are typically added as an internal standard (though the approach has also been used without incorporating an isotopic internal standard) (41). Monitoring more than one MS-MS transition for each target species provides a powerful approach to quantify a predetermined set of proteins for multiple samples, and it can potentially offer precise and accurate, absolute quantification. Each target protein is cleaved to yield peptides, many of which have a unique sequence (that is, signature, or “proteotypic” peptides). A stable, isotope-labeled version of each signature peptide, designed to be identical to the tryptic peptides generated during digestion, is added at a fixed concentration to each sample, to serve as an internal standard. Because the labeled peptides are coeluted with the target peptide, the internal standards enter the mass spectrometer at the same time as the sample-derived peptides, and therefore they can be concomitantly analyzed by MS-MS. Typically, this approach is performed on a triple-quadrupole mass spectrometer or a hybrid (for example, a quadrupole combined with time-of-flight [TOF] or orbital ion trap analyzer). The target peptide concentration is determined by measuring its observed signal response relative to that of the stable-isotope internal standard. Absolute concentrations can be calculated referring to a calibration curve prepared at the same time. (Calibration curves must be generated for each target peptide in the sample.) With thoughtful selection of the target peptides, it is possible to quantify a specific protein or even a modified form of that protein. No antibody is required, and the process can be performed simultaneously on multiple — even hundreds of — peptides. Therefore, multiple proteins can be quantified in a single LC–MS-MS run. A variant of this process, known as parallel reaction monitoring (PRM), allows simultaneous monitoring of all product ions of a target peptide, rather than only a few predetermined transitions (42).

Critical Considerations
Selection of the specific peptides is a central issue because they should be diagnostic of the full target protein; use of just one or two peptides can lead to overestimations of proteins (15). (As discussed previously, target peptides could be common to known variants of the same protein.) Other important considerations address the possibility of incomplete digestion and the fact that sensitivity can be limited in the case of low-abundance proteins without an isolation or enrichment step. A major benefit of this strategy is that cost-effective, precise, and accurate analysis is possible without access to immunoreagents. Yet the approach can prove costly because of the requirement for multiple, stable, isotope-labeled peptides for each target protein. It can
also prove time-consuming, because of the need to analyze the potentially complex MRM data.

Stable-Isotope Standard Capture with Antipeptide Antibodies
Stable-isotope standard capture with antipeptide antibodies (SISCAPA) is essentially the same procedure as that described above, except that it incorporates a specific, antipeptide antibody capture step for the signature peptide and its companion internal standard (43). The additional step enriches the sample for the target peptide and stable isotope standard. At the same time, it provides an opportunity to deplete the sample of interferents, including other peptides generated during the digestion. Importantly, because the internal standard is a perfect mimic of the target peptide, the peptide-to-internal standard ratio is preserved throughout the workup process. Extensive washing can be undertaken, to remove other peptides and clean the sample, without introducing additional variability in the results. The sample is then resolved by a short reversed-phase LC separation and analyzed by LC–MS–MS. Ions characteristic of the target peptide and its corresponding internal standard are monitored in MRM mode. From the signature peptide-to-internal standard ratio, the concentration of the peptide can be calculated by reference to a calibration curve.

Critical Evaluation

Antibody quality is important. So, too, is the selection of the specific peptides. The use of a single peptide is fraught with the problems discussed above. The limit of detection is improved because of the opportunity to trap and enrich the target peptides, but the cost and complexity of the approach is increased by the inclusion of this step.

Conclusions
MS quantification is not a trivial undertaking, even for small molecules. When the task at hand is the quantification of hundreds to thousands of proteins in a single sample, the complexity of the analysis is even greater. Shortcuts compromise the process and lead to unsatisfactory and irreproducible results. Nevertheless, with proper care, reflection upon sources of variance, and attention to generating reproducible results, multicomponent protein quantification fit for specific purposes are possible.

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References
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This article originally appeared in LCGC as an installment of the “MS—The Practical Art” column, which is edited by Kate Yu, PhD, of Waters, in Milford, Massachusetts. She has a wealth of experience applying LC–MS technologies to various fields such as metabolite identification, metabolomics, quantitative bioanalysis, natural products, and environmental applications.

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Reference:

(7) D. Stoll et al., Front Biosci. 7, c13–32 (2002).
(23) B. Zybailov et al., J. Proteome Res. 5(9), 2339–2347 (2006).
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The use of a mass spectrometer in quantitative analysis exploits its exquisite selectivity and sensitivity as a detector, allowing a signal to be ascribed with high certainty to a particular chemical entity, even when that chemical is present in a sample at low concentration. In what is often referred to as an increase in the ratio of signal to noise (S/N), the selectivity of the mass analyzer (or analyzer combinations) decreases the contribution of background ions to the measured signal, producing a net gain in sensitivity. By operating at enhanced resolution, or by adopting two stages of mass analysis (for example, tandem mass spectrometry [MS-MS]), the specificity is enhanced in a way that is unparalleled by other present-day analytical methods. This
article abstracts information previously published by the authors in a book entitled *The Principles of Quantitative Mass Spectrometry* (1), combining it with new information that will be included in a planned second edition.

Quantitative analysis typically utilizes at least one scanning mass analyzer (that is, a quadrupole, but occasionally a magnetic sector) operated so as to maximize the efficiency of the ion-counting process and, thereby, increase the signal (and S/N) for specific ions or ion transitions of interest. This is often referred to as an increase in the duty-cycle because the mass spectrometer now spends significantly more time counting ions of interest and wastes no time acquiring data on other ions of little or no interest. For some instruments, notably those incorporating ion-trap or time-of-flight (TOF) analyzers, the collection of a full mass spectrum has no effect on the efficiency with which a single ion can be measured; thus, with ion trap and TOF analyzers, there is no sensitivity advantage inherent in the selected-ion monitoring (SIM) experiment. While these analyzers may not deliver the same lower limit of detection and precision of a quadrupole or sector analyzer, the acquisition of the full mass spectrum often increases the certainty of the determination. As illustrated in Figure 1, with scanning instruments, full spectrum measurements can be abandoned in favor of more selective modes of measurement: monitoring the intensity of an ion characteristic of a compound, as is the case with SIM; several ions characteristic of a compound, as is the case with multiple-ion monitoring; or one or more characteristic ion transitions, such as single-reaction monitoring (SRM) or multiple-reaction monitoring (MRM), respectively. As used in the latter phrases, the term *reaction* indicates that a chemical change is involved: one or more selected (precursor) ions undergo collisional dissociation to yield one or more characteristic product ions. Only ion pairs that undergo this particular type of transition (or transitions) yield a measured signal (or signals).

The general approach to quantitative analysis is actually almost independent of the nature of the sample, the analyte (or analytes) of interest, and the separation and detection device. In any given setting, the measurement simply aims to provide the most suitable estimate, in a statistical sense, of the amount of analyte present in a sample. Some special considerations apply, however, when a mass spectrometer is used as a quantitative tool, and they are discussed below. In addition, some of the key terms that appear throughout this installment are defined in the glossary near the end of the text. These definitions help readers who are unfamiliar with the use of these terms in a scientific context to understand their very precise definitions when used in an analytical setting.

**Quantitative Relationships and Mass Spectrometry**

Traditional measuring devices link concentration with such properties as absorption of ultraviolet light (spectroscopic tech-
niques), absorption of visible light (colorimetric techniques), or current (electrical techniques). For most instrumental methods, over a limited range of concentration or amount, there is a linear response to some physical stimulus with respect to the amount of analyte specified. While ideally the relationship would be linear, in practice the curve may deviate from linearity at the lowest and highest concentrations. On the basis of this fundamental relationship, the approach commonly used with any instrumental technique is the calibration-curve method. If the analysis of a series of standards is shown to be reproducible and rugged under well-defined conditions, a linear range and the relationship between the response and the concentration can be defined. Moreover, the response for each unknown can be translated into a concentration or amount by referring to that relationship (or calibration curve).

To perform quantitative analysis with a mass spectrometer, a relationship must first be established between an observed ionic signal for a compound and the amount introduced into the system, typically expressed as a concentration. In practice, absolute ion currents do not correlate well with the absolute amount of analyte: variations between instruments (or even for a given instrument over time) and sample composition preclude the establishment of a universal relationship that could be carried from one setting to another.

We can, however, expect that for two separate samples containing the same analyte and introduced into the mass spectrometer under identical conditions, the relationship between the signal intensity or response, $R$, observed for each will be in direct proportion to their respective concentrations, $C$. Thus, $R_1/R_2 = C_1/C_2$. Rearranging this equation such that $R_1 = (C_1/C_2)R_2$ means that if the concentration of the analyte in one sample is known, the concentration of the analyte in the second can be calculated.

In quantitative mass spectrometry, because the response of a mass spectrometer fluctuates with time and also as a consequence of other components in a matrix, the internal standard calibration curve method is frequently used. With the addition of a chemical mimic of the analyte added to all samples and standards at a fixed and known concentration, the signals for the analyte and the internal standard are measured, and the ratio $R/R_{IS}$ is determined for each point. The ratio $R/R_{IS}$ versus concentration is plotted for all standards, yielding a calibration curve. Thereafter, for all unknowns, the concentration of the target analyte is determined by referencing the calibration curve. Evaluation of the quality of the regression is sometimes facilitated by using a ratio transform: replacing the concentration of the standard as the x-axis variable with the ratio of concentration of the standard to the (fixed) concentration of the internal standard and replotting the data. Having both axes given as ratios yields a regression line for which the slope should always equal 1.00 and the intercept always zero. One advantage to this
Quantifying proteins

approach is that statistical tests are readily available to assess the significance of slope deviations from 1.00. In addition, by centering the unit ratio [that is, when the analyte concentration equals the internal standard concentration] within the range of measurements and then restricting the total range of the analysis to about two orders of magnitude, ratios of high precision result because of matched S/N of each value at each point on the standard curve.

The standard addition method, an alternative approach to quantification, is typically adopted when matrix effects are pronounced; analyte concentrations are at or near the limit of detection; the sample set is unique or diverse in composition; or when an analyte-free matrix is unavailable. The method of standard addition involves dividing a sample containing an unknown amount of analyte into two (or more) portions, after which a known amount of that analyte (often referred to as a spike) is added to one of these. The two samples are analyzed, and the analyte response in the spiked sample is compared to that in the unspiked sample. The larger response in the spiked sample is ascribed to the (additional) amount of analyte in the sample before the spike. The response provides a calibration point to determine the amount of analyte in the original sample.

A linear response with concentration (or amount) is assumed with this two-point (or more) determination.

Steps in a Typical MS Quantitative Assay

Although the steps vary, depending on the analyte and the matrix, the scheme described below is typical of what is used for the majority of small-molecule quantitative determinations based on MS. The objective is to generate two completely independent signals: one for the analyte and the other for the internal standard. The internal standard, if carefully selected and added early in the process, not only improves precision in the mass analysis steps, but also accounts for analyte loss during sample manipulation, work-up, and introduction (typically liquid chromatography [LC] or gas chromatography [GC]).
Preparation of Standards
Absolute quantification requires that relative levels be converted to absolute levels by reference to the calibration curve. As described above, the curve (or mathematical relationship between amount and response) is generated from data obtained by analyzing the standard samples that contain differing known amounts of the analyte of interest. The standards that constitute this curve are prepared in the same matrix and worked up at the same time as the samples.

Solubilizing the Analyte
When working with solid samples, a critical step is to dissolve the analyte of interest completely in a suitable solvent to create a stock solution that can be used to spike the calibration standards. It is also important to remove particulate debris from the stock that might otherwise interfere with the analysis.

Addition of the Internal Standard
A fixed amount of an appropriate internal standard is added to each sample: standard, quality control (QC), and unknown.

Sample Work-Up (Extraction and Concentration)
The objective of sample work-up is to remove as many of the interfering substances as possible from the sample matrix, thus generating a relatively clean solution containing the highest possible concentration of the analyte per unit volume.

Sample Analysis
Frequently, sample analysis involves introducing the sample into a separations device (for example, GC or LC) coupled directly to the mass spectrometer. The mass spectrometer monitors at least two separate signals for all samples: one characteristic of the analyte and the other characteristic of the internal standard.

Regression of Calibrator Responses
The mathematical function that best fits the relationship between concentration and response (or, more correctly, response ratio) in the set of standard samples is determined.

Calculation of the Concentrations of the Analyte in All Samples
The ratio of the peak intensities for the analyte and the internal standard in each unknown sample is determined, and that ratio is converted to an absolute concentration or amount by reference to the calibration curve.

Evaluation of Data
Data evaluation is based on additional samples included in the analysis set, such as matrix blanks and quality-control samples. It is important to note the response of a sample containing no analyte, to ensure that no interferences occur within the matrix. It is also important to determine the appropriateness of the regression by calculating concentrations for samples of known composition. These test samples are often referred to as QCs.
The Need for Assay Validation
Quantitative analysis methods that rely on MS frequently undergo validation, a process that demonstrates the ability of an assay in toto to achieve its purpose. That purpose is to quantify analyte concentrations with a defined degree of accuracy and precision. Thus, the validation process demonstrates whether an analytical method performs as intended.

Validation ensures that an assay will produce quantitative data uncompromised in quality by errors in procedure. The primary objective is to assess the method’s accuracy and precision: the value obtained must be a (statistically) good estimate of the true value, and repetitive measurements must yield values within the statistical distribution of good estimates of the true value. Secondarily, validation will establish the concentration range over which the method is reliable (the upper and lower limits of quantification), the selectivity (the presence or absence of interfering peaks), the amount of analyte recovered from a sample during its preparation for analysis, and the stability of the analyte in samples under defined conditions of storage.

Overview of Validation Procedure
Extrapolation of values beyond the range of the calibration curve is generally not acceptable. Such values would lie outside the linear range of the assay. Thus, for
Quantifying Biopharmaceuticals in Biological Samples

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower limit of quantification (LLOQ)</td>
<td>The lowest point on the standard curve.</td>
</tr>
<tr>
<td>Matrix</td>
<td>The collection of all chemical species (background) among which the analyte is found. For biological samples, matrices can include plasma, serum, urine, cerebrospinal fluid, tissue, and milk; for environmental samples, typical matrices include water, soil, and air.</td>
</tr>
<tr>
<td>Matrix blank</td>
<td>Sample of the matrix of interest known to be free of the analyte of interest.</td>
</tr>
<tr>
<td>Outlier</td>
<td>Sample with anomalously high or low predicted concentration.</td>
</tr>
<tr>
<td>Precision</td>
<td>The reproducibility, or repeatability, of a measurement. Reproducibility can apply to replicate measurements of a single sample or single measurements of like samples. The scatter in results that occurs when repeat determinations are made.</td>
</tr>
<tr>
<td>Predicted concentration</td>
<td>Estimate of concentration determined from the line generated by regression of calibrator responses.</td>
</tr>
<tr>
<td>Quality control (QC) samples</td>
<td>Samples of the matrix of interest containing a known amount of the analyte of interest. QC samples are used to test for the accuracy and precision of concentration determinations.</td>
</tr>
<tr>
<td>Range</td>
<td>Interval over which acceptable accuracy, linearity, and precision are achieved.</td>
</tr>
<tr>
<td>Ratio measurement</td>
<td>Response of an analyte relative to the response of an internal standard in a given sample. The use of a ratio in quantification accounts for factors such as sample-to-sample variability in the mass spectrometer’s response.</td>
</tr>
<tr>
<td>Reference standard</td>
<td>A sample of analyte for which the purity is defined. The ideal reference standard is in a stable form and of the highest purity achievable.</td>
</tr>
<tr>
<td>Regression</td>
<td>Mathematical relationship that is established between a measured response and known concentration of reference standard.</td>
</tr>
<tr>
<td>Sample set</td>
<td>The ensemble of samples included in a single batch or run. This includes calibrators (typically a minimum of six concentrations, in duplicate), quality control samples (typically three concentrations, in triplicate), matrix blanks, and unknowns.</td>
</tr>
<tr>
<td>Sample work-up or preparation</td>
<td>Procedures necessary to separate the analyte from some of its matrix components so that it becomes possible to measure the analyte's specific response in the mass spectrometer. Increased work-up minimizes interferences but can result in analyte loss and decreases in precision. Some work-up steps may lead to contamination or artifactual formation.</td>
</tr>
<tr>
<td>Selectivity (or specificity)</td>
<td>The ability to measure the response arising from one analyte independent of any other sample component (or components).</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>The lowest possible amount of analyte that can be determined with acceptable precision and accuracy.</td>
</tr>
<tr>
<td>Speed (of analysis or assay)</td>
<td>The time to analyze one sample; the time to complete a set of determinations; or, the sample-analysis time plus the total development time of the analytical method.</td>
</tr>
<tr>
<td>Stability</td>
<td>An index of the propensity for the chemical integrity of the analyte to change under various conditions. For example, analyte stability may be influenced by storage at room temperature, −20 °C, or −70 °C; during freezing or thawing; or during sample work-up and analysis.</td>
</tr>
<tr>
<td>Standard curve</td>
<td>Range of reliable response of the assay; that is, the range of concentrations over which accurate determinations of concentrations of unknowns can be made.</td>
</tr>
<tr>
<td>Upper limit of quantification (ULOQ)</td>
<td>The highest point on the standard curve.</td>
</tr>
</tbody>
</table>

Quantifying proteins, the lowest and highest calibrators constituting the curve define the limits of quantification. The lower limit of quantification (LOQ or LLOQ) is the smallest amount or lowest concentration quantifiable that is consistent with the accuracy and precision required by the assay. Operationally, the International
Union of Pure and Applied Chemistry (IUPAC) defines LOQ as the concentration that gives rise to a signal 10 times the standard deviation of the matrix blank and three times that of the limit of detection (LOD) (2). The upper limit of quantification (ULQ or ULOQ) may be determined by the physical–linear boundary of the regression line, potential for contamination of samples at low concentration, ion-suppression effects at high concentration, or simply by an experiment’s parameters. A recent discussion of these and related topics can be found in reference 3.

Accuracy measures the closeness with which an individual measurement approaches the true value. Because all actual measurements are estimates of the true value, only by producing replicate measurements of the concentration of a single sample (repeatability or the precision of the measurement) and the second is associated with repetitive (typically 3–6) preparations of a single homogeneous sample (reproducibility or precision of the method).

Because they provide different information, the precision of the measurement and method are often calculated and reported separately. The errors associated with the method, in toto, exceed in number those arising from instrument variation alone. The difference between the two is a measure of the errors associated with sample preparation. It should be noted that the error associated with any step is a function of the random errors contributed by all of the components and that the overall random error is, therefore, dominated by the least
precise step or component.

A statistical assessment of the variability of an assay is often calculated as a part of the validation process. This analysis of variance (ANOVA) produces values for two types of precision: within-day precision (that is, intra-run or intra-laboratory precision, sometimes referred to as the repeatability) and between-day precision (that is, inter-run, between-run, or inter-laboratory precision, sometimes referred to as the reproducibility).

Because more steps and more variables are involved in an assay process that extends over several days, the within-run relative standard deviation (RSD) is generally less than the between-run RSD. Nevertheless, fortuitous cancellation of errors between days can (very) occasionally result in a between-day precision value less than that of the within-day precision value. It should be noted that the precision decreases as the analyte concentration decreases, ultimately reaching unacceptable values to the point at which the measured signal approaches the LOQ. Characterization of the precision of the method over the entire concentration range, therefore, requires that the assessment be done at several different concentrations.

Additional Considerations

The combination of detection, identification, and measurement of target compounds, especially at low concentrations, is one of the most challenging tasks that mass spectrometrists undertake. In these situations — where a need exists to justify the choice of a particular method or substantiate the data obtained — the issue of fitness for purpose is crucial.

The term fitness for purpose describes “. . . the property of data produced by a measurement process that enables the user of the data to make technically correct decisions for a stated purpose” or “. . . the magnitude of the uncertainty associated with a measurement in relation to the needs of the application area” (4–6). Yet fitness for purpose can also be described as the process by which the nature of a task is allowed to define the requirements of the method. Work that is primarily quantitative requires measurement at a specific level or within a specific range, and assay performance standards can be set to define a “zone of uncertainty” above or below a particular decision point, as shown in Figure 2. For work that blends qualitative and quantitative analyses, a decision point can be set at the center of the zone of uncertainty with accuracy and precision indicated by expressions of associated confidence intervals.

The goal is always to choose experimental conditions and mathematical manipulations that bring the mean result closer to the true mean and to minimize the confidence interval around the measurement, increasing accuracy and precision. When choosing the analytical approach, considerations such as cost, time, and the available instrumentation...
are major factors. The key to performing successful quantitative analyses, however, is to ensure that a chosen analytical method fits the specific purpose and to provide a level of uncertainty in measurement that lies within acceptable limits.

To ensure that the uncertainty associated with the results has been acknowledged and accepted, it is often useful, at the outset of planned work, to do as follows:

- Define the analytical problem, and consider the needs of all interested parties.
- Evaluate the perceived risk associated with the analyte and the consequences associated with the result.
- Balance thoroughness with time and resources.
- Define the tolerance or uncertainty.
- Specify limits for accuracy, precision, and false identification.

• Estimate the expected concentrations, and choose the range of calibrators accordingly.

Only in this way can answers be confidently given to questions posed by those who would use the data to make decisions.

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Liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) has recently become a more and more popular alternative to traditional ligand-binding assays for the quantitative determination of biopharmaceuticals. LC–MS–MS offers several advantages such as improved accuracy and precision, better selectivity, and generic applicability without the need for raising analyte-directed antibodies. Here we discuss the technical requirements for a successful LC–MS–MS method for the quantitation of biopharmaceuticals and evaluate the advantages and disadvantages compared to ligand-binding assays.

The development of protein-based pharmaceuticals, or biopharmaceuticals, is by far the fastest growing part of the pharmaceutical industry today. With over 1500 biopharmaceuticals in clinical development and more and more companies shifting their R&D efforts towards this sophisticated and relatively profitable class of drugs, the pharmaceutical landscape has changed beyond recognition compared to 20 or even 10 years ago. As a result, the field of bioanalysis that supports drug development by measuring the concentrations of...
drugs or relevant endogenous molecules in biological samples has also seen many changes. The quantitative determination of biopharmaceuticals has traditionally been the domain of ligand-binding assays, such as ELISA. However, in the past few years there has been a clear increase in the application of alternative analytical platforms, in particular, liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS), which has been the workhorse for small-molecule bioanalysis for over 20 years (1–5).

Over the past decade, there have been many advances in the LC–MS–MS-based quantitation of biopharmaceuticals, both from an analytical and a conceptual point of view. In this article, an overview is given of the many aspects of this field of analytical research by reference to a selection of recent applications.

**Protein Digestion**
Tandem mass spectrometry remains the detection technique of choice for the quantitative determination of biopharmaceuticals because of its sensitivity and its widespread availability in the pharmaceutical and related industries. However, the use of LC–MS–MS to quantify biopharmaceuticals is more complex than for small molecules because it is not directly compatible with molecules with a mass above around 5000 Da. The ions of larger analytes are distributed over many different charge states and usually do not readily fragment and this considerably reduces sensitivity.

Therefore, a typical step in the analysis is the (enzymatic) digestion of a biopharmaceutical into a mixture of smaller peptides, followed by the analysis of the digest and quantitation of one or more so-called *signature peptides* as a measure for the intact protein. Digestion is usually performed using the enzyme trypsin, which cleaves the amino acids chain in proteins after a lysine or arginine. Trypsin is popular because it is readily available at a reasonable price and can cleave proteins into peptides of a size (500–2000 Da) that is well suited for MS–MS detection.

Protein digestion enormously increases the complexity of a biological sample. Matrices such as plasma contain proteins at a total concentration of around 80 mg/mL and, when no further clean-up of the sample is performed, each of these proteins is cleaved into a series of peptides that are all of a similar size and have more or less comparable physicochemical and analytical properties. Therefore, it is often challenging to detect low concentrations of a signature peptide in a digest, because of the presence of so many endogenous peptides, which all consist of combinations of the same 20 amino acids and often occur at much higher levels than the signature peptide itself.

Despite the selective nature of MS–MS detection, chromatograms of digested biological samples often contain many background peaks originating from endogenous peptides that show a response at the mass transition of the signature peptide. Figure 1 shows this effect for a
fixed concentration of digested salmon calcitonin in the presence of increasing amounts of digested plasma (6). The selectivity of the method is clearly affected by the presence of endogenous background peptides. As a result, method sensitivity is also heavily impacted — in this case the achievable lower limit of quantitation (LLOQ) increases 100-fold, from 0.2 ng/mL (60 pM) in the absence of

Figure 1: LC–MS–MS (m/z 561.9 to m/z 204.0) chromatograms of a signature peptide of 2 ng/mL salmon calcitonin in samples containing increasing amounts of human plasma digest. Analyte peak at 2.9 min. Adapted and reproduced with permission from Analytical Chemistry 85, K.J. Bronsema, R. Bischoff, and N.C. van de Merbel, High-Sensitivity LC–MS/MS Quantification of Peptides and Proteins in Complex Biological Samples: The Impact of Enzymatic Digestion and Internal Standard Selection on Method Performance, 9528–9535 (2013) © American Chemical Society.
matrix peptides to 20 ng/mL (6 nM) in the presence of 50% of digested plasma.

A review of current literature (1, 4) shows that a typical LLOQ for a biopharmaceutical in plasma or serum, only treated by digestion, is in the high ng/mL to low µg/mL range (corresponding to low nM levels for many proteins). Figure 2 shows an example chromatogram for a signature peptide of recombinant human alpha-glucosidase at its LLOQ of 0.5 µg/mL (5 nM) in human plasma (7).

**Signature Peptide Selection**

The possibilities for selecting a proper signature peptide are usually rather limited. First and foremost, it is essential that the selected signature peptide has a unique amino acid sequence that does not naturally occur in any of the endogenous matrix proteins. Selection of a non-unique signature peptide results in an overestimation of analyte concentrations, because the same amino acid sequence that is released from endogenous proteins would contribute to the overall signal. This often disqualifies a large number of the theoretical signature peptides, particularly for biopharmaceuticals with a high degree of similarity to endogenous proteins, such as humanized antibodies (if these need to be quantified in human plasma).

In addition, other criteria are applied to ensure robustness of the LC–MS–MS assay. Peptides containing unstable amino acids, such as methionine and tryptophan that can be oxidized, or glutamine and asparagine that can be deamidated, are usually disregarded to avoid losses during analysis, although forced oxidation of a signature peptide to a stable oxidized product has been successfully used (8). Similarly, peptides with (variable) post-translational modifications — such as O- or N-glycosylated amino acids — are typically excluded because these would introduce undesirable heterogeneity.

In addition, peptides that are too small, too large, too polar, or too hydrophobic might cause analytical problems because
of adsorption, sub-optimal chromatographic behaviour, or limited selectivity and sensitivity. In the end, there may be just a few out of the many potential signature peptides that can be successfully used in practice.

Protein Extraction
An obvious way to improve selectivity and sensitivity of an LC–MS–MS method is to remove interfering matrix proteins prior to digestion, which can be achieved by applying immunocapture (IC) techniques. Magnetic beads or other resins are coated with a protein that displays a high binding affinity towards the analyte, typically an antibody raised against the analyte or the pharmacological target to which a biopharmaceutical is directed. By mixing the sample with a suspension of the beads or passing it through a cartridge filled with the resin, the analyte is selectively isolated from the complex sample. This approach is particularly popular for the quantitation of endogenous proteins such as biomarkers, for which well-characterized immunological reagents are widely available.

One example is an LC–MS–MS method for parathyroid

![Figure 3: LC–MS–MS (m/z 729.0 to m/z 942.4) chromatograms of the signature peptide of 10 ng/mL rhTRAIL in human serum and the corresponding blanks, pretreated with SCX or IMAC before digestion. Adapted and reproduced with permission from Bioanalysis 7(6), D.Wilffert, R. Bischoff, and N.C. van de Merbel, Antibody-free workflows for protein quantification by LC-MS/MS, 763–779 (2015) © Future Science Ltd.](image)

![Figure 4: LC–MS–MS (m/z 752.0 to m/z 773.3) chromatograms of the signature peptide of 10 ng/mL of a nanobody in human plasma (a) without or (b) with solid-phase extraction of the plasma digest. Analyte peak at 4.6 min. Adapted and reproduced with permission from Bioanalysis 7(1), K.J. Bronsema, R. Bischoff, M.P. Bouche, K. Mortier, and N.C. van de Merbel, High-sensitivity quantitation of a Nanobody® in plasma by single-cartridge multidimensional SPE and ultra-performance LC-MS/MS, 53–64 (2015) © Future Science Ltd.](image)
hormone (PTH) in human serum. A sample of 1 mL was treated by IC with polystyrene beads coated with murine anti-PTH antibodies and the trapped analyte digested with trypsin. The IC treatment allowed the quantitation of PTH down to 40 pg/mL (4 pM) in serum, which shows the enormous clean-up potential of this approach. A completely 15N-labelled form of PTH was added to the sample as an internal standard at the very beginning of the sample handling procedure. In general, it is desirable that a stable-isotope-labelled or other closely related form of the protein analyte be included in the method as an internal standard, to correct for the variability of the extraction procedure. This is also one of the drawbacks of extracting a biological sample before digestion, because such a protein-based internal standard can usually only be obtained by biotechnological means, which may be difficult, if not impossible.

The disadvantages associated with the use of immunological reagents — such as their potentially limited availability, varying quality, and the interference of matrix proteins with the extraction efficiency — have prompted researchers to investigate alternative so-called antibody-free extraction approaches. An interesting technique is immobilized-metal affinity chromatography (IMAC), which is based on the interaction of metal ions, such as Ni²⁺, with amino acids that feature strong electron donor groups, such as histidine. Proteins with such amino acids on their surface will be selectively captured by IMAC resins. As an example, the biopharmaceutical recombinant human tumour necrosis factor-related apoptosis-inducing ligand (rhTRAIL) has been quantified in human and mouse serum down to 20 ng/mL (340 pM) by removing 95% of matrix proteins, while recovering >70% of the analyte with IMAC.

Another technique is solid-phase extraction (SPE) with ion-exchange materials, which separates proteins based on their isoelectric point (pI). Proteins with a relatively high pI bear a net positive charge and can be trapped on a cation-exchange resin at neutral or slightly alkaline pH, at which many endogenous proteins with a lower pI will be negatively charged and thus not be captured. The extraction of rhTRAIL with strong-cation exchange (SCX) SPE was found to have a similar clean-up potential to IMAC, with an analyte recovery of 70% and a protein removal efficiency of 99%. As an illustration, Figure 3 shows chromatograms obtained for 10 ng/mL (170 pM) of rhTRAIL in human serum, which was extracted by SCX or IMAC, followed by trypsin digestion and LC–MS–MS analysis of the signature peptide.

Peptide Extraction
Removal of interfering matrix components is also possible after digestion, that is, at the peptide level. This approach has some distinct advantages. From a practical point of view the optimization of an SPE procedure is more straightforward because of the wide availability of a range of materials that are commonly used for
small-molecule extractions and because of the more predictable extraction behavior of smaller peptides compared to that of intact proteins.

The accuracy and precision of extractions may be influenced by protein-protein interactions in samples (such as binding of a biopharmaceutical to its target or to anti-drug antibodies), or the occurrence of aggregates. If a sample is first subjected to digestion, these interactions will no longer influence the extraction because all proteins will have been cleaved to peptides that are much less likely to bind to one another with a high affinity.

No less importantly, peptide extraction does not need a protein-based internal standard; it can instead perform very well when using a stable-isotope labelled form of the signature peptide (4,6), which is considerably less expensive and easier to obtain. It may, however, be difficult to achieve sufficient selectivity because the peptides in a plasma digest are much more similar to each other than the plasma proteins were before digestion. Again, the highest selectivity and sensitivity is achieved by applying immunocapture, which in this case uses immobilized antibodies raised against the signature peptide. This approach is most widespread in the field of biomarker analysis, where the number of analytes is relatively limited and assays are relevant to many research groups around the globe. Large clean-up efficiencies can be achieved in this way, as was reported for the endogenous proteins α1-antichymotrypsin (1453-fold enrichment relative to matrix proteins) and TNF-α (573-fold enrichment) (10).

IC at the peptide level is less popular in biopharmaceutical analysis, probably because of the general drawbacks of antibody-based reagents with regard to availability and batch-to-batch reproducibility. A more generic approach for peptide extraction from a digest is to use conventional ion-exchange SPE, but this needs to be carefully optimized to obtain sufficient selectivity. A digest of a protein-rich biological sample (such as plasma) contains a multitude of peptides, which all have carboxylic and amine groups, and the signature peptide can only be separated from the excess of
endogenous background peptides if its pI value is sufficiently different. Typically, the pH and ionic strength of the loading, washing, and elution steps need to be carefully optimized for a selective extraction.

A biopharmaceutical nanobody was quantified down to 10 ng/mL (360 pM) in rabbit and human plasma by trypsin digestion followed by SPE on a weak-anion exchange phase (11). The signature peptide contained three carboxylic acid groups and was strongly retained by the positively charged SPE phase at pH 5; many endogenous peptides with less negative charges were not trapped during sample loading or were removed from the SPE material by a washing step with 300 mM sodium chloride. The mixed-mode SPE phase, which also contained reversed-phase groups, was subsequently neutralized at a high pH and the (relatively polar) signature peptide was eluted, while some less polar endogenous peptides remained bound by reversed-phase interactions. In this way, two dimensions of selectivity (ion exchange and reversed phase) were used to isolate the signature peptide from the plasma digest. Figure 4 illustrates that many interfering peaks were removed from the chromatogram with this approach and that selectivity was clearly improved. Of course, cation-exchange SPE can be applied in the same way in case the signature peptide has multiple positive charges, and even reversed-phase SPE might be an option if the signature peptide is particularly hydrophobic.

**Combined Protein and Peptide Extraction**

As illustrated above, generic protein or peptide extractions typically result in LLOQs in the low ng/mL (mid to high pM)
range, while IC extraction at the protein or peptide level enables quantitation down to mid pg/mL (low to mid pM) concentrations. If more sensitivity is required, one option is to combine protein and peptide extractions. Excellent selectivity and sensitivity can be reached even without antibody-based extraction materials, as was shown for rhTRAIL in saliva (12). After IMAC extraction of the protein analyte and trypsin digestion, the digest was further purified using SPE on a SCX cartridge. Because of the presence of four basic amino acids in the signature peptide, the digest was acidified before loading onto the SPE phase. The peptide was then trapped and endogenous peptides were removed by washing with 200 mM sodium chloride. After elution at alkaline pH, the signature peptide was quantified using LC–MS–MS. As shown in Figure 5, a TRAIL concentration as low as 0.2 ng/mL (3.4 pM) could be quantified in both dog and human saliva. In principle, protein or peptide extractions can be combined in many ways and as long as the separation mechanisms are orthogonal, improved selectivity and sensitivity can be expected compared to a single-extraction approach.

The ultimate combination of protein and peptide extraction is IC of the protein analyte followed by digestion and IC of the signature peptide. Although this requires two specifically raised antibodies and is by no means a generic approach, it can result in impressive sensitivities. The biomarker interleukin-21 (IL-21) was quantified in human serum and monkey tissues with an LLOQ of 0.78 pg/mL (0.05 pM). This was achieved by combining off-line magnetic bead-based protein extraction using an anti-IL-21 antibody with on-line enrichment of the signature peptides using immobilized anti-peptide antibodies (13). Figure 6 shows representative chromatograms. It is important to realize that the obtained LLOQ corresponds to a molar concentration of the protein, which is five orders of magnitude lower than that shown in Figure 2 (digestion only). This convincingly demonstrates the enormous clean-up capability of this combination of techniques.

**LC–MS–MS versus ELISA**

Compared to ligand-binding assays, LC–MS–MS has a number of analytical advantages such as a larger linear dynamic range; (usually) higher accuracy and precision because of the possibility to apply internal standards (4); the ability to quantify multiple analytes simultaneously; and the fact that it does not necessarily require immunological reagents (5). The last point can be especially critical, because such reagents may be problematic to obtain or show a large batch-to-batch variability, which makes comparison of results between laboratories or over a longer period of time difficult, if not impossible. The disadvantages of LC–MS–MS include its generally higher operational cost; more limited sample throughput; and less favourable concentration sensitivity. In addition, with the digestion step that is
generally needed for LC–MS–MS, the three-dimensional structure of a protein analyte is lost and the analytical principle is therefore not related to the complex molecular structure of a protein, which determines its pharmacological activity.

Now that more and more reports are appearing that compare newly developed LC–MS–MS methods with existing ELISAs for the same protein analyte, it is becoming increasingly clear that both techniques do not always give superimposable concentration results (14,15). Although in the world of small-molecule quantitation, two different results for the same sample would be seen as proof that at least one of them is incorrect, this is not necessarily true for biopharmaceuticals. It should be realized that, in contrast to small molecules, LC–MS–MS as well as ELISA only use a small part of the protein molecule for the actual quantitation, the signature peptide and the binding epitope, respectively, and this may represent as little as a few percent of the entire molecule. Furthermore, both techniques are based on quite different (bio)chemical principles, to which the structurally complex and often heterogeneous biopharmaceuticals may respond in different ways. Thus, neither LC–MS–MS nor ELISA should be regarded as the ultimate quantitation technique for biopharmaceuticals, but rather as complementary tools for obtaining quantitative information about this complicated but very interesting class of compounds.

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