QUALITATIVE AND QUANTITATIVE CHARACTERIZATION OF PROTEIN BIOTHERAPEUTICS WITH LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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In the last decade, the advancement of liquid chromatography mass spectrometry (LC/MS) techniques has enabled their broad application in protein characterization, both quantitatively and qualitatively. Owing to certain important merits of LC/MS techniques (e.g., high selectivity, flexibility, and rapid method development), LC/MS assays are often deemed as preferable alternatives to conventional methods (e.g., ligand-binding assays) for the analysis of protein biotherapeutics. At the discovery and development stages, LC/MS is generally employed for two purposes absolute quantification of protein biotherapeutics in biological samples and qualitative characterization of proteins. For absolute quantification of a target protein in bio-matrices, recent work has led to improvements in the efficiency of LC/MS method development, sample treatment, enrichment and digestion, and high-performance low-flow-LC separation. These advances have enhanced analytical sensitivity, specificity, and robustness. As to qualitative analysis, a range of techniques have been developed to characterize intramolecular disulfide bonds, glycosylation, charge variants, primary sequence heterogeneity, and the drug-to-antibody ratio of antibody drug conjugate (ADC), which has enabled a refined ability to assess product quality. In this review, we will focus on the discussion of technical challenges and strategies of LC/MS-based quantification and characterization of biotherapeutics, with the emphasis on the analysis of antibody-based biotherapeutics such as monoclonal antibodies (mAbs) and ADCs. © 2016 Wiley Periodicals, Inc. Mass Spec Rev

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I. INTRODUCTION

Benefiting from the technical breakthroughs in recombinant genetic engineering and manufacturing, development of biotherapeutics for disease treatments has thrived in the last decades, especially the monoclonal antibody (mAb), which have garnered substantial interest from the biopharmaceutical industry and academia. The high target specificity and minimal toxicity of mAb render it among one of the most-promising categories of marketed drugs for cancer therapies (Jemal et al., 2011). Currently, more than 40 mAbs have been approved and more than 300 mAb candidates are in the development pipeline (Wang, Wang, & Balthasar, 2008b; Li & Zhu, 2010; Ecker, Jones, & Levine, 2015).

The importance of bioanalysis in the initial discovery and preclinical/clinical developments of biotherapeutics has been well-recognized (Dudal et al., 2014). Accurate, sensitive, selective, robust, and high throughput quantification is essential to obtain fundamental temporal data for pharmacokinetic (PK), pharmacodynamic (PD), and toxicokinetic (TK) analyses (Dudal et al., 2014; Zhang, Olah & Zeng, 2014; Chilewski & Jiang, 2015). Ligand-binding assays (LBAs) and liquid chromatography mass spectrometry (LC/MS/MS)-based strategies represent the mainstream analytical methods to collect such data, and their quantitative performances have been comprehensively reviewed in previous reports (Ezan & Bitsch, 2009; Hoofnagle & Wener, 2009; An, Zhang, & Qu, 2014a). Whereas LBA is probably still considered as the gold standard for biotherapeutics analysis, LC/MS-based strategies have emerged as promising alternatives to LBA, and have been successfully applied for the quantitative analysis of several biotherapeutics (Li, Fast, & Michael, 2011, 2012; Zheng, Bantog, & Bayer, 2011; Duan et al., 2012a; Ouyang et al., 2012; Yuan et al., 2012; Furlong et al., 2014).

Qualitative characterization with high accuracy, sensitivity, and selectivity is also essential to support development and evaluation of biotherapeutics. The impending expiration of patents for many blockbuster biologic drugs will trigger the rapid development of biosimilar products (Calo-Fernandez & Martinez-Hurtado, 2012; Colbert et al., 2014). Meaningful

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assessment of comparability of biosimilar products will require evaluation of primary sequence, post-translational modifications (PTMs), higher-order structure, product-related impurity species, etc.; LC/MS might be an ideal analytical strategy to employ for such assessments. Furthermore, extensive efforts have been directed toward the modification of biotherapeutic molecules in order to produce novel entities with desired features including lower toxicity, higher affinity to target antigen, smaller size, better *in vivo* stability, improved tissue penetration, lower immunogenicity, etc. These endeavors have resulted in dozens of different new protein scaffolds, such as Fab/Fc domain-modified mAb, antibody drug conjugates (ADC), nanobodies, and bispecific scaffolds (AlDeghaither, Smaglo, & Weiner, 2015). In order for optimal development, assessment, and production of these novel structures, reliable approaches for qualitative characterization are required (Houde et al., 2009; Liu, De Felippis, & Huang, 2013b, 2015; Shah et al., 2014; Huang et al., 2015; Wang et al., 2015).

In the following sections, we will discuss the current status of development for mAb and mAb derivatives. The review of technical rationale, challenges, and solutions for LC/MS-based quantitative and qualitative characterization for protein therapeutics will be focused, followed by illustrations of representative applications.

II. THE STATE-OF-THE-ART DEVELOPMENT OF ANTIBODY-BASED BIOTHERAPEUTICS

The past decade has witnessed a tremendous investment in the development of mAb-based biotherapeutics, most prominently in the treatment of oncological and auto-immune diseases, with expansions to other fields such as treatment of bacteremia, stroke, myocardial infarction, osteoporosis, and hyperlipidemia (Beck et al., 2010; Olinger et al., 2012). Dozens of mAbs have been approved by FDA and EMA for clinical treatment of human diseases (Li & Zhu, 2010), and the worldwide market sales of the biotherapeutics have reached nearly $75 billion in 2014. It is predicted that this number will climb to $94 billion by 2017 (Ecker, Jones, & Levine, 2015). Following the advent of hybridoma technology, and the report of the first mAb by Kohler and Milstein (1975), the development of therapeutic mAbs might be described in three distinct, but overlapping, phases.

The first phase applied standard hybridoma technology, as introduced by Kohler and Milstein (1975), to develop murine mAbs. This phase of development led to some success, including the FDA approval of Orthoclone® OKT3 (muromonab-CD3) in 1986, thus laying the foundation for further development of mAbs. However, the first generation of mAbs suffered from many disadvantages, including immunogenicity (human anti-murine antibody response), short half-life, and poor efficacy (inability to elicit robust human immune effector activity), which are largely attributed to the nature of murine mAb (Khazaeli, Conry, & LoBuglio, 1994). Additionally, the genetic instability and low-yielding characteristics of many hybridomas also hindered the development of mAbs (Li & Zhu, 2010). These disadvantages substantially dampened enthusiasm in mAbs until technical breakthroughs pushed mAb development to the second phase.

The second phase commenced with the development of humanization strategies, including the development of chimeric antibodies, where ~65% of the rodent sequence associated with the parent murine mAb was replaced with human sequence (via recombinant DNA technologies), and CDR-grafted antibodies, where ~90–95% of the murine sequence was replaced with human IgG sequence (Robinson, Weiner, & Adams, 2004). Further development of humanization strategies led to the generation of transgenic mice and antibody phage display (APD) technologies, which allowed generation of fully humanized monoclonal antibodies (Schirrmann et al., 2011). This second, and still current, phase of mAb development has yielded tremendous success, with over 30 humanized mAbs approved for therapeutic use (worldwide). However, even for fully humanized antibodies, problems, and limitations often exist, including insufficient potency, selectivity, and efficacy (AlDeghaither, Smaglo, & Weiner, 2015).

Thus, the third phase of development emerged to engineer mAbs and to develop mAb derivatives, with improved pharmacokinetics, pharmacodynamics, efficacy, and safety. Many novel constructs have been introduced, including mAbs with single amino acid substitutions (e.g., to enhance binding to effector cells or to enhance binding to the IgG protection receptor, FcRn (Chames et al., 2009)), ADC, where small-molecule drugs are chemically linked to mAb) (Teicher & Chari, 2011), and also including mAb derivatives that, in many cases, differ substantially from IgG mAb in terms of structural characteristics (e.g., including molecular weight and valence). Several novel constructs have been introduced (Fig. 1), including: (i) single-chain variable fragments (scFv, ~25 kDa), which contain the variable domains of mAb light and heavy chains that are linked by a short peptide; (ii) nanobodies and domain antibodies (dAbs) that contain a single variable domain (~11–15 kDa) (Muyldermans et al., 1994, 2013); and (iii) bispecific constructs such as Bispecific T cell Engagers (BiTE), which bind T-cell surface proteins (e.g., CD3) and tumor antigens (e.g., CD19, EpCAM, or EGFR) to elicit T-cell immunity (Baeuerle, Kufer, & Bargou, 2009). These novel constructs were developed to achieve several desirable features over IgG mAbs. First, they can be used as "bricks" to build more flexible and adapted biotherapeutical constructs; second, these agents are often thermotolerant and often exhibit less immunogenicity than conventional mAbs (Robinson, Weiner, & Adams, 2004); third, some of these molecules can penetrate tissues more effectively than a mAb. Owing to these attractive features, tremendous efforts have been placed on the development this next generation of biotherapeutics (AlDeghaither, Smaglo, & Weiner, 2015).

In summary, a large variety of biotherapeutics with diverse structures, functions, and properties have been developed in the past decades. Given the favorable safety/efficacy features of biotherapeutics and the rapid progress of biotechnology, we expect that the upward trend of biotherapeutics development will continue into the future. To keep up with this trend, development of advanced qualitative and quantitative analytical methods with sufficient selectivity, accuracy, sensitivity, and throughput, is urgently critical. The following sections will discuss the challenges and current strategies of quantification and qualitative analysis of protein therapeutics by LC-MS in detail.

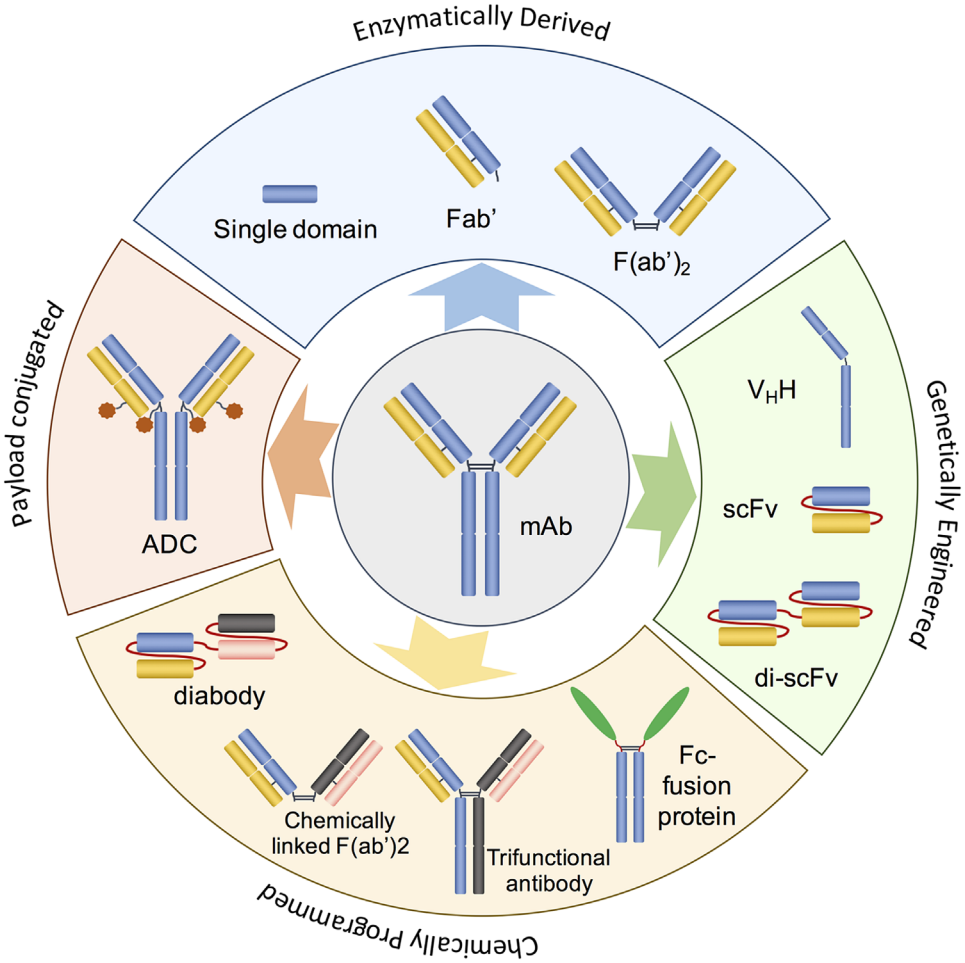

FIGURE 1. Simplified structures of major forms of biotherapeutics derived from mAb.

III. QUANTIFICATION OF BIOTHERAPEUTICS IN BIOMATRICES

A. Current Quantitative Techniques

LBA, based on immunocapture and detection, is the most-frequently practiced method for protein quantification for several decades. Currently, LBA is utilized for PK, PD, and TK studies of biotherapeutics via specific capture with target antigen or anti-idiotypic antibodies (Damen et al., 2009b; Kim et al., 2012; Leary et al., 2013). Because the most-commonly used LBA method, enzyme-linked immunosorbent assay (ELISA) is often considered as the gold standard for protein quantification and poses features such as high sensitivity and throughput. However, several major limitations are associated with this approach (Ezan & Bitsch, 2009; Pendley & Shankar, 2011; Geist et al., 2013; Zheng et al., 2014): (i) the method depends on high-quality critical reagents to obtain selectivity and it is highly challenging to achieve sufficient selectivity that unambiguously identify specific amino acid sequenes; (ii) the development process is often time-consuming and cost-prohibitive; (iii) the linear range of the calibration curve is narrow (typically within 100-fold), which is inadequate to cover the dynamic range required for a regular PK study; (iv) high susceptibility to matrix interference and the method is usually matrix-selective; for example, a method developed for plasma samples cannot be easily transferred for use in tissue samples. Given these drawbacks, substantial efforts have been devoted in developing alternative methods.

LC/MS has long been a powerful method for selective (sequence-level with bottom-up strategy), accurate, sensitive, and rapid analysis of small-molecule drugs and biomarkers (Hsieh & Korfmacher, 2006), and has recently been introduced for the quantification of protein drugs. Although it is possible to quantify proteins with LC/MS on the intact-protein level, the vast majority of current methods are performed on peptide level via LC/MS operated in the selected reaction monitoring (SRM) mode (Heudi et al., 2008; Pan et al., 2009; Duan et al., 2012a; Furlong et al., 2012; Ouyang et al., 2012; Yuan et al., 2012; Li et al., 2013), largely because peptide-level protein quantification with LC/MS provides substantially higher sensitivity, accuracy, and robustness than protein-level quantification (Blackburn, 2013; Hopfgartner, Lesur, & Varesio, 2013). Consequently, the discussion of protein drug quantification in the following sections will be emphasized on LC-SRM. The rationales and characteristics of LC-SRM had been discussed in previous reviews (Damen, Schellens, & Beijnen, 2009; van den Broek, Niessen, & van Dongen, 2013; An, Zhang, & Qu, 2014a).

LC-SRM has several attractive features over LBA, including rapid method development, wider dynamic range, minimal matrix dependence (e.g., a developed quantitative method can be readily transferred among plasma and different tissues), and high multiplexing ability (Huang et al., 2005; Jemal & Xia, 2006; Duan et al., 2012a; Li et al., 2012; Liu et al., 2013a; van den Broek, Niessen, & van Dongen, 2013).

Although LC-SRM has already been demonstrated to be a highly promising method for biotherapeutics analysis, an optimal strategy with high sensitivity, selectivity, throughput, and robustness remains elusive. Figure 2 illustrates the performance of LBA and various LC/MS-based methods for protein-drug analysis. For example, low-flow-LC/MS (e.g., nano-LC/MS) and immunoaffinity (IA)-LC/MS have been developed to significantly enhance sensitivity (Ackermann & Berna, 2007; Gama, Collins, & Bottoli, 2013), but at the cost of compromised reproducibility and throughput, as well as longer time for method development, unless a well-established streamlined workflow is available. The following section will discuss analytical challenges on quantification with LC/MS approach and recent technical advancements that attempt to enhance selectivity, sensitivity, robustness, and throughput by addressing specific issues along the workflow.

B. Analytical Challenges in Quantification

Rapid progression of the discovery and development of biotherapeutics necessitates methods for quantification in biomatrices (tissues and bodily fluids) with sufficient specificity, accuracy, sensitivity, reproducibility, and throughput (Leary et al., 2013; Bults, van de Merbel, & Bischoff, 2015; Chilewski & Jiang, 2015; Shen, Liu, & Zhao, 2015); nonetheless, such method has been challenging to achieve and some key difficulties are discussed below.

First, the most-prominent challenge is insufficient sensitivity. The high potency and specificity of biotherapeutics often enables the use of relatively low therapeutic doses that result in low systemic concentrations of drug. Moreover, tissue concentrations are often tens or hundreds of times lower than plasma concentrations, and thus require ultra-sensitive methods (Duan et al., 2012a; Neubert et al., 2012). LBA methods are often not readily adaptable to tissue analysis; however, LC/MS assays is more easily applied, because LC/MS is less prone to matrix-related interference. However, for LC/MS-based protein quantification, insufficient sensitivity is a major concern due to two reasons: (i) the response of electrospray ionization (ESI)-MS is dependent on the molarity rather than mass concentration of analytes (Fred Banks, 1996; Mitulovic et al., 2003); hence, the large molecular weight (MW) of a therapeutic protein poses a substantial disadvantage; (ii) because of the high protein contents in most biomatrices (e.g., typically 30–80 mg/mL proteins in plasma and 30–200 mg/g proteins in various tissues (Addis et al., 1940; Zaia, Verri & Zaia, 2000; Zaias et al., 2009), it is always necessary to dilute a biosample (often >10-fold) before digestion and LC/MS analysis. The combination of these two factors significantly decreases sensitivity for LC/MS-based protein analysis. For a hypothetical example: if the limit of quantification (LOQ) of a small-molecule drug (MW = 200) was 0.5 ng/mL in plasma by LC/MS, then assuming a mAb drug has the same molar response as the small-molecule and

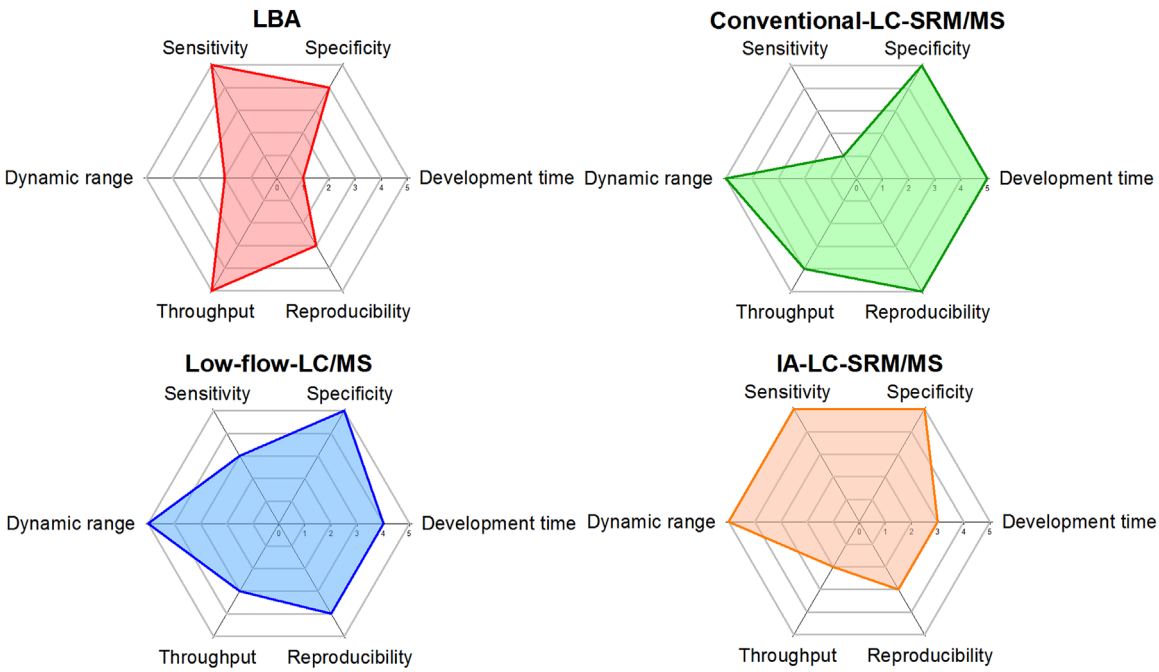

FIGURE 2. Radar chart illustration of the performances of typical LC-SRM-based methods and LBA for quantification of biotherapeutics. For each specification, scores of 0–5 represent low to high performance. For example, although conventional-flow LC-SRM method is superior to LBA in terms of development time and wider dynamic range, it falls short in sensitivity; while methods such as low-flow-LC and IA-LC-SRM can significantly improve sensitivity, the throughput and reproducibility are compromised compared with conventional LC/SRM-MS.

assuming that plasma samples require 15-fold dilution before LC/MS analysis, the LOQ of the mAb becomes >2.5 μg/mL, which is not sensitive enough for a typical PK study.

Second, in typical plasma or tissue samples, a protein drug often presents at relatively low levels among a highly complex matrix proteome that encompasses thousands of different proteins. Consequently, it is challenging for any analytical method to selectively quantify the target protein in biomatrices, especially when considerable endogenous interferences exist. The heterogeneous *in vivo* biotransformations (modifications, truncation, metabolism, etc.) of the drug further complicate the problem (Hagman et al., 2008; Jenkins, Murphy, & Tyther, 2008; Ezan, Dubois, & Becher, 2009; Pendley & Shankar, 2011; van den Broek, Niessen, & van Dongen, 2013). Moreover, in many pharmaceutical investigations, it is important to selectively quantify biotherapeutics in various forms, (e.g., free vs. bound, fragmented vs. intact, etc.), which is difficult to achieve with either LBA or LC/MS alone.

Third, highly accurate determination of the absolute levels of a protein drug in bio-matrices is critical for drug discovery, development and evaluation; for example, PK and TK studies. Nonetheless, quantitative accuracy of protein drug can be compromised by insufficient selectivity and limitations in analytical methods. For example, LC/MS and LBA both target a limited epitope or sequence domain rather than the entire protein. It would be difficult to discriminate metabolic/degradation fragments containing the selected epitope or sequence from the intact protein; for LC/MS technique, the conversation of protein to signature peptide is not 100%. Consequently, the signal response of the epitope or sequence may not properly reflect the quantity of intact protein, unless optimal calibration and validation procedures are used to avoid severe quantitative biases (Nouri-Nigjeh et al., 2014).

Finally, matrix effect might severely interfere with sample preparation and analysis of protein drugs (van den Broek, Niessen, & van Dongen, 2013). It is well-known that quantitative performance of LBA is often profoundly affected by biological matrices (Pendley & Shankar, 2011; Gorovits et al., 2014). Although LC/MS has enhanced selectivity over LBA, matrix effect are also a pronounced problem for LC/MS-based methods. Specifically, a large amount of matrix peptides can be generated from a plasma or tissue sample, which might cause ion suppression along with other non-protein matrix components (e.g., lipid and phospholipids (King et al., 2000; Shen et al., 2005).

C. Emerging Technical Advances to Improve the Performance of LC-SRM-Based Quantification of Biotherapeutics

This section discusses the recently developed approaches that improve method development, specificity, accuracy, sensitivity, robustness, and throughput for protein biotherapeutics quantification. These methods are summarized in Figure 3.

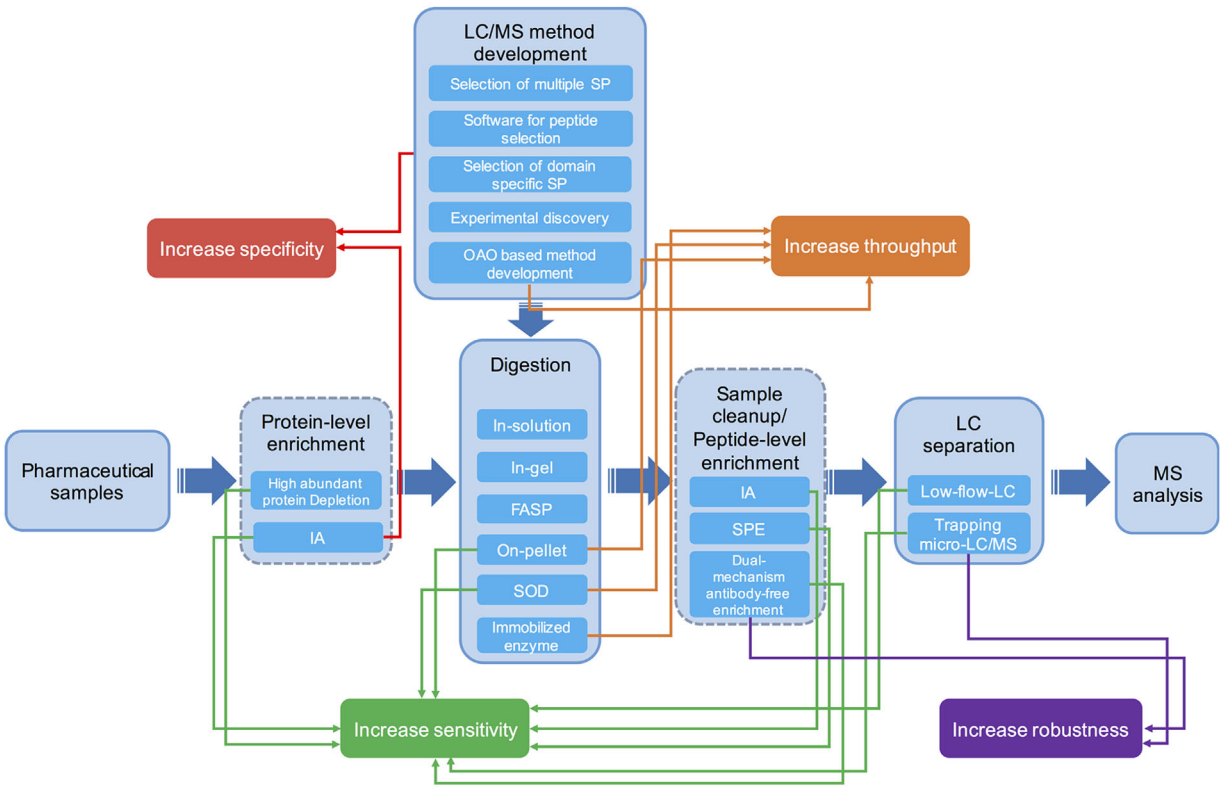

FIGURE 3. General workflow for LC/MS quantification of biotherapeutics in biomatrices and the summary of recent technical advances improving the performances. LC/MS method development is often the first step, since the SP selection and sensitivity/selectivity evaluation in this step often determines sample preparation procedures. Steps framed in dash line represents steps that may be optional depending on the needs of a specific project.

1. *Advancements in Quantitative Method Development*

Though it is relatively easy to develop a LC-SRM method to quantify of biotherapeutics, it remains challenging to establish optimized methods that afford the reliability, sensitivity, and reproducibility required for collection of PK/PD and TK data. For example, the initial step to establish a LC-SRM-based quantitative method is to determine the optimal signature peptide (SP) that can quantitatively represent the target biotherapeutic. This step is critical to ensure specific, sensitive, and robust quantification; nonetheless, selection of the best SP that carries high specificity, sensitivity, and stability among many enzymatic and domain choices is not straightforward. To address this need, a number of *in silico* prediction and experimental approaches, or a combination of the two, have been established over the past several years.

In silico prediction approaches are widely employed to facilitate the selection of SPs and method development with minimal wet-lab efforts. Popular tools in this regard include Skyline, PeptideAtlas, and MRMaid (Mead et al., 2009; Cham Mead, Bianco, & Bessant, 2010; Maclean et al., 2010; Halquist & Thomas Karnes, 2011; Rauh, 2012). These approaches substantially expedite and simplify method-development process, and greatly promote the application of LC/SRM-MS to quantify proteins. Reliance on *in silico* prediction alone might not achieve an optimal method development, because such an approach might not completely predict the efficiencies for enzymatic cleavage, ionization/fragmentation, and peptide stability, nor matrix-dependent features such as chemical interferences of a particular peptide candidate (Pan et al., 2009; Cao et al., 2010; Duan et al., 2012a). To address this problem, we developed an experimental-based procedure that enables rapid and streamlined method development for sensitive, selective, and reliable quantification of mAbs in multiple tissue matrices and plasma (Cao et al., 2010; Duan et al., 2012a). The workflow for this procedure is shown in Figure 4. A high-throughput, on-the-fly orthogonal array optimization (OAO) approach was employed, which can develop the optimal LC/SRM-MS method for many SP candidates within a ***single*** LC/SRM-MS analysis. This method enables extensive evaluation of all potential SP candidates (>20) in target matrices, and thus ensures the discovery of the best SP carrying

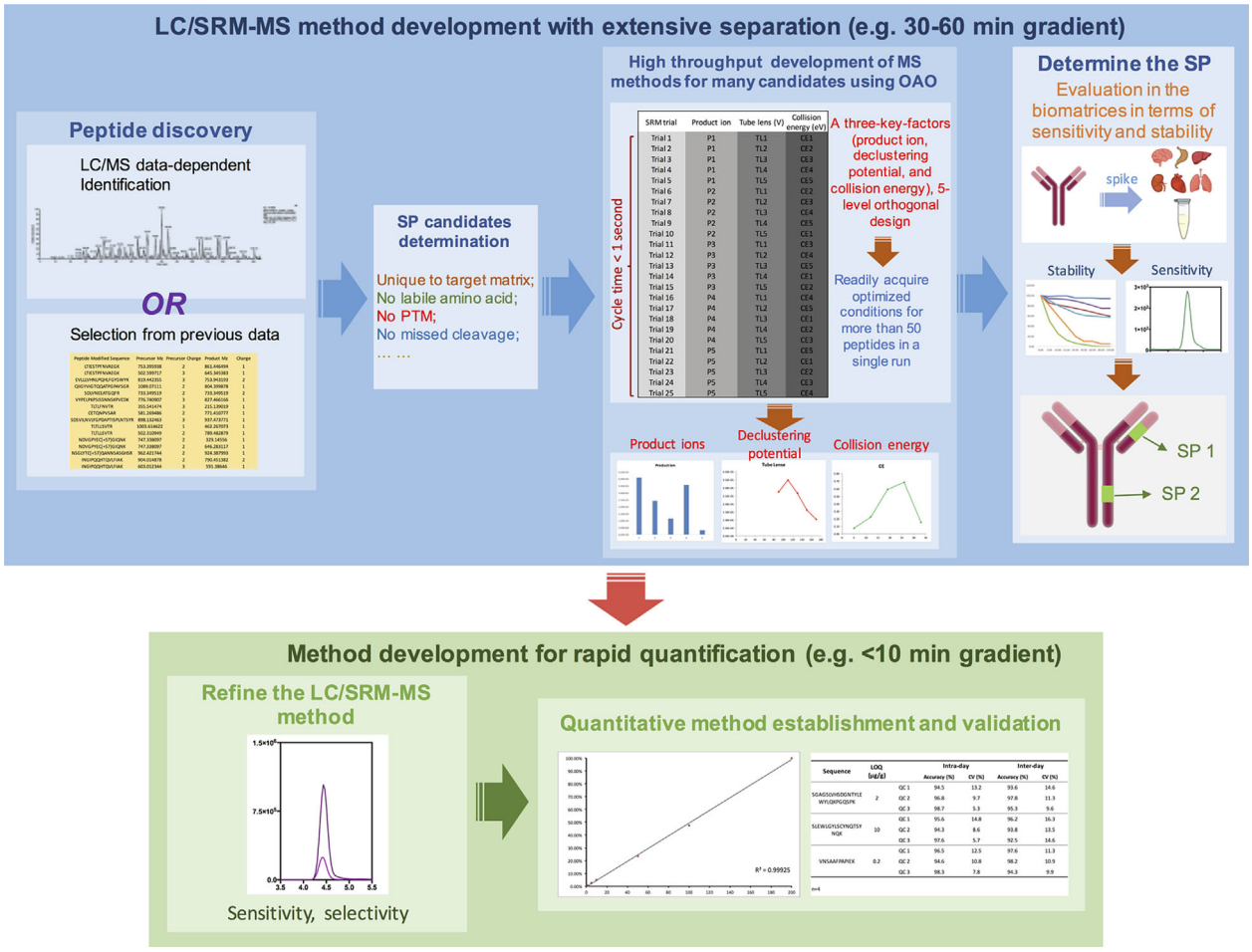

FIGURE 4. The procedure of an on-the-fly Orthogonal Array Optimization strategy, an experimental procedure for high-throughput and accurate development of LC/SRM-MS method (Cao et al., 2010; Duan et al., 2012b). This approach is capable of reliable development of LC/SRM-MS methods for many SP candidates in a single run; then the candidates are evaluated in digested matrixes for sensitivity and stability to determine the optimal SP; two SP are selected for quantification to enhance quantitative reliability.

the optimal selectivity and sensitivity in specific pharmaceutical samples. In previous works, moreover, this strategy does not need synthesis of multiple potential SP candidates in order to select a SP, and thus saves both costs and time.

Given the limitations of prediction models, more software packages combine *in silico* prediction and experimental optimization for method development. For example, Skyline, the most widely used tool for LC/SRM-MS method development, employs a comprehensive *in silico* prediction for potential SP candidates and SRM parameters, followed by extensive experimental validation of these candidates (Maclean et al., 2010). Such strategies efficiently increase the chance of success compared with those that rely on prediction alone.

2. *Improvement of Specificity*

The selectivity of a quantitative method is profoundly affected by the selection of the SP. Choosing a peptide with a sequence unique to the target species and free of endogenous interference is necessary to ensure specificity. For the same biotherapeutics molecule, the choice of an optimal SP is highly species-dependent. For instance, for analysis of a human mAb in a preclinical animal model, many SP candidates can be chosen from the human IgG constant region; nonetheless, the same drug can only be quantified with SP from the very few domains within the variable regions for clinical investigation with human subjects. Software packages such as Skyline greatly facilitate selection of SP using the combination of *in silico* prediction and experimental validation (Bereman et al., 2012), as described above.

Because the selected SP domain might be modified, truncated, or exist in various metabolite/catabolite fragments, use of one SP for quantification might carry the risk of poor selectivity (Hoofnagle & Wener, 2009). We and others have addressed this problem by utilizing two SPs from distinct domains to ensure the specific quantification of protein molecules with structural integrity (Li, Fast, & Michael, 2011; Duan et al., 2012a; van den Broek, Niessen, & van Dongen, 2013). Examination of the discrepancy between the quantitative results obtained independently from two SPs, the above-mentioned problems can be readily detected (Duan et al., 2012a, b; Jenkins et al., 2015). Additionally, the use of multiple SPs can also help to obtain information on the different forms of a protein drug *in vivo* (e.g., intact and catabolic products). For example, if the monitored peptide is from an epitope that is susceptible to *in vivo* proteolysis, then quantitative data of this SP may reveal the *in vivo* and *in vitro* stability (Hager et al., 2013). On the other hand, if an SP is from a relatively stable region, then the corresponding quantitative result likely reflects intact and truncated forms. In this way, the combination of a primary SP with multiple secondary SPs from different regions enables selective evaluation of the structural integrity of the protein analyte (Jenkins et al., 2015).

Moreover, recent work demonstrated that the hybrid LBA-LC/MS method (i.e., reagent enrichment of the target biotherapeutics followed by LC/SRM-MS analysis) can enable highly selective analysis due to the combined specificity from the two methods (Hager et al., 2013). Lee and co-workers successfully used this method to selectively quantify free and total mAb (Lee et al., 2011).

3. *Improvement of Sensitivity*

As mentioned above, insufficient sensitivity is the most-prominent challenge for LC/SRM-MS-based quantification of biotherapeutics. In most cases, conventional LC/SRM-MS might not achieve comparable sensitivity to a well-developed LBA method (Keshishian et al., 2009). Although the sensitivity of LBA depends on the quality and specificity of critical reagents, sensitivity of LC/SRM-MS is determined by not only the characteristics of the SP, but also factors like sample preparation (e.g., pretreatment, enrichment, and digestion) and chromatographic approaches. Correspondingly, efforts to enhance the sensitivity for LC/SRM-MS mainly focus on the improvement of sample preparation or chromatography, as discussed below.

a. Efficient production of an SP

Because the vast majority of LC/SRM-MS-based methods for protein quantification rely on the analysis of proteolytic peptides, it is important to achieve high recovery of the SP from the target protein via enzymatic digestion. Three conventional methods are used for this purpose, including in-solution-digestion (Olsen et al., 2006), gel clean-up followed by in-gel-digestion (Shevchenko et al., 2006), and filter-aided-sample-preparation (FASP) (Wisniewski et al., 2009). The in-solution-digestion is quite straightforward to use; proteins are digested directly in diluted biological samples, with or without mild denaturation with urea (Li et al., 2013). One drawback of this approach is that all matrix/buffer components remain in the digest, which necessitates a following cleanup step such as solid phase extraction and often leads to loss of peptides (Choksawangkarn et al., 2012; van den Broek, Niessen, & van Dongen, 2013). On the contrary, in-gel-digestion efficiently removes matrix components and simplifies the samples (Havlis & Shevchenko, 2004); however the procedure is relatively laborious and time-consuming, which limits large-scale applications such as those required in PK analysis. Most importantly, the incomplete digestion and gel-absorption of peptides often result in low peptide recovery. The FASP method also enables clean digestion by removing small-molecule matrix/buffer components with a molecular-weight-based cutoff filter, followed by digestion of the retained proteins on the filter (Wisniewski et al., 2009). However, certain compounds (e.g., phospholipids) that potentially undermine digestion and LC/MS analysis cannot be completely removed (Hustoft et al., 2011), and the approach often suffers from low and variable peptide recoveries likely due to in-filter absorption of protein/peptides (Choksawangkarn et al., 2012).

To address these problems, our laboratory developed a straightforward precipitation/on-pellet-digestion method that affords high and reproducible protein/peptide recoveries for both proteomic profiling and absolute quantification in complex tissue/plasma samples (Duan et al., 2009, 2012a; Tu et al., 2012, 2013; Nouri-Nigjeh et al., 2014). Several independent studies also demonstrated comparable or better peptide recovery with on-pellet digestion comparing to in-solution digestion for targeted protein quantification (Ouyang et al., 2012; Yuan et al., 2012).

More recently, we devised an optimized surfactant-aided-precipitation/on-pellet-digestion procedure (SOD) that provides high and reproducible peptide recovery with a rapid digestion (An et al., 2015a). Briefly, a relatively high concentration of surfactant (or mixture of surfactants) was used for extensive denaturation, reduction, and alkylation of target proteins, which also inactivates endogenous protease-inhibiting proteins and helps to eliminate matrix compounds such as lipids. An organic solvent precipitation was employed to effectively remove detergents and endogenous compounds, followed by a rapid complete on-pellet-digestion. The method offerred substantially higher sensitivity and reproducibility than other approaches to quantify mAbs in plasma and tissues (Fig. 5). Because of the extensive denaturation with surfactants and precipitation, complete digestion of mAb was achieved in only 45 min at 37˚C. Moreover, the 37˚C-digestion afforded significantly higher digestion efficiency than higher temperatures, a common practice to speed up digestion. Finally, the efficient sample cleanup with SOD affords a highly robust sample preparation (An et al., 2015a).

b. Increase the relative concentration of target: Depletion and enrichment

One of the most-efficient approaches to improve sensitivity is to preferentially increase the relative concentration of the target in a biological sample. To this end, two categories of methods have been developed: depletion of matrix proteins and enrichment of the target. The latter category includes methods to enrich at protein- or peptide-levels, or both.

Depletion of high-abundance matrix components before digestion might considerably increase sensitivity, because such a strategy not only increases the ratio of target protein versus total proteins in sample, but also might benefit downstream sample preparation and LC/SRM-MS analysis by enabling more-efficient digestion, higher loading capacity, and decreased chemical noises and ion suppression (Anderson & Hunter, 2006; Liu et al., 2014; Shen, Liu, & Zhao, 2015). Due to technical

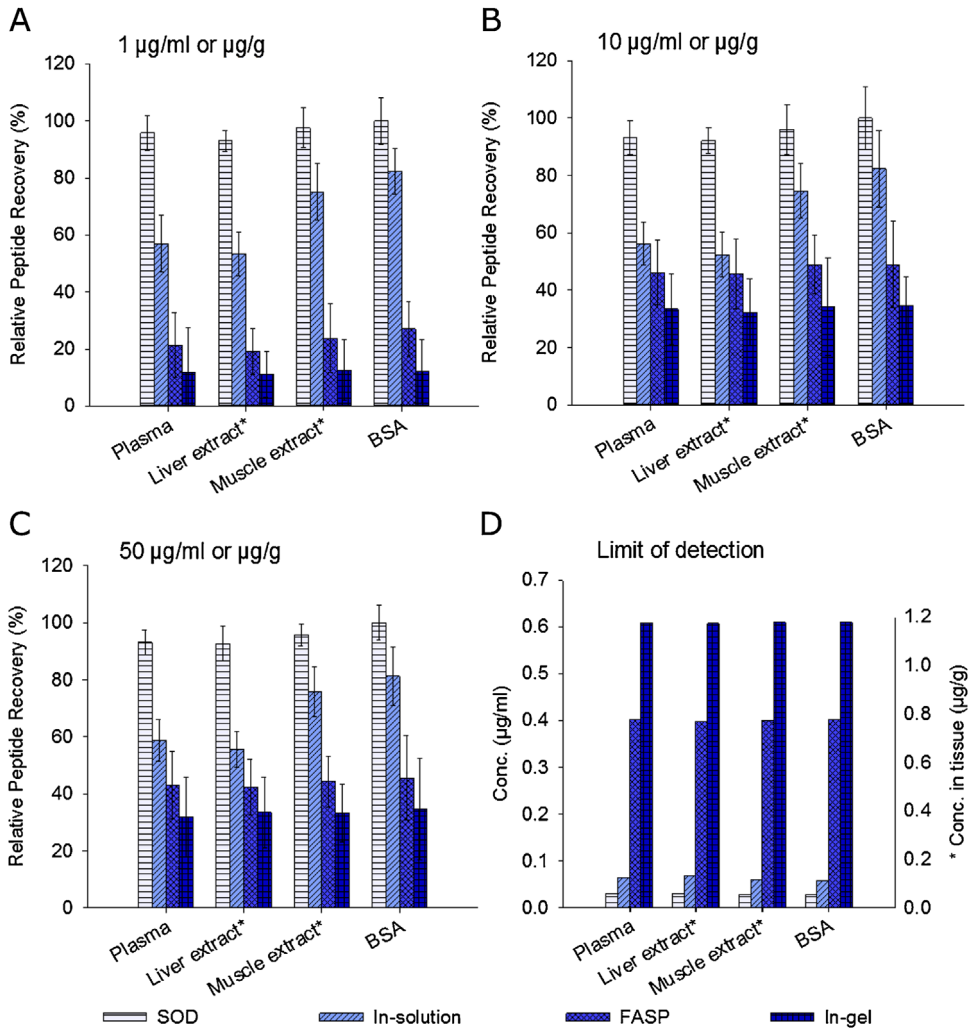

FIGURE 5. Comparison of the peptide recovery, reproducibility, and LOD of SOD versus three prevalent approaches, in-solution-digestion, in-gel-digestion, and FASP in different matrices. The LOD was defined as the concentration yielding an S/N = 3 (D). (Reprinted with permission from (An et al., 2015a), copyright, 2015, Analytical Chemistry).

limitations, such strategies are mainly applied for plasma/serum samples. Anderson and Hunter (2006) used a multiple affinity removal system ("MARS") to deplete the six highest-abundance proteins from human plasma. Liu et al. (2014) established a simple and cost-effective method to remove albumin from plasma sample by precipitation with mixture of isopropanol and trichloroacetic acid. The authors removed albumin successfully, while retain the targeted therapeutic protein (BMS-C), which resulted in a four to fivefold improvement on sensitivity. Shi et al. (2012b) established a tandem IgY14-Supermix depletion method to remove the top 14 high-abundance and up to 60 moderate-abundance plasma proteins, to enable analysis of low-abundance proteins in human plasma. All of those studies demonstrated that high-abundance protein depletion improves the limitation of detection (LOD).

LC/SRM-MS analysis after immunoaffinity (IA) enrichment of a target protein is an efficient approach to improve sensitivity. Quite a number of methods have been developed based on this philosophy (Ackermann & Berna, 2007; Dubois et al., 2008; Fernandez Ocana et al., 2012; Li et al., 2012; Neubert et al., 2013; Xu et al., 2014), and several typical works are exemplified here. Ho et al. (2011) realized a highly sensitive quantification of five exogenous insulins in equine plasma (LOD of 0.05 ng/mL) with IA enrichment and LC/MS analysis. Dubois et al. (2008) used a specific antibody to capture cetuximab from human plasma prior to LC/SRM-MS detection to result in a LOQ comparable to those achieved with ELISA methods. Li et al. (2012) established an immnocapture strategy to enable highly sensitive analysis of four IgG1 and four IgG2 mAbs. Though such strategies are sometimes dubbed as the "hybrid LBA" (LB-LC/MS), it should be well-recognized that, unlike traditional LBA methods, a highly selective IA reagent is generally not necessary because of the high selectivity of the downstream LC/SRM-MS. Consequently, less costs and time are usually required for method development, compared to a conventional LBA method (Onami et al., 2014; Xu et al., 2014). For instance, Xu et al. (2014) reported a successful immunocapture-LC/MS method that used two anti-idiotypic reagents to sensitively investigate mAbs in human serum. Interestingly, the very same reagents did not work for LBA. Moreover, when properly designed, IA capture of proteins can reveal valuable activity and structural information along with quantitative results. Detailed benefits of IA-LC-SRM were discussed in a previous review (Neubert, Palandra, & Fernandez Ocana, 2014). As an example, LB-LC/MS had been adopted in a biotransformation study of Fc-fusion protein, which facilitated the identification and quantification of intact and truncation products *in vivo* (Hager et al., 2013).

Although protein-level enrichment promises to improve LC/MS sensitivity, and the critical reagent is often relatively easy to produce, one potential drawback is variability in the enrichment step, which might not be perfectly quantitative and reproducible (Li et al., 2012). The enrichment might introduce bias and variation, which often necessitates a stable-isotope-labeled (SIL) full-length protein internal standard (IS) to be spiked in before enrichment. SIL proteins are expensive to produce and often not available for a biotherapeutics. Recently, some exciting developments have been made to address this reproducibility issue. For example, using a streamlined, reproducible procedure for protein capture, denaturation, reduction and rapid digestion, Neubert et al. (2013) and Keyang et al. achieved excellent quantitative accuracy and precision (<15% CV and error) (Xu et al., 2014). By comparison, enrichment at peptide-levels is often less liable to the variation problem, because a SIL signature peptide IS, which is readily obtained, can be used prior to enrichment. Although protein-level enrichment does not work well in circumstances such as in tissue extracts that contain highly denaturing buffers, peptide-level enrichment is less likely to suffer from this problem as it is often straightforward to render a digest antibody-friendly. One important paradigm for this technique is the "stable isotope standards and capture by anti-peptide antibodies" (SISCAPA) developed by Anderson et al. (2004), which employs an anti-peptide antibody to enrich the target peptide from the digest. In one application, the authors reported 1,800–18,000-fold peptide enrichment of α-1-antichymotrypsin and lipopolysaccharide-binding protein from a human plasma digest (Anderson et al., 2009). Several other reports that described optimized peptide IA enrichment technique also demonstrated significant sensitivity improvement (Whiteaker et al., 2010, 2012; Kuhn et al., 2012). More recent works employed both protein- and peptide-level enrichment to further enhance sensitivity. Neubert et al. (2013) developed an ultra-sensitive method for human β-nerve growth factor, utilizing sequential protein and peptide IA captures and achieved an LOQ of 7.03 pg/mL in human plasma. Despite the above merits of the peptide IA enrichment, one major problem is that generation of an efficient anti-peptide antibody is often challenging largely due to the low immunogenicity of a peptide (Shen, Liu, & Zhao, 2015).

Currently, an antibody-free enrichment method is under development. One approach is fractionation with high-pH or strong cation exchange (SCX) chromatography (Shi et al., 2012a; Yang et al., 2012; Betancourt et al., 2013). Though these methods could significantly increase sensitivity, extensive HPLC fractionation is required, which severely limits the throughput. Yuan et al. used SPE to increase the abundance of target peptides to enable high-throughput enrichment (Yuan et al., 2013). We developed a method for dual-mechanisms enrichment of SP in complex digestion with sequential SCX and high-pH-RP separations on the same cartridge. The method selectively enriched the target SP, eliminated the majority of matrix components, and increased sensitivity by >20-fold (An et al., 2015b).

c. The use of low-flow-LC/MS

Utilization of low-flow-LC generally increase the sensitivity of LC-SRM-based quantification (Arnold & Needham, 2013; Gama, Collins, & Bottoli, 2013; Lassman & Fernandez-Metzler, 2014). ESI is the most-used interface for LC/MS-based protein quantification, which is a concentration-dependent detector as discussed previously. The use of low-flow-LC/MS efficiently boosts signal intensity by increasing peak concentration and ionization efficiency. Lesur, Varesio, and Hopfgartner (2010) developed a capillary LC-SRM method to quantify tryptic peptides of a recombinant human mAb utilizing a capillary column with 1 mm inner diameter (ID) to enhance sensitivity. Liu et al. (2011) established a low-flow LC-SRM method (flow rate at 50 μL/min) combined with IA enrichment; the method achieved sensitive quantification of recombinant mAb in monkey serum. To further improve sensitivity over these, we have developed a sensitive and reproducible nano-flow LC/SRM-MS strategy (flow rate at 250 nL/min) that typically

lowered the LOQ by 30- to 50-fold compared to a conventional-flow LC/SRM-MS. A unique flow configuration enabled consistent and relatively robust analysis of plasma and tissue samples (Duan et al., 2012b). A number of other reports also employed nano-flow-LC/SRM-MS for sensitive quantification of proteins (Li et al., 2012; Neubert et al., 2013). It is noteworthy that most of these low-flow-LC based methods have been coupled to procedures that simplify the matrix prior to LC/MS (e.g., SDS–PAGE or IA separation to remove matrix components), in order to ensure sensitivity and robustness. This is largely because the loading capacity of complex biological samples on low-flow-LC is limited (Lassman & Fernandez-Metzler, 2014). To address this limit, we recently developed a trapping micro-LC/MS (Tμ-LC/MS) method to enable loading of large amounts of biological samples without exceeding the system capacity. A selective trapping/delivery approach strategically eliminated most of hydrophobic and hydrophilic matrix components/peptides and concentrated the target peaks prior to the downstream micro-flow-LC/MS analysis. The method is straightforward, and achieves high sensitivity comparable to a nano-LC/MS, with the comparable robustness and throughput to conventional-flow-LC/MS (Zhang et al., 2015). These features render the technology applicable to large-scale analysis where high sensitivity is essential.

4. Improvement of Throughput Through Sample Preparation

Analytical throughput is frequently an important consideration for the selection of bioanalytical platform for a preclinical or clinical study. The major limitations of the throughput of LC-SRM-based protein analysis are associated with the speeds of sample preparation and LC/MS analysis, and sample preparation is often one of the main rate-limiting steps. For example, in some preparation procedures, enzymatic digestion must be run overnight to reach completeness (Capelo et al., 2010; Hustoft et al., 2011; Choksawangkarn et al., 2012). Several accelerated digestion approaches have been reported. For example, Hervey, Strader, and Hurst (2007) showed that 80% acetonitrile could attain completion of digestion of protein samples in 1 hr. Wang et al. (2008a) established an IR-assisted on-plate proteolysis approach, which can achieve efficient protein digestion in 5 min. Lesur, Varesio, and Hopfgartner (2010) described digestion of a therapeutic human mAb in 30 min with microwave-assisted protocol. In addition, Yang et al. (2010) have reported a pressure-assisted tryptic digestion, which achieved a higher peptide recovery in 30 min at 37°C than overnight atmospheric digestion. It should be noted that digestion of a therapeutic protein such as an mAb is generally more difficult than other proteins due to the unique, complex structure of IgG (Yuan et al., 2012). With the aforementioned on-pellet-digestion protocol, Yuan et al. (2012) observed efficient release of the selected target peptides after 30 min for the model mAb. With the SOD protocol described above, we achieved highly efficient and reproducible digestion of several humanized mAbs in plasma and tissues within 45 min, to significantly shorten the sample-preparation time and maintain very high and reproducible peptide recovery (An et al., 2015a). Moreover, technologies such as immobilized enzymatic digestion are developed and commercialized (e.g., the Flash Digest™ kit by Perfinity and SMART Digest™ kit by Thermo), which could complete the digestion within 1 hr. The applicability of such methods to absolute quantification of biotherapetuics in biological samples has yet to be evaluated.

Additionally, developments on high-throughput LC/MS anlaysis have also been made in recent year. These techniques were discussed in other sections in this review.

5. Improvement of Robustness

Regarding the LC/MS analysis, usually a high-flow chromatography (e.g., flow rate >200 μL/min) is used to achieve robust analysis. Nonetheless these approaches suffer from low sensitivity. As mentioned in a previous section, we developed a Tμ-LC/MS method enabling highly sensitive analysis (e.g., LOD of 15–45 ng/g for mAbs in various tissues without enrichment) with high throughput (<10 min/sample) and good robustness (continuous analysis of >800 samples) recently (Zhang et al., 2015). In addition to such on-line strategy to increase robustness, SPE-based off-line cleanup post-digestion was also developed and utilized for protein analysis with LC/MS-based method (Yang et al., 2007; Heudi et al., 2008; Winther et al., 2009; Yuan et al., 2013), which could remove matrix components before LC/MS analysis to enhance robustness. In this regard, we developed a dual-mechanism, antibody-free enrichment method, to specifically enrich target peptide while significantly eliminating the sample matrix (An et al., 2015b). This method substantially removed matrix components and non-target peptides to greatly promotes analytical robustness.

6. Improvement of Accuracy

In many applications of LC/SRM-MS-based assays, such as biomarker validation, the quantitative accuracy in terms of absolute levels (e.g., concentrations in plasma or tissues) might not be highly critical, as long as good accuracy for relative quantification (e.g., how many-fold change between groups) is achieved. By comparison, highly accurate absolute quantification is required in other applications, such as the assay of plasma samples for PK/PD investigations (DeSilva et al., 2012; Chilewski & Jiang, 2015); however, this requirement is occasionally underappreciated.

Selections of calibrator and IS profoundly affect the accuracy for absolute quantification of proteins. For instance, synthesized peptides as the calibrator and SIL peptides as IS were usually employed for LC/MS-based protein quantification (Bronsema, Bischoff, & van de Merbel, 2012; van den Broek, Niessen, & van Dongen, 2013), largely due to the fact that peptide standards and IS are easily available at a reasonable price. It turns out that the accuracy of this kind of calibration method is low because the errors and biases in pretreatment and digestion procedures are not assessed appropriately (van den Broek, Niessen, & van Dongen, 2013; Nouri-Nigjeh et al., 2014). We have observed a severely negative bias for a peptide-level calibration method in quantification of an mAb in plasma (Nouri-Nigjeh et al., 2014); we deduced that the assumption of complete peptide recovery during sample treatment and enzymatic digestion might not be true (Picotti et al., 2009). Extended-peptides that contain an SP sequence with three to six amino acids residues on N- and C-termini have been introduced to compensate for digestion efficiency (Ocana & Neubert, 2010;

Rauh, 2012; Simpson & Beynon, 2012; Kushnir et al., 2013). Although use of such extended-peptide calibrators could adjust for the digestion efficiency to some extent, differences in protease accessibility and digestion rates between these extended-peptides and the intact protein still make the correction inadequate (Brownridge & Beynon, 2011; Nouri-Nigjeh et al., 2014). Full-length protein calibrator and SIL full length protein IS are the most ideal standards for absolute quantification, and could completely correct the variance and bias during sample pre-treatment, digestion, and analysis (Brun et al., 2007; Heudi et al., 2008; Li et al., 2012; Picard et al., 2012). However, because SIL-proteins are expensive to produce and often not available for the target protein, our laboratory and others have practiced accurate quantification of regulatory proteins and protein drugs in plasma and tissues using "hybrid" calibration strategies (Cao et al., 2010; Duan et al., 2012a,b; Jiang et al., 2013). With full-length protein calibrator and SIL- peptide or extended peptide IS, the hybrid calibration strategy can achieve precise and accurate quantification as long as a highly reproducible sample preparation is achieved (Nouri-Nigjeh et al., 2014). As a result, the hybrid calibration represents a cost-effective alternative to protein-level calibration.

D. Applications in Biotherapeutics Quantification

In the past decade, many studies on LC-SRM-based protein quantification have been reported. Here, we show some representative studies with an emphasis on quantification of mAb and ADC.

1. Plasma PK Study

LC-SRM method had been utilized in PK studies across different species. Heudi et al. (2008) developed a quantitative method to characterize a candidate mAb in marmoset serum from a PK study, and used SPE cleanup after digestion and a SIL-full-length protein as IS. That report is one of the earliest works on LC/MS-based quantification of protein therapeutics. By comparing with the results from a specific LBA, the authors suggested that their LC/MS method quantified total mAb rather than the free form. Li et al. (2012) developed a universal LC-SRM approach to quantify a variety of therapeutic mAbs based on the use of a full-length SIL mAb as the universal IS in rat and monkey serum. The method was successfully applied in a rat PK study. The authors employed the same critical reagent for LBA and immunocapture followed by LC/MS quantification, and found that the PK profiles obtained by the two methods were similar. With an albumin-depletion approach, Hagman et al. (2008) developed an LC/SRM-MS method to quantify a human mAb in the serum of cynomolgus monkey with improved sensitivity. The work proved the advantages of LC/MS-based method over LBA in terms of reduced inter-assay variance and time required for method development and validation. Dubois et al. (2008) captured cetuximab via its pharmacological target, followed by low-flow-LC-QTOF-MS/MS analysis in human plasma, resulting in sensitive quantification of the target mAb in clinical samples, and achieved a LOQ as low as 20 ng/mL. We established a sensitive nano-LC/SRM-MS method for quantification of a chimeric mAb (cT84.66) in mouse serum. Because of the high sensitivity and selectivity achieved, the method was successfully applied to low-dose preclinical PK study via intravenous and subcutaneous injections (Duan et al., 2012b). Ouyang et al. (2012) described an LC/SRM-MS for reproducible analysis of a mAb drug candidate in monkey plasma, which was facilitated by on-pellet-digestion protocol. The drug was quantified by monitoring one main SP and another confirmatory peptide simultaneously. The identical PK profiles of the two peptides suggested the high likelihood that the protein remained intact in circulation.

2. Determination of Tissue Distribution

To understand the ADME of biotherapeutics and evaluate safety and efficacy, it is important to investigate tissue levels. Moreover, the exposure of the drug at the target site is also of high interest, and thereby the quantification of biotherpeutics in tissues along with target antigen expression level is critical.

Compared to biotherapetuics quantification in plasma, in which the protein can be directly quantified, tissue analysis requires additional treatments and presents more challenges. Optimal procedures for tissue sample procurement, storage, extraction, clean-up, and digestion need to be developed; it could be difficult to correct and normalize biases and variations in these procedures and maintain high efficiency and reproducibility (Duan et al., 2012a; Neubert et al., 2012). One example among these unique challenges is the removal of residual blood from tissue. Because the circulating levels of a biotherapeutics are often tens or hundreds-times higher than tissue levels unless a tissue-specific target exists, residual blood must be effectively eliminated from the tissue to avoid a severe positive bias; however, this removal might be difficult to achieve without losing tissue-associated drug (Duan et al., 2012a). With a robust on-pellet digestion method coupled with nano-LC/SRM-MS, and an OAO strategy to facilitate method development, we demonstrated a sensitive quantification of two mAbs (8c2 and cT84.66) in major tissues (e.g., brain, heart, liver, spleen, kidney, and lung) with LOQ in the range of 0.15–0.30 μg/g (Duan et al., 2012a). The method has been used to evaluate tissue distribution of mAbs at "steady-state" after long-term, multiple-dosing regimens. The results suggested that knocking out FcγR did not significantly affect the tissue exposure of 8c2 in mice. Recently, Sleczka et al. (2014) established a sensitive LC-MS/MS assay for human mAb quantification in mouse tissues, which utilized a generic immunoaffinity enrichment to achieve an LOQ of 20 ng/mL in a variety of animal tissues. With this method, the authors concluded that there was no difference in tissue penetration between a novel molecule and ustekinumab.

3. Rapid Quantification to Facilitate Discovery and Development of Biotherapeutics

To expedite the discovery and development of biotherapeutics, rapid quantitative methods are often important. These methods include fast method development, high-throughput analysis (discussed above), and speedy screening of promising compounds.

At the initial development stage, it is important to quickly develop a bioanalytical platform to rapidly screen drug candidates for further development. A number of strategies to expedite method development are discussed above. Furthermore, in order to shorten the assay development time,

Furlong et al. (2012) developed a generic LC-MS/MS assay to quantify human mAbs or human Fc-fusion proteins in preclinical studies, which used "universal IgG4" peptides (Furlong et al., 2012, 2014). The authors selected the tryptic peptide TVAAPSVFIFPPSDEQLK from the light chain (κ class) and VVSVLTVLHQDWLNGK from the heavy chain (IgG1 and IgG4 subclasses) as common surrogate peptides for quantification, which are peptides shared by humanized IgG4 therapeutic mAbs. The method was applied to mAb quantification in human plasma samples with the presence of anti-drug antibody (ADA).

The multiplexing capacity of LC/MS enabled rapid compound screening. For example, cassette-dosing of multiple drugs to the same set of animals greatly reduced animal use and improved efficiency and speed of preclinical investigations. Jiang et al. (2013) used an LC/SRM-MS method to simultaneously quantify two co-administrated mAbs, which showed good quantitative performance for both targets in monkey plasma. The LC-SRM acquired PK profiles agreed well with those obtained by separate LBA analyses for each of the molecules. Li et al. (2014) reported quantification of four mAbs after subcutaneous co-administration, with LOQ of 0.1–0.5 μg/mL in plasma. The method obtained PK parameters for the four mAbs in a single study. Xu et al. (2014) developed an IA capture-aided LC/MS method to simultaneously quantify two mAbs in clinical study. Their results showed an evident dose-dependent response for both mAbs.

4. Application of Hybrid LBA-LC-MS Analysis

Hybrid LBA, or named as LB-LC/MS, refers to the application of LBA and LC-MS techniques successively or in parallel depending on the purpose of the study. (Ackermann, 2012; Neubert, Palandra, & Fernandez Ocana, 2014) The sequential approach is often employed to improve sensitivity, as discussed in previous sections. Additionally, sequential utilization of LBA and LC/MS methods helps to measure specific types of target in biomatrices. For instance, Neubert et al. (2008) developed a magnetic bead-based immunoprecipitation method to quantify an anti-drug antibody (ADA) with the existence of therapeutic proteins in monkey and human plasma, and considerably facilitated the immunogenicity investigation of an mAb. The parallel use of LBA and LC-MS can also reveal specific pharmaceutical information such as biological activity and molecular integrity of biotherapeutics (Szapacs, Urbanski, & Kehler, 2010). For example, LBA and LC/MS have been applied to determine separately the concentrations of mAbs and unconjugated drugs in ADC bioanalysis (Kaur, 2013). In a PK investigation, the free mAb, free targets, and mAb-target complexes can be measured with LBA; meanwhile, total mAbs in matrices can be measured by LC/MS. Fernandez Ocana et al. (2012) established an LC/MS-based work flow to quantify free and total anti-MadCAM mAb (PF-00547, 659) in human serum with anti-ID antibody and protein G enrichment strategies respectively. LBA and LC/MS could be used to obtain information about biotransformation, such as measuring intact drugs or metabolites/catabolites. Hager et al. (2013) developed differential ELISA and LBA-LC/MS methods to localize the specific labile sites on model Fc-fusion proteins. Using this strategy, they concluded that the re-engineered protein FGF21 RT was more resistant to degradation *in vivo* compared to the original form.

IV. QUALITATIVE CHARACTERIZATION

A. Challenges and Currently Available Strategies

Biotherapeutics are highly complex products that must be characterized by specifications such as primary sequence, secondary structure, and PTMs. In addition, various impurities with micro-heterogeneity in size, charge, and structure could be produced by enzymatic or spontaneous degradation and chemical modifications during production, purification, and storage processes (Srebalus Barnes & Lim, 2007). The most frequently occurring modifications of protein therapeutics are glycosylation, C-terminal lysine truncation, N-terminal glutamine cyclization, methionine oxidation, asparagine deamidation, aspartate isomerization, and hydrolysis (Harris et al., 2001; Lyubarskaya et al., 2006; Neill et al., 2015). Because these modifications might alter the stability of the product, half-life, and activity (Robinson & Jones, 2011), accurate and reproducible analytical platforms that can reliably evaluate these specifications in a high-throughput manner are required during development and production. Furthermore, such platforms are also critical in the development and production of biosimilars. Theoretically, it is impossible to produce absolutely identical copies of biotherapeutics between a biosimilar and its reference product, let alone that even for the same product lot-to-lot variations often occur. The goal of characterization is to confirm that there is no existing variant that alters safety, potency, and efficacy. For biosimilars, it is important to monitor amino acid sequence, PTMs, as well as tertiary and quaternary structure, in order to verify biological similarity (Xie et al., 2010; Chen et al., 2013).

MS-based technology, especially the use of high-resolution/-accuracy measurement, is a promising approach for biotherapeutics characterization; small shifts in mass caused by modifications such as oxidation (+16 Da), deamidation (+1 Da), and reduction of disulfide bonds (+2 Da) could be readily detectable (Parker et al., 2010). However, an optimal strategy to meet all the requirements remains challenging for a number of causes. To name a few: (i) biotherapeutics often have very large molecule weight with complex structures that contain multiple disulfide bonds and modifications (Fornelli et al., 2012). Consequently, the target is highly heterogeneous and often contains multiple species with various modifications, some with small mass shifts as low as 1 Da. Moreover, different modifications could occur simultaneously in one molecule; (ii) analysis of biotherapeutics on the intact-protein level (top-down, discussed below) is often required for accurate and comprehensive characterization, but often suffers from problems associated with low sensitivity, incomplete coverage, and ambiguous sequencing information; (iii) analysis of proteins in their native form is important in a number of circumstances (e.g., characterization of protein conformation or certain individual species of ADC), but difficult to achieve with ESI-MS (Rosati et al., 2013; Thompson, Rosati, & Heck, 2014); (iv) although it is theoretically possible to directly infuse the preparation solution to a mass spectrometer, separation strategies are often necessary to remove buffer components (e.g., salt) and to isolate different

species in a mixture, especially to identify low-abundance target species (Xiu et al., 2014). Separation of intact proteins or large protein fragments with good resolution has always been challenging (Huang et al., 2015). Many traditional protein separation techniques such as ion-exchange chromatograph, isoelectric focusing, and size-exclusion chromatography are incompatible with ESI-MS (Birdsall et al., 2015).

Thus far, numerous techniques have been developed to characterize protein therapeutics. Here, we roughly divide them into three categories based on the MS techniques used: (i) peptide-mapping strategy that analyzes target proteins after an extensive proteolytic digestion; (ii) middle-down strategy that characterizes large fragments produced by disulfide reduction or/and limited digestion with enzymes such as Lys-C (Gadgil et al., 2006), papain (Vlasak et al., 2009), and IdeS protease (FabRICATOR) (Fornelli et al., 2014); (iii) top-down strategy, which characterizes the intact protein by MS, often under native conditions. Details of each technique are discussed below.

B. Techniques for LC/MS-Based Biotherapeutics Characterization

1. Peptide-Mapping Strategy (Bottom-Up)

Characterization of biotherapeutics on the peptide level is recognized as the method of choice to identify and quantify site-specific chemical modifications (Wan et al., 1999; Ren et al., 2008), which is able to sensitively detect modifications or mutations with accurate localization information and excellent sequence coverage (Xie et al., 2010). Briefly, after enzymatic digestion, the proteolytic peptides are separated on a liquid chromatography column followed by MS analysis. For example, Chen et al. (2013) established a peptide mapping and sequencing method for sensitive characterization of biosimilars. They have achieved 100% sequence coverage for two model mAb molecules, with information on disulfide linkages, glycosylations, and other chemical modifications such as deamidation, oxidation, dyhydration, and K-clipping. This work also demonstrated the utility of electron-transfer dissociation (ETD) to analyze sequences and modifications. ETD can provide complementary fragmentation specificity compared to collision-induced dissociation (CID), especially for highly charged peptides and peptides with labile modifications (Syka et al., 2004; Wang et al., 2009; Tu et al., 2011; Chen et al., 2013; Sarbu, Ghiulai, & Zamfir, 2014). For instance, ETD can retain the labile glycosidic linkage and facilitate identification of glycosylation locations in biotherapeutics (Christiansen et al., 2010). A number of studies used ETD to localize glycosylation, disulfide bonds, and other modifications (Wu et al., 2009; Wang et al., 2011; Houel et al., 2014), more details are in the following sections. Because the peptide-mapping method analyzes protein after extensive proteolysis, efficient enzymatic digestion as well as optimized LC separation are also critical to achieve high sequence coverage, sensitivity, and selectivity, as discussed in the above sections.

2. Middle-Down Strategy

The middle-down strategy relies on the analysis of large fragments generated from biotherapeutics molecules. Compared with the bottom-up approach, middle-down uses much larger fragments to enable more comprehensive characterization of a protein drug; moreover, it exhibits higher sensitivity, reproducibility, and robustness than analysis of an intact protein. The middle-down approach is often used in conjunction with other sample-preparation and chromatographic methods to achieve an extensive analysis. For a few examples: Tang et al. (2013) employed Hydrogen-Deuterium (H/D) exchange MS approach on pepsin-generated $F(ab')_2$, Fab, and Fc fragments to investigate the charge variants of a human IgG1 antibody. Birdsall et al. (2015) used a two-dimensional chromatography coupled to Q-TOF mass spectrometer to analyze sub-domains of a cysteine-conjugated ADC. The first dimension was hydrophobic interaction chromatography (HIC) and the second dimension was the RP chromatography. This configuration enabled the conjunction of HIC to LC/MS for protein therapeutics analysis. After solvent and heat denaturation, dissociated subunits separated with HIC were subsequently characterized with RPLC/MS with abundant information. Using digestion with IdeS, An et al. (2014b) developed a middle-down strategy to characterize domain-specific modifications such as oxidation, charge heterogeneity, and glycoform profile, which has been successfully applied in the characterization of IgGs. In another study, with limited proteolysis and reduction in a simple protocol, the authors produced three fragments of 25 kDa to identify and quantify the site-specific methionine oxidation in IgG (Pipes et al., 2010).

3. Top-Down Strategy

Generally, top-down MS analysis characterizes an intact proteins, which has the potential to analyze complete protein sequence and localization of the type of PTMs, amino acid substitutions, and C- or/and N-terminal truncations for specific protein species at the protein level (Ren et al., 2009; Cui, Rohrs, & Gross, 2011; Wang et al., 2015). That being said, top-down strategies often suffer from low sensitivity due to the low ionization efficiency of large proteins (Valeja et al., 2010; Woods et al., 2013) and low sequence coverage due to the inability of fragmentation method to uniformly cleave each peptide bond given the high complexity of biotherapeutics (Liu & Schey, 2008; Wu et al., 2013). Moreover low reproducibility due to the high background caused by various matrix components such as salt, high abundance protein, lipids, etc., is often a prominent issue (Fornelli et al., 2012). Take glycosylation as an example, not only the various glycoforms, but also the additional structure features, including sequence variance, linkage, and branching of glycans render the characterization of intact proteins very challenging. As a result, enzymatic removal of glycans with PNGase F is used as a common approach to reduce the complexity of recombinant protein therapeutics (Hansen et al., 2010; Fornelli et al., 2012). However, the introduced PNGase F could co-elute with an intact protein on a typical RPLC system, which might suppress target signals. Huang et al. (2015) implemented ion mobility (IM) with RP-LC/MS to measure the drug-to-antibody ratio (DAR) of intact ADC. This strategy allowed the separation of ions of different sizes/shapes in the gas phase to significantly improve signal-to-noise ratios (S/N). The combination of IM and RPLC enabled chromatography separation of PNGase F to improve the quality of DAR measurement. Several other separation techniques have also been utilized to facilitate top-down

analysis. For example, Liu et al. (2015) reported a UHPLC-MS method to characterize of free thiol variants of IgG1. The author claimed that diphenyl stationary phase with small particle size and wide pore size posed excellent performance to separate intact mAbs. Recently, Hengel et al. (2014) developed a native LC-MS method to characterize drug load distribution of cycteine-linked ADC after administration to rats. After capturing the ADC with specific affinity reagents, they have successfully measured drug load distribution from an *in vivo* source. Wang et al. (2015) utilized the combination of top-down and middle-down LC/MS for the characterization of drug-product-related impurities in products of anti-*Clostridium difficile* IgG1 mAb. Cation-exchange liquid chromatography was employed to separate drug from impurities prior to MS analysis. As discussed above, low sensitivity is usually observed in top-down protein characterization. In some examples, low sensitivity in top-down MS analysis of large protein was addressed by averaging time-domain transients recorded in different LC-MS/MS experiments before signal processing (Fornelli et al., 2012). In other studies, fractionation strategies, such as filter-aided fractionation was utilized to increase the sensitivity for protein characterization in complex sample (Kellie et al., 2012; Fagerquist et al., 2014). Kim et al. (2012) found that use of narrow ID columns combined with an innovative Low Protein Oxidation (LPOx) configuration yielded significantly increased sensitivity for intact protein analysis in clinical samples. In addition, multiple fragmentation approaches such as ETD and electron capture dissociation (ECD) have been used to improve the sequence coverage for top-down protein analysis (Ahlf et al., 2012; Mao et al., 2013; Tran et al., 2015).

C. Applications

1. Analysis of Glycosylation

As discussed before, the biotherpeutics produced by a recombinant system are heterogeneous. Glycosylation is a common PTM for mAb produced by mammalian cells, and various glycoforms represent the most ubiquitous sources of heterogeneity from batch to batch or different systems (including structural and site difference). For instance, the differences of glycosylation patterns between recombinant products and native proteins derived from human are tremendous (Fenaille et al., 2008; Houel et al., 2014). Because glycosylation influences protein folding, protein stability, and biological activity, it is critical to accurately characterize glycosylation for biotherapeutics during development or production (i.e., quality control) (Sola & Griebenow, 2009; Zheng, Bantog, & Bayer, 2011). Quite a number of mass spectrometric methods are available for glycosylation profiling, which were thoroughly compared in a previous article (Reusch et al., 2015). Traditionally, HILIC has been widely used for glycosylation characterization. In one study, HILIC/MS with a quadrupole TOF mass spectrometer was utilized for detailed characterization of a marketed therapeutic mAb, Rituxan® (Shang et al., 2014). In another example, Houel et al. (2014) utilized the HILIC-UPLC-fluorescence detection (FLR) method to analysis the released N- and O-glycan from etanercept. The N-glycans on peptides of etanercept were analyzed with UPLC-HILIC-FLR and exoglycosydase digestion array, while O-glycans were analyzed by UPLC-HILIC-FLR and LC-MS^E and ETD. In another work, through an electrospray quadrupole ion-mobility time-of-flight mass spectrometry (ESI-Q-IM-TOF) platform, the author acquired the global glycoprofiles as well as glycan structure at each glycosylation sites for transtuzumab from different batches with combination of analysis at protein, domain, and peptide levels (Damen et al., 2009a). Oh et al. (2013) used MALDI-LC/MS for glycan profiling and nano-LC/MS/MS for detailed glycan structural analysis, respectively. The developed platform had well-characterized the glycosylation of recombinant erythropointin and facilitated the evaluation of the products from different batches.

2. Disulfide-Bond Profiling

Disulfide bond (DSB) is one of the most important specifications for biotherapeutics because they directly affect the higher-order structures of the molecules and alter the stability, safety, and efficacy (Berkowitz et al., 2012; Liu & May, 2012). A number of bottom-up LC/MS-based approaches have been reported for DSB analysis (Mhatre, Woodard, & Zeng, 1999; Gorman, Wallis, & Pitt, 2002; Zhang, Pan, & Chen, 2009; Beck et al., 2013; Wiesner et al., 2015). In detail, Wang et al. (2011) developed a LC/MS-based peptide-mapping strategy for DSB characterization, which utilized the combination of ETD and CID fragmentations to study disulfide stability of biotherapeutics from different sources. The author characterized disulfide bonds, including inter-/intra-chain and scrambling disulfides, for three mAbs (anti-HER2, anti-CD11a, and GLP-1 with IgG-Fc fusion protein). Switzer et al. (2015) developed a LC-electrochemistry (EC)-MS platform to characterize protein DSBs with a bottom-up workflow. The authors have identified and localized the DSBs in β-lactoglobulin and ribonuclease B. In another example, Wu et al. (2009) used ETD to determine disulfide linkages in recombinant human growth hormone (Nutropin), a therapeutic mAb, and tissue plasminogen activator (Activase), respectively. In addition, several top-down based strategies have also been established for DSB analysis. In one example, Nguyen et al. (2011) used top-down MS-based method to analyze DSB, which facilitated the discovery of the linear structure of cyclotides. In another study, Peng et al. (2012) established top-down MS approach combined with affinity purification and partial reduction to characterize human salivary α-amylase, which precisely mapped DSB positions on the analyte (Nicolardi et al., 2014).

3. Charge Variants

Some of the common chemical modifications could alter the surface-charge properties of proteins that contribute to the heterogeneity of biotherapeutic products. The charge variants can be resolved with chromatographic methods such as IEC and IEF (Antes et al., 2007; Khawli et al., 2010). It is critical to study the local structural changes of the charge variants to understand the change of PK/PD properties of these heterogeneity species. With H/D exchange techniques coupled with middle-down MS analysis, Tang et al. (2013) investigated the local and sub-global/global unfolding of the charge variants of a human IgG1 mAb, which made possible the further study of biofunctions of the product. Asparagine (Asn) deamidation and Aspartic acid (Asp) isomerization are common chemical modifications that result in charge heterogeneity in

biotherapeutics (Perkins et al., 2000). Sreedhara et al. (2012) employed IEC, HIC, peptide mapping, and LC/MS strategies to analyze heterogeneous products derived from Aspartate residues in mAb. The authors discovered the degradation profiles for peptides with labile Asp. Huang et al. (2015) used trypsin and Glu-C to digest the protein sample sequentially, followed by RP-HPLC-MS/MS anslysis to determine deamidation of humanized IgG1 mAb at the low-nanogram level in biological matrix. Ponniah et al. (2015) investigated the acidic species of a mAb generated by different conditions with LC/MS after separation with weak cation-exchange chromatography.

4. Characterization of Overall Stability and Biotransformation for ADC

Development of ADC is one of the hottest topics in the field of discovery of biotherapeutics. Evaluation of heterogeneity of ADC especially the payload distribution, including DAR values (0–8) and localization (heavy chain or light chain) (Wakankar et al., 2011), is of particular importance to the design and quality control of an ADC product, because these specifications impact safety and efficacy of the products (McDonagh et al., 2006). However, DAR-value determination is challenging due to the complexity of payload itself, in addition to other frequent PTMs and chemical modifications. Huang et al. (2015) used IM-LC/MS to characterize DAR values of intact ADC after removal of glycans. According to the analysis results for an unconjugated mAb, a site-specific ADC, and a random conjugated ADC, IM-LC/MS showed excellent separation of a PNGase F and ADC mixture, which can increase the analytical sensitivity. The author further evaluated six lots of site-specific ADC with two different DARs to prove the robustness and efficiency of utilization of this strategy to characterize ADC. Birdsall et al. (2015) used LC/LC/QTOF-MS to analyze the cysteine-conjugated ADC. The structures of each species from HIC peaks were elucidated with downstream RP chromatography-MS in an efficient and rapid manner. Their method have successfully characterized the DAR-value distribution and sub-unit structure of each species under denaturing conditions for the model IgG1-based ADC from three batches with different drug-load levels. In another example, Xu et al. (2011) developed an intact ADC characterization strategy, which used specific critical reagent to capture the ADC from plasma before LC/MS analysis (Xu et al., 2011, 2013). Their strategy directly characterized the drug-release process *in vitro* and *in vivo* to facilitate the understanding of stability and activity of ADC. Valliere-Douglass, McFee, and Salas-Solano (2012) described a non-denaturing LC/MS-based method to determine of drug distribution in ADCs based on ion abundance from the deconvoluted spectrum of interchain cysteine-conjugated ADCs.

V. CONCLUSION AND FUTURE PERSPECTIVES

In recent years, LC/MS has become a powerful method for the quantitative and qualitative characterization of biotherapeutics across a wide range of matrices, such as in formulated products, bodily fluids, and tissues. As a result, it is widely applied in every stage across the research and development of biotherapeutics, from discovery to development and post-market monitoring. LC/MS is often considered a promising alternative or complementary approach to the prevalently-used LBA, primarily because it enables timely development of methods with wide dynamic range, excellent selectivity, and reproducibility, and delivers reliable analysis in almost all biomatrices. Further improvements in accuracy, sensitivity, and throughput might allow LC/MS to displace LBA as the leading approach for quantification of biologic drugs in bio-matrices. By combining with a wide range of sample preparation, chromatographic, and mass spectrometric technologies, a large number of quantitative and qualitative strategies have been established and used in various applications to greatly facilitate investigations like product quality control, pre-clinical and clinical investigation, and PK profiling. Among many of these applications, LC/MS is uniquely advantageous over other approaches; to give a few example: LC/MS strategy is currently the most-reliable means to quantify protein drugs in tissues (Duan et al., 2012a; An, Zhang, & Qu, 2014a); LC/MS-based characterizations are becoming mandatory for sequence determination, PTM identification, and structure characterization in development and production of biotherapeutics products, due to its ability to provide detailed information (Alvarez et al., 2011; Oh et al., 2013; Wang et al., 2015). In addition, LC/MS based novel strategies, such as native-state Hydrogen exchange, native top-down MS, protein painting, and etc., have been established to investigate protein thermodynamic and conformational dynamic, higher-order structure characterization, native protein–protein interfaces, and so on (Luchini, Espina, & Liotta, 2014; Boeri Erba & Petosa, 2015; Witten et al., 2015), these methods could facilitate the discovery and development of novel protein therapeutics.

LC/MS techniques still face substantial challenges associated with analytical sensitivity and throughput, as well as technical difficulties in analysis of tissues. Furthermore, new approaches must be developed to specifically analyze different forms of biotherapeutics that result from *in vivo* interactions and biotransformation, such as free versus bound drugs, immune-complex, metabolic and catabolic products, and modified forms. To address these needs, various tools such as LBA (i.e., LB-LC/MS), electrophoresis, affinity chromatography, etc. can be coupled to LC/MS for quantification. This trend is still going strong as this article is being composed. As a result, in the next several years we will witness an explosion of exciting new LC/MS-based approaches to substantially improve analysis of biotherapeutics in highly complex samples, including but not limited to novel sample preparation, separation, and LC/MS techniques.

ABBREVIATIONS

ADC	antibody-drug conjugates
APD	antibody phage display
CID	collision-induced dissociation
DAR	drug-to-antibody ratio
DSB	disulfide bond
ELISA	enzyme-linked immunosorbent assay
ETD	electron-transfer dissociation
ESI	electrospray ionization
FASP	filter aided sample preparation
HIC	hydrophobic interaction chromatography
IS	internal standards
LBA	ligand-binding assays

LC	liquid chromatograph
LOD	limit of detection
LOQ	limit of quantification
mAb	monoclonal antibodies
MS	mass spectrometry
OAO	orthogonal-array-optimization
PD	pharmacodynamic
PK	pharmacokinetics
PTM	post-translational modifications
SCX	strong-cation exchange
SIL	stable isotope labeled
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
S/N	signal-to-noise ratios
SP	signature peptide
SRM	selected-reaction monitoring
TK	toxicokinetics
TOF	time-of-flight

REFERENCES

Ackermann B, Berna M. 2007. Coupling immunoaffinity techniques with MS for quantitative analysis of lowabundance protein biomarkers. Proteomics 4:175–186.

Ackermann B. 2012. Understanding the role of immunoaffinity-based mass spectrometry methods for clinical applications. Clin Chem 58:1620–1622.

Addis T, Lee DD, Lew W, Poo LJ. 1940. The protein content of the organs and tissues at different levels of protein consumption. J Nutr 19:199–205.

Ahlf DR, Compton PD, Tran JC, Early BP, Thomas PM, Kelleher NL. 2012. Evaluation of the compact high-field orbitrap for top-down proteomics of human cells. J Proteome Res 11:4308–4314.

AlDeghaither D, Smaglo BG, Weiner LM. 2015. Beyond peptides and mAbs-current status and future perspectives for biotherapeutics with novel constructs. J Clin Pharmacol 55(Suppl 3):S4–S20.

Alvarez M, Tremintin G, Wang J, Eng M, Kao YH, Jeong J, Ling VT, Borisov OV. 2011. On-line characterization of monoclonal antibody variants by liquid chromatography-mass spectrometry operating in a two-dimensional format. Anal Biochem 419:17–25.

An B, Zhang M, Qu J. 2014a. Toward sensitive and accurate analysis of antibody biotherapeutics by liquid chromatography coupled with mass spectrometry. Drug Metab Dispos 42:1858–1866.

An Y, Zhang Y, Mueller HM, Shameem M, Chen X. 2014b. A new tool for monoclonal antibody analysis: Application of IdeS proteolysis in IgG domain-specific characterization. MAbs 6:879–893.

An B, Zhang M, Johnson RW, Qu J. 2015a. Surfactant-aided precipitation/on-pellet-digestion (SOD) procedure provides robust and rapid sample preparation for reproducible, accurate and sensitive LC/MS quantification of therapeutic protein in plasma and tissues. Anal Chem 87:4023–4029.

An B, Zhang M, Qu Y, ASMS, JQ. St. Louis, May 2015 2015b; 167.

Anderson L, Hunter CL. 2006. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol Cell Proteomics 5:573–588.

Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. 2004. Mass spectrometric quantitation of peptides and proteins using stable isotope standards and capture by anti-peptide antibodies (SISCAPA). J Proteome Res 3:235–244.

Anderson NL, Jackson A, Smith D, Hardie D, Borchers C, Pearson TW. 2009. SISCAPA peptide enrichment on magnetic beads using an in-line bead trap device. Mol Cell Proteomics 8:995–1005.

Antes B, Amon S, Rizzi A, Wiederkum S, Kainer M, Szolar O, Fido M, Kircheis R, Nechansky A. 2007. Analysis of lysine clipping of a humanized Lewis-Y specific IgG antibody and its relation to Fc-mediated effector function. J Chromatogr B Analyt Technol Biomed Life Sci 852:250–256.

Arnold DW, Needham SR. 2013. Micro-LC-MS/MS: The future of bioanalysis. Bioanalysis 5:1329–1331.

Baeuerle PA, Kufer P, Bargou R. 2009. BiTE: Teaching antibodies to engage T-cells for cancer therapy. Curr Opin Mol Ther 11:22–30.

Beck A, Wurch T, Bailly C, Corvaia N. 2010. Strategies and challenges for the next generation of therapeutic antibodies. Nat Rev Immunol 10:345–352.

Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianferani S. 2013. Characterization of therapeutic antibodies and related products. Anal Chem 85:715–736.

Bereman MS, MacLean B, Tomazela DM, Liebler DC, MacCoss MJ. 2012. The development of selected reaction monitoring methods for targeted proteomics via empirical refinement. Proteomics 12:1134–1141.

Berkowitz SA, Engen JR, Mazzeo JR, Jones GB. 2012. Analytical tools for characterizing biopharmaceuticals and the implications for biosimilars. Nat Rev Drug Discov 11:527–540.

Betancourt LH, De Bock PJ, Staes A, Timmerman E, Perez-Riverol Y, Sanchez A, Besada V, Gonzalez LJ, Vandekerckhove J, Gevaert K. 2013. SCX charge state selective separation of tryptic peptides combined with 2D-RP-HPLC allows for detailed proteome mapping. J Proteomics 91:164–171.

Birdsall RE, Shion H, Kotch FW, Xu A, Porter TJ, Chen W. 2015. A rapid on-line method for mass spectrometric confirmation of a cysteine-conjugated antibody-drug-conjugate structure using multidimensional chromatography. MAbs 7:1036–1044.

Blackburn M. 2013. Advances in the quantitation of therapeutic insulin analogues by LC-MS/MS. Bioanalysis 5:2933–2946.

Boeri Erba E, Petosa C. 2015. The emerging role of native mass spectrometry in characterizing the structure and dynamics of macromolecular complexes. Protein Sci 24:1176–1192.

Bronsema KJ, Bischoff R, van de Merbel NC. 2012. Internal standards in the quantitative determination of protein biopharmaceuticals using liquid chromatography coupled to mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 893–894:1–14.

Brownridge P, Beynon RJ. 2011. The importance of the digest: Proteolysis and absolute quantification in proteomics. Methods 54:351–360.

Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M, Vandenesch F, Garin J. 2007. Isotope-labeled protein standards: Toward absolute quantitative proteomics. Mol Cell Proteomics 6:2139–2149.

Bults P, van de Merbel NC, Bischoff R. 2015. Quantification of biopharmaceuticals and biomarkers in complex biological matrices: A comparison of liquid chromatography coupled to tandem mass spectrometry and ligand binding assays. Expert Rev Proteomics 12:355–374.

Calo-Fernandez B, Martinez-Hurtado JL. 2012. Biosimilars: Company strategies to capture value from the biologics market. Pharmaceuticals (Basel) 5:1393–1408.

Cao J, Gonzalez-Covarrubias V, Straubinger RM, Wang H, Duan X, Yu H, Qu J, Blanco JG. 2010. A rapid, reproducible, on-the-fly orthogonal array optimization method for targeted protein quantification by LC/MS and its application for accurate and sensitive quantification of carbonyl reductases in human liver. Anal Chem 82:2680–2689.

Capelo JL, Carreira RJ, Fernandes L, Lodeiro C, Santos HM, Simal-Gandara J. 2010. Latest developments in sample treatment for 18O-isotopic labeling for proteomics mass spectrometry-based approaches: A critical review. Talanta 80:1476–1486.

Cham Mead JA, Bianco L, Bessant C. 2010. Free computational resources for designing selected reaction monitoring transitions. Proteomics 10:1106–1126.

Chames P, Van Regenmortel M, Weiss E, Baty D. 2009. Therapeutic antibodies: Successes, limitations and hopes for the future. Br J Pharmacol 157:220–233.

Chen SL, Wu SL, Huang LJ, Huang JB, Chen SH. 2013. A global comparability approach for biosimilar monoclonal antibodies using LC-tandem MS based proteomics. J Pharm Biomed Anal 80:126–135.

Chilewski SD, Jiang H. 2015. Choosing the right bioanalytical assay platform(s) to support the PK assessment of protein biotherapeutic programs. Bioanalysis 7:1197–1199.

Choksawangkarn W, Edwards N, Wang Y, Gutierrez P, Fenselau C. 2012. Comparative study of workflows optimized for in-gel, in-solution, and on-filter proteolysis in the analysis of plasma membrane proteins. J Proteome Res 11:3030–3034.

Christiansen MN, Kolarich D, Nevalainen H, Packer NH, Jensen PH. 2010. Challenges of determining O-glycopeptide heterogeneity: A fungal glucanase model system. Anal Chem 82:3500–3509.

Colbert A, Umble-Romero A, Prokop S, Chow VF, Wong T, DeSimone D, Zhou L, Pederson S. 2014. Bioanalytical strategy used in development of pharmacokinetic (PK) methods that support biosimilar programs. MAbs 6:1178–1189.

Cui W, Rohrs HW, Gross ML. 2011. Top-down mass spectrometry: Recent developments, applications and perspectives. Analyst 136:3854–3864.

Damen CW, Schellens JH, Beijnen JH. 2009. Bioanalytical methods for the quantification of therapeutic monoclonal antibodies and their application in clinical pharmacokinetic studies. Hum Antibodies 18:47–73.

Damen CW, Chen W, Chakraborty AB, van Oosterhout M, Mazzeo JR, Gebler JC, Schellens JH, Rosing H, Beijnen JH. 2009a. Electrospray ionization quadrupole ion-mobility time-of-flight mass spectrometry as a tool to distinguish the lot-to-lot heterogeneity in N-glycosylation profile of the therapeutic monoclonal antibody trastuzumab. J Am Soc Mass Spectrom 20:2021–2033.

Damen CW, de Groot ER, Heij M, Boss DS, Schellens JH, Rosing H, Beijnen JH, Aarden LA. 2009b. Development and validation of an enzyme-linked immunosorbent assay for the quantification of trastuzumab in human serum and plasma. Anal Biochem 391:114–120.

DeSilva B, Garofolo F, Rocci M, Martinez S, Dumont I, Landry F, Dicaire C, Szekely-Klepser G, Weiner R, Arnold M, Bansal S, Bateman K, Bauer R, Booth B, Davis S, Dudal S, Gouty D, Grundy J, Haidar S, Hayes R, Jemal M, Kaur S, Kelley M, Knutsson M, Le Blaye O, Lee J, Lowes S, Ma M, Mitsuoka T, Neto JT, Nicholson R, Ormsby E, Sailstad J, Stevenson L, Tang D, Welink J, Viswanathan CT, Wang L, Woolf E, Yang E. 2012. 2012 white paper on recent issues in bioanalysis and alignment of multiple guidelines. Bioanalysis 4:2213–2226.

Duan X, Young R, Straubinger RM, Page B, Cao J, Wang H, Yu H, Canty JM, Qu J. 2009. A straightforward and highly efficient precipitation/on-pellet digestion procedure coupled with a long gradient nano-LC separation and Orbitrap mass spectrometry for label-free expression profiling of the swine heart mitochondrial proteome. J Proteome Res 8:2838–2850.

Duan X, Abuqayyas L, Dai L, Balthasar JP, Qu J. 2012a. High-throughput method development for sensitive, accurate, and reproducible quantification of therapeutic monoclonal antibodies in tissues using orthogonal array optimization and nano liquid chromatography/selected reaction monitoring mass spectrometry. Anal Chem 84:4373–4382.

Duan X, Dai L, Chen SC, Balthasar JP, Qu J. 2012b. Nano-scale liquid chromatography/mass spectrometry and on-the-fly orthogonal array optimization for quantification of therapeutic monoclonal antibodies and the application in preclinical analysis. J Chromatogr A 1251:63–73.

Dubois M, Fenaille F, Clement G, Lechmann M, Tabet JC, Ezan E, Becher F. 2008. Immunopurification and mass spectrometric quantification of the active form of a chimeric therapeutic antibody in human serum. Anal Chem 80:1737–1745.

Dudal S, Staack RF, Stoellner D, Fjording MS, Vieser E, Pascual MH, Brudny-Kloeppel M, Golob M. 2014. How the bioanalytical scientist plays a key role in interdisciplinary project teams in the development of biotherapeutics—A reflection of the European bioanalysis forum. Bioanalysis 6:1339–1348.

Ecker DM, Jones SD, Levine HL. 2015. The therapeutic monoclonal antibody market. MAbs 7:9–14.

Ezan E, Bitsch F. 2009. Critical comparison of MS and immunoassays for the bioanalysis of therapeutic antibodies. Bioanalysis 1:1375–1388.

Ezan E, Dubois M, Becher F. 2009. Bioanalysis of recombinant proteins and antibodies by mass spectrometry. Analyst 134:825–834.

Fagerquist CK, Zaragoza WJ, Sultan O, Woo N, Quinones B, Cooley MB, Mandrell RE. 2014. Top-down proteomic identification of Shiga toxin 2 subtypes from Shiga toxin-producing *Escherichia coli* by matrix-assisted laser desorption ionization-tandem time of flight mass spectrometry. Appl Environ Microbiol 80:2928–2940.

Fenaille F, Groseil C, Ramon C, Riande S, Siret L, Chtourou S, Bihoreau N. 2008. Mass spectrometric characterization of N- and O-glycans of plasma-derived coagulation factor VII. Glycoconj J 25:827–842.

Fernandez Ocana M, James IT, Kabir M, Grace C, Yuan G, Martin SW, Neubert H. 2012. Clinical pharmacokinetic assessment of an anti-MAdCAM monoclonal antibody therapeutic by LC-MS/MS. Anal Chem 84:5959–5967.

Fornelli L, Damoc E, Thomas PM, Kelleher NL, Aizikov K, Denisov E, Makarov A, Tsybin YO. 2012. Analysis of intact monoclonal antibody IgG1 by electron transfer dissociation Orbitrap FTMS. Mol Cell Proteomics 11:1758–1767.

Fornelli L, Ayoub D, Aizikov K, Beck A, Tsybin YO. 2014. Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Anal Chem 86:3005–3012.

Fred Banks J, Jr. 1996. High-sensitivity peptide mapping using packed-capillary liquid chromatography and electrospray ionization mass spectrometry. J Chromatogr A 743:99–104.

Furlong MT, Ouyang Z, Wu S, Tamura J, Olah T, Tymiak A, Jemal M. 2012. A universal surrogate peptide to enable LC-MS/MS bioanalysis of a diversity of human monoclonal antibody and human Fc-fusion protein drug candidates in pre-clinical animal studies. Biomed Chromatogr 26:1024–1032.

Furlong MT, Titsch C, Xu W, Jiang H, Jemal M, Zeng J. 2014. An exploratory universal LC-MS/MS assay for bioanalysis of hinge region-stabilized human IgG4 mAbs in clinical studies. Bioanalysis 6:1747–1758.

Gadgil HS, Bondarenko PV, Pipes GD, Dillon TM, Banks D, Abel J, Kleemann GR, Treuheit MJ. 2006. Identification of cysteinylation of a free cysteine in the Fab region of a recombinant monoclonal IgG1 antibody using Lys-C limited proteolysis coupled with LC/MS analysis. Anal Biochem 355:165–174.

Gama MR, Collins CH, Bottoli CB. 2013. Nano-liquid chromatography in pharmaceutical and biomedical research. J Chromatogr Sci 51:694–703.

Geist BJ, Egan AC, Yang TY, Dong Y, Shankar G. 2013. Characterization of critical reagents in ligand-binding assays: Enabling robust bioanalytical methods and lifecycle management. Bioanalysis 5:227–244.

Gorman JJ, Wallis TP, Pitt JJ. 2002. Protein disulfide bond determination by mass spectrometry. Mass Spectrom Rev 21:183–216.

Gorovits B, McNally J, Fiorotti C, Leung S. 2014. Protein-based matrix interferences in ligand-binding assays. Bioanalysis 6:1131–1140.

Hager T, Spahr C, Xu J, Salimi-Moosavi H, Hall M. 2013. Differential enzyme-linked immunosorbent assay and ligand-binding mass spectrometry for analysis of biotransformation of protein therapeutics: Application to various FGF21 modalities. Anal Chem 85:2731–2738.

Hagman C, Ricke D, Ewert S, Bek S, Falchetto R, Bitsch F. 2008. Absolute quantification of monoclonal antibodies in biofluids by liquid chromatography-tandem mass spectrometry. Anal Chem 80:1290–1296.

Halquist MS, Thomas Karnes H. 2011. Quantitative liquid chromatography tandem mass spectrometry analysis of macromolecules using signature peptides in biological fluids. Biomed Chromatogr 25:47–58.

Hansen R, Dickson AJ, Goodacre R, Stephens GM, Sellick CA. 2010. Rapid characterization of N-linked glycans from secreted and gel-purified monoclonal antibodies using MALDI-ToF mass spectrometry. Biotechnol Bioeng 107:902–908.

Harris RJ, Kabakoff B, Macchi FD, Shen FJ, Kwong M, Andya JD, Shire SJ, Bjork N, Totpal K, Chen AB. 2001. Identification of multiple sources of charge heterogeneity in a recombinant antibody. J Chromatogr B Biomed Sci Appl 752:233–245.

Havlis J, Shevchenko A. 2004. Absolute quantification of proteins in solutions and in polyacrylamide gels by mass spectrometry. Anal Chem 76:3029–3036.

Hengel SM, Sanderson R, Valliere-Douglass J, Nicholas N, Leiske C, Alley SC. 2014. Measurement of in vivo drug load distribution of cysteine-linked antibody-drug conjugates using microscale liquid chromatography mass spectrometry. Anal Chem 86:3420–3425.

Hervey WJ 4th, Strader MB, Hurst GB. 2007. Comparison of digestion protocols for microgram quantities of enriched protein samples. J Proteome Res 6:3054–3061.

Heudi O, Barteau S, Zimmer D, Schmidt J, Bill K, Lehmann N, Bauer C, Kretz O. 2008. Towards absolute quantification of therapeutic monoclonal antibody in serum by LC-MS/MS using isotope-labeled antibody standard and protein cleavage isotope dilution mass spectrometry. Anal Chem 80:4200–4207.

Ho EN, Wan TS, Wong AS, Lam KK, Stewart BD. 2011. Doping control analysis of insulin and its analogues in equine urine by liquid chromatography-tandem mass spectrometry. J Chromatogr A 1218:1139–1146.

Hoofnagle AN, Wener MH. 2009. The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry. J Immunol Methods 347:3–11.

Hopfgartner G, Lesur A, Varesio E. 2013. Analysis of biopharmaceutical proteins in biological matrices by LC-MS/MS II. LC-MS/MS analysis. Trac-Trends Anal Chem 48:52–61.

Houde D, Arndt J, Domeier W, Berkowitz S, Engen JR. 2009. Characterization of IgG1 conformation and conformational dynamics by hydrogen/deuterium exchange mass spectrometry. Anal Chem 81:2644–2651.

Houel S, Hilliard M, Yu YQ, McLoughlin N, Martin SM, Rudd PM, Williams JP, Chen W. 2014. N- and O-glycosylation analysis of etanercept using liquid chromatography and quadrupole time-of-flight mass spectrometry equipped with electron-transfer dissociation functionality. Anal Chem 86:576–584.

Hsieh YS, Korfmacher WA. 2006. Increasing speed and throughput when using HPLC-MS/MS systems for drug metabolism and pharmacokinetic screening. Curr Drug Metab 7:479–489.

Huang L, Lu J, Wroblewski VJ, Beals JM, Riggin RM. 2005. In vivo deamidation characterization of monoclonal antibody by LC/MS/MS. Anal Chem 77:1432–1439.

Huang RY, Deyanova EG, Passmore D, Rangan V, Deshpande S, Tymiak AA, Chen G. 2015. Utility of ion mobility mass spectrometry for drug-to-Antibody ratio measurements in antibody-drug conjugates. J Am Soc Mass Spectrom 26:1791–1794.

Hustoft HK, Reubsaet L, Greibrokk T, Lundanes E, Malerod H. 2011. Critical assessment of accelerating trypsination methods. J Pharm Biomed Anal 56:1069–1078.

Jemal M, Xia YQ. 2006. LC-MS development strategies for quantitative bioanalysis. Curr Drug Metab 7:491–502.

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. 2011. Global cancer statistics. CA Cancer J Clin 61:69–90.

Jenkins N, Murphy L, Tyther R. 2008. Post-translational modifications of recombinant proteins: Significance for biopharmaceuticals. Mol Biotechnol 39:113–118.

Jenkins R, Duggan JX, Aubry AF, Zeng J, Lee JW, Cojocaru L, Dufield D, Garofolo F, Kaur S, Schultz GA, Xu K, Yang Z, Yu J, Zhang YJ, Vazvaei F. 2015. Recommendations for validation of LC-MS/MS bioanalytical methods for protein biotherapeutics. AAPS J 17:1–16.

Jiang H, Zeng J, Titsch C, Voronin K, Akinsanya B, Luo L, Shen H, Desai DD, Allentoff A, Aubry AF, Desilva BS, Arnold ME. 2013. Fully validated LC-MS/MS assay for the simultaneous quantitation of coadministered therapeutic antibodies in cynomolgus monkey serum. Anal Chem 85:9859–9867.

Kaur S. 2013. Bioanalysis special focus issue on antibody-drug conjugates. Bioanalysis 5:981–983.

Kellie JF, Catherman AD, Durbin KR, Tran JC, Tipton JD, Norris JL, Witkowski CE, II., Thomas PM, Kelleher NL. 2012. Robust analysis of the yeast proteome under 50 kDa by molecular-mass-based fractionation and top-down mass spectrometry. Anal Chem 84:209–215.

Keshishian H, Addona T, Burgess M, Mani DR, Shi X, Kuhn E, Sabatine MS, Gerszten RE, Carr SA. 2009. Quantification of cardiovascular biomarkers in patient plasma by targeted mass spectrometry and stable isotope dilution. Mol Cell Proteomics 8:2339–2349.

Khawli LA, Goswami S, Hutchinson R, Kwong ZW, Yang J, Wang X, Yao Z, Sreedhara A, Cano T, Tesar D, Nijem I, Allison DE, Wong PY, Kao YH, Quan C, Joshi A, Harris RJ, Motchnik P. 2010. Charge variants in IgG1: Isolation, characterization, in vitro binding properties and pharmacokinetics in rats. MAbs 2:613–624.

Khazaeli MB, Conry RM, LoBuglio AF. 1994. Human immune response to monoclonal antibodies. J Immunother Emphasis Tumor Immunol 15:42–52.

Kim W, Lee JE, Li XF, Kim SH, Han BG, Lee BI, Kim JK, Choi K, Kim HJ. 2012. Quantitative measurement of anti-aquaporin-4 antibodies by enzyme-linked immunosorbent assay using purified recombinant human aquaporin-4. Mult Scler 18:578–586.

King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. 2000. Mechanistic investigation of ionization suppression in electrospray ionization. J Am Soc Mass Spectrom 11:942–950.

Kohler G, Milstein C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497.

Kuhn E, Whiteaker JR, Mani DR, Jackson AM, Zhao L, Pope ME, Smith D, Rivera KD, Anderson NL, Skates SJ, Pearson TW, Paulovich AG, Carr SA. 2012. Interlaboratory evaluation of automated, multiplexed peptide immunoaffinity enrichment coupled to multiple reaction monitoring mass spectrometry for quantifying proteins in plasma. Mol Cell Proteomics 11:M111 013854.

Kushnir MM, Rockwood AL, Roberts WL, Abraham D, Hoofnagle AN, Meikle AW. 2013. Measurement of thyroglobulin by liquid chromatography-tandem mass spectrometry in serum and plasma in the presence of antithyroglobulin autoantibodies. Clin Chem 59:982–990.

Lassman ME, Fernandez-Metzler C. 2014. Applications of low-flow LC-SRM for the analysis of large molecules in pharmaceutical R & D. Bioanalysis 6:1859–1867.

Leary BA, Lawrence-Henderson R, Mallozzi C, Fernandez Ocana M, Duriga N, O'Hara DM, Kavosi M, Qu Q, Joyce AP. 2013. Bioanalytical platform comparison using a generic human IgG PK assay format. J Immunol Methods 397:28–36.

Lee JW, Kelley M, King LE, Yang J, Salimi-Moosavi H, Tang MT, Lu JF, Kamerud J, Ahene A, Myler H, Rogers C. 2011. Bioanalytical approaches to quantify "total" and "free" therapeutic antibodies and their targets: Technical challenges and PK/PD applications over the course of drug development. AAPS J 13: 99–110.

Lesur A, Varesio E, Hopfgartner G. 2010. Accelerated tryptic digestion for the analysis of biopharmaceutical monoclonal antibodies in plasma by liquid chromatography with tandem mass spectrometric detection. J Chromatogr A 1217:57–64.

Li J, Zhu Z. 2010. Research and development of next generation of antibody-based therapeutics. Acta Pharmacol Sin 31:1198–1207.

Li F, Fast D, Michael S. 2011. Absolute quantitation of protein therapeutics in biological matrices by enzymatic digestion and LC-MS. Bioanalysis 3:2459–2480.

Li H, Ortiz R, Tran L, Hall M, Spahr C, Walker K, Laudemann J, Miller S, Salimi-Moosavi H, Lee JW. 2012. General LC-MS/MS method approach to quantify therapeutic monoclonal antibodies using a common whole antibody internal standard with application to preclinical studies. Anal Chem 84:1267–1273.

Li H, Ortiz R, Tran LT, Salimi-Moosavi H, Malella J, James CA, Lee JW. 2013. Simultaneous analysis of multiple monoclonal antibody biotherapeutics by LC-MS/MS method in rat plasma following cassette-dosing. AAPS J 15:337–346.

Li L, Gardner I, Dostalek M, Jamei M. 2014. Simulation of monoclonal antibody pharmacokinetics in humans using a minimal physiologically based model. AAPS J 16:1097–1109.

Liu H, May K. 2012. Disulfide bond structures of IgG molecules: Structural variations, chemical modifications and possible impacts to stability and biological function. MAbs 4:17–23.

Liu Z, Schey KL. 2008. Fragmentation of multiply-charged intact protein ions using MALDI TOF-TOF mass spectrometry. J Am Soc Mass Spectrom 19:231–238.

Liu H, Manuilov AV, Chumsae C, Babineau ML, Tarcsa E. 2011. Quantitation of a recombinant monoclonal antibody in monkey serum by liquid chromatography-mass spectrometry. Anal Biochem 414:147–153.

Liu G, Ji QC, Dodge R, Sun H, Shuster D, Zhao Q, Arnold M. 2013a. Liquid chromatography coupled with tandem mass spectrometry for the bioanalysis of proteins in drug development: Practical considerations in assay development and validation. J Chromatogr A 1284:155–162.

Liu Q, De Felippis MR, Huang L. 2013b. Method for characterization of PEGylated bioproducts in biological matrixes. Anal Chem 85:9630–9637.

Liu G, Zhao Y, Angeles A, Hamuro LL, Arnold ME, Shen JX. 2014. A novel and cost effective method of removing excess albumin from plasma/serum samples and its impacts on LC-MS/MS bioanalysis of therapeutic proteins. Anal Chem 86:8336–8343.

Liu H, Jeong J, Kao YH, Zhang YT. 2015. Characterization of free thiol variants of an IgG1 by reversed phase ultra high pressure liquid chromatography coupled with mass spectrometry. J Pharm Biomed Anal 109:142–149.

Luchini A, Espina V, Liotta LA. 2014. Protein painting reveals solvent-excluded drug targets hidden within native protein-protein interfaces. Nat Commun 5:4413.

Lyubarskaya Y, Houde D, Woodard J, Murphy D, Mhatre R. 2006. Analysis of recombinant monoclonal antibody isoforms by electrospray ionization mass spectrometry as a strategy for streamlining characterization of recombinant monoclonal antibody charge heterogeneity. Anal Biochem 348:24–39.

Maclean B, Tomazela DM, Abbatiello SE, Zhang S, Whiteaker JR, Paulovich AG, Carr SA, Maccoss MJ. 2010. Effect of collision energy optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. Anal Chem 82:10116–10124.

Mao Y, Valeja SG, Rouse JC, Hendrickson CL, Marshall AG. 2013. Top-down structural analysis of an intact monoclonal antibody by electron capture dissociation-fourier transform ion cyclotron resonance-mass spectrometry. Anal Chem 85:4239–4246.

McDonagh CF, Turcott E, Westendorf L, Webster JB, Alley SC, Kim K, Andreyka J, Stone I, Hamblett KJ, Francisco JA, Carter P. 2006. Engineered antibody-drug conjugates with defined sites and stoichiometries of drug attachment. Protein Eng Des Sel 19:299–307.

Mead JA, Bianco L, Ottone V, Barton C, Kay RG, Lilley KS, Bond NJ, Bessant C. 2009. MRMaid, the web-based tool for designing multiple reaction monitoring (MRM) transitions. Mol Cell Proteomics 8:696–705.

Mhatre R, Woodard J, Zeng C. 1999. Strategies for locating disulfide bonds in a monoclonal antibody via mass spectrometry. Rapid Commun Mass Spectrom 13:2503–2510.

Mitulovic G, Smoluch M, Chervet JP, Steinmacher I, Kungl A, Mechtler K. 2003. An improved method for tracking and reducing the void volume in nano HPLC-MS with micro trapping columns. Anal Bioanal Chem 376:946–951.

Muyldermans S, Atarhouch T, Saldanha J, Barbosa JA, Hamers R. 1994. Sequence and structure of VH domain from naturally occurring camel heavy chain immunoglobulins lacking light chains. Protein Eng 7:1129–1135.

Muyldermans S. 2013. Nanobodies: Natural single-domain antibodies. Annu Rev Biochem 82:775–797.

Neill A, Nowak C, Patel R, Ponniah G, Gonzalez N, Miano D, Liu H. 2015. Characterization of recombinant monoclonal antibody charge variants using OFFGEL fractionation, weak anion exchange chromatography, and mass spectrometry. Anal Chem 87:6204–6211.

Neubert H, Grace C, Rumpel K, James I. 2008. Assessing immunogenicity in the presence of excess protein therapeutic using immunoprecipitation and quantitative mass spectrometry. Anal Chem 80:6907–6914.

Neubert H, Fountain S, King L, Clark T, Weng Y, O'Hara DM, Li W, Leung S, Ray C, Palandra J, Ocana MF, Chen J, Ji C, Wang M, Long K, Gorovits B, Fluhler E. 2012. Tissue bioanalysis of biotherapeutics and drug targets to support PK/PD. Bioanalysis 4:2589–2604.

Neubert H, Muirhead D, Kabir M, Grace C, Cleton A, Arends R. 2013. Sequential protein and peptide immunoaffinity capture for mass spectrometry-based quantification of total human beta-nerve growth factor. Anal Chem 85:1719–1726.

Neubert H, Palandra J, Fernandez Ocana M. 2014. Quantification of biotherapeutic targets: New opportunities with immunoaffinity LC-MS/MS. Bioanalysis 6:1731–1733.

Nguyen GK, Zhang S, Wang W, Wong CT, Nguyen NT, Tam JP. 2011. Discovery of a linear cyclotide from the bracelet subfamily and its disulfide mapping by top-down mass spectrometry. J Biol Chem 286:44833–44844.

Nicolardi S, Deelder AM, Palmblad M, van der Burgt YE. 2014. Structural analysis of an intact monoclonal antibody by online electrochemical reduction of disulfide bonds and Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 86:5376–5382.

Nouri-Nigjeh E, Zhang M, Ji T, Yu H, An B, Duan X, Balthasar J, Johnson RW, Qu J. 2014. Effects of calibration approaches on the accuracy for LC-MS targeted quantification of therapeutic protein. Anal Chem 86:3575–3584.

Ocana MF, Neubert H. 2010. An immunoaffinity liquid chromatography-tandem mass spectrometry assay for the quantitation of matrix metalloproteinase 9 in mouse serum. Anal Biochem 399:202–210.

Oh MJ, Hua S, Kim BJ, Jeong HN, Jeong SH, Grimm R, Yoo JS, An HJ. 2013. Analytical platform for glycomic characterization of recombinant erythropoietin biotherapeutics and biosimilars by MS. Bioanalysis 5:545–559.

Olinger GG, Jr., Pettitt J, Kim D, Working C, Bohorov O, Bratcher B, Hiatt E, Hume SD, Johnson AK, Morton J, Pauly M, Whaley KJ, Lear CM, Biggins JE, Scully C, Hensley L, Zeitlin L. 2012. Delayed treatment of Ebola virus infection with plant-derived monoclonal antibodies provides protection in rhesus macaques. Proc Natl Acad Sci USA 109:18030–18035.

Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127:635–648.

Onami I, Ayabe M, Murao N, Ishigai M. 2014. A versatile method for protein-based antigen bioanalysis in non-clinical pharmacokinetics studies of a human monoclonal antibody drug by an immunoaffinity liquid chromatography-tandem mass spectrometry. J Chromatogr A 1334:64–71.

Ouyang Z, Furlong MT, Wu S, Sleczka B, Tamura J, Wang H, Suchard S, Suri A, Olah T, Tymiak A, Jemal M. 2012. Pellet digestion: A simple and efficient sample preparation technique for LC-MS/MS quantification of large therapeutic proteins in plasma. Bioanalysis 4:17–28.

Pan S, Aebersold R, Chen R, Rush J, Goodlett DR, McIntosh MW, Zhang J, Brentnall TA. 2009. Mass spectrometry based targeted protein quantification: Methods and applications. J Proteome Res 8:787–797.

Parker CE, Mocanu V, Mocanu M, Dicheva N, Warren MR. 2010. Mass spectrometry for post-translational modifications. In: Alzate O, editor., Neuroproteomics. Boca Raton, FL.

Pendley C, Shankar G. 2011. Bioanalytical interferences in immunoassays for antibody biotherapeutics. Bioanalysis 3:703–706.

Peng Y, Chen X, Sato T, Rankin SA, Tsuji RF, Ge Y. 2012. Purification and high-resolution top-down mass spectrometric characterization of human salivary alpha-amylase. Anal Chem 84:3339–3346.

Perkins M, Theiler R, Lunte S, Jeschke M. 2000. Determination of the origin of charge heterogeneity in a murine monoclonal antibody. Pharm Res 17:1110–1117.

Picard G, Lebert D, Louwagie M, Adrait A, Huillet C, Vandenesch F, Bruley C, Garin J, Jaquinod M, Brun V. 2012. PSAQ standards for accurate MS-based quantification of proteins: From the concept to biomedical applications. J Mass Spectrom 47:1353–1363.

Picotti P, Bodenmiller B, Mueller LN, Domon B, Aebersold R. 2009. Full dynamic range proteome analysis of *S. cerevisiae* by targeted proteomics. Cell 138:795–806.

Pipes GD, Campbell P, Bondarenko PV, Kerwin BA, Treuheit MJ, Gadgil HS. 2010. Middle-down fragmentation for the identification and quantitation of site-specific methionine oxidation in an IgG1 molecule. J Pharm Sci 99:4469–4476.

Ponniah G, Kita A, Nowak C, Neill A, Kori Y, Rajendran S, Liu H. 2015. Characterization of the acidic species of a monoclonal antibody using weak cation exchange chromatography and LC-MS. Anal Chem 87:9084–9092.

Rauh M. 2012. LC-MS/MS for protein and peptide quantification in clinical chemistry. J Chromatogr B Analyt Technol Biomed Life Sci 883–884:59–67.

Ren D, Pipes G, Xiao G, Kleemann GR, Bondarenko PV, Treuheit MJ, Gadgil HS. 2008. Reversed-phase liquid chromatography-mass spectrometry of site-specific chemical modifications in intact immunoglobulin molecules and their fragments. J Chromatogr A 1179:198–204.

Ren D, Pipes GD, Hambly D, Bondarenko PV, Treuheit MJ, Gadgil HS. 2009. Top-down N-terminal sequencing of Immunoglobulin subunits with electrospray ionization time of flight mass spectrometry. Anal Biochem 384:42–48.

Reusch D, Haberger M, Falck D, Peter B, Maier B, Gassner J, Hook M, Wagner K, Bonnington L, Bulau P, Wuhrer M. 2015. Comparison of methods for the analysis of therapeutic immunoglobulin G Fc-glycosylation profiles-Part 2: Mass spectrometric methods. MAbs 7:732–742.

Robinson CJ, Jones C. 2011. Quality control and analytical techniques for biopharmaceuticals. Bioanalysis 3:81–95.

Robinson MK, Weiner LM, Adams GP. 2004. Improving monoclonal antibodies for cancer therapy. Drug Dev Res 61:172–187.

Rosati S, van den Bremer ET, Schuurman J, Parren PW, Kamerling JP, Heck AJ. 2013. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified orbitrap. MAbs 5:917–924.

Sarbu M, Ghiulai RM, Zamfir AD. 2014. Recent developments and applications of electron transfer dissociation mass spectrometry in proteomics. Amino Acids 46:1625–1634.

Schirrmann T, Meyer T, Schutte M, Frenzel A, Hust M. 2011. Phage display for the generation of antibodies for proteome research, diagnostics and therapy. Molecules 16:412–426.

Shah B, Jiang XG, Chen L, Zhang Z. 2014. LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins. J Am Soc Mass Spectrom 25:999–1011.

Shang TQ, Saati A, Toler KN, Mo J, Li H, Matlosz T, Lin X, Schenk J, Ng CK, Duffy T, Porter TJ, Rouse JC. 2014. Development and application of a robust N-glycan profiling method for heightened characterization of monoclonal antibodies and related glycoproteins. J Pharm Sci 103:1967–1978.

Shen JX, Motyka RJ, Roach JP, Hayes RN. 2005. Minimization of ion suppression in LC-MS/MS analysis through the application of strong cation exchange solid-phase extraction (SCX-SPE). J Pharm Biomed Anal 37:359–367.

Shen JX, Liu G, Zhao Y. 2015. Strategies for improving sensitivity and selectivity for the quantitation of biotherapeutics in biological matrix using LC-MS/MS. Expert Rev Proteomics 12:125–131.

Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1:2856–2860.

Shi T, Fillmore TL, Sun X, Zhao R, Schepmoes AA, Hossain M, Xie F, Wu S, Kim JS, Jones N, Moore RJ, Pasa-Tolic L, Kagan J, Rodland KD, Liu T, Tang K, Camp DG, II., Smith RD, Qian WJ. 2012a. Antibody-free, targeted mass-spectrometric approach for quantification of proteins at low picogram per milliliter levels in human plasma/serum. Proc Natl Acad Sci USA 109:15395–15400.

Shi T, Zhou JY, Gritsenko MA, Hossain M, Camp DG, II., Smith RD, Qian WJ. 2012b. IgY14 and SuperMix immunoaffinity separations coupled with liquid chromatography-mass spectrometry for human plasma proteomics biomarker discovery. Methods 56:246–253.

Simpson DM, Beynon RJ. 2012. QconCATs: Design and expression of concatenated protein standards for multiplexed protein quantification. Anal Bioanal Chem 404:977–989.

Sleczka BG, Mehl JT, Shuster DJ, Lewis KE, Moore R, Vuppugalla R, Rajendran S, D'Arienzo CJ, Olah TV. 2014. Quantification of human mAbs in mouse tissues using generic affinity enrichment procedures and LC-MS detection. Bioanalysis 6:1795–1811.

Sola RJ, Griebenow K. 2009. Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci 98:1223–1245.

Srebalus Barnes CA, Lim A. 2007. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. Mass Spectrom Rev 26:370–388.

Sreedhara A, Cordoba A, Zhu Q, Kwong J, Liu J. 2012. Characterization of the isomerization products of aspartate residues at two different sites in a monoclonal antibody. Pharm Res 29:187–197.

Switzar L, Nicolardi S, Rutten JW, Oberstein SA, Aartsma-Rus A, van der Burgt YE. 2015. In-depth characterization of protein disulfide bonds by online liquid chromatography-electrochemistry-mass spectrometry. J Am Soc Mass Spectrom 27(1):50–58.

Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF. 2004. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc Natl Acad Sci USA 101:9528–9533.

Szapacs ME, Urbanski JJ, Kehler JR, Wilson R, Boram SL, Hottenstein CS, Citerone DR. 2010. Absolute quantification of a therapeutic domain antibody using ultraperformance liquid chromatography-mass spectrometry and immunoassay. Bioanalysis 2:1597–1608.

Tang L, Sundaram S, Zhang J, Carlson P, Matathia A, Parekh B, Zhou Q, Hsieh MC. 2013. Conformational characterization of the charge variants of a human IgG1 monoclonal antibody using H/D exchange mass spectrometry. MAbs 5:114–125.

Teicher BA, Chari RV. 2011. Antibody conjugate therapeutics: Challenges and potential. Clin Cancer Res 17:6389–6397.

Thompson NJ, Rosati S, Heck AJ. 2014. Performing native mass spectrometry analysis on therapeutic antibodies. Methods 65:11–17.

Tran BQ, Barton C, Feng J, Sandjong A, Yoon SH, Awasthi S, Liang T, Khan MM, Kilgour DP, Goodlett DR, Goo YA. 2015. Comprehensive glycosylation profiling of IgG and IgG-fusion proteins by top-down MS with multiple fragmentation techniques. J Proteomics 134:93–101.

Tu C, Li J, Young R, Page BJ, Engler F, Halfon MS, Canty JM, Jr., Qu J. 2011. Combinatorial peptide ligand library treatment followed by a dual-enzyme, dual-activation approach on a nanoflow liquid chromatography/orbitrap/electron transfer dissociation system for comprehensive analysis of swine plasma proteome. Anal Chem 83:4802–4813.

Tu C, Li J, Bu Y, Hangauer D, Qu J. 2012. An ion-current-based, comprehensive and reproducible proteomic strategy for comparative characterization of the cellular responses to novel anti-cancer agents in a prostate cell model. J Proteomics 77:187–201.

Tu C, Li J, Jiang X, Sheflin LG, Pfeffer BA, Behringer M, Fliesler SJ, Qu J. 2013. Ion current based proteomic profiling of the retina in a rat model of smith-lemli-opitz syndrome. Mol Cell Proteomics 12(12): 3583–3598.

Valeja SG, Tipton JD, Emmett MR, Marshall AG. 2010. New reagents for enhanced liquid chromatographic separation and charging of intact protein ions for electrospray ionization mass spectrometry. Anal Chem 82:7515–7519.

Valliere-Douglass JF, McFee WA, Salas-Solano O. 2012. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. Anal Chem 84:2843–2849.

van den Broek I, Niessen WM, van Dongen WD. 2013. Bioanalytical LC-MS/MS of protein-based biopharmaceuticals. J Chromatogr B Analyt Technol Biomed Life Sci 929:161–179.

Vlasak J, Bussat MC, Wang S, Wagner-Rousset E, Schaefer M, Klinguer-Hamour C, Kirchmeier M, Corvaia N, Ionescu R, Beck A. 2009. Identification and characterization of asparagine deamidation in the light chain CDR1 of a humanized IgG1 antibody. Anal Biochem 392:145–154.

Wakankar A, Chen Y, Gokarn Y, Jacobson FS. 2011. Analytical methods for physicochemical characterization of antibody drug conjugates. MAbs 3:161–172.

Wan M, Shiau FY, Gordon W, Wang GY. 1999. Variant antibody identification by peptide mapping. Biotechnol Bioeng 62:485–488.

Wang S, Bao H, Zhang L, Yang P, Chen G. 2008a. Infrared-assisted on-plate proteolysis for MALDI-TOF-MS peptide mapping. Anal Chem 80:5640–5647.

Wang W, Wang EQ, Balthasar JP. 2008b. Monoclonal antibody pharmacokinetics and pharmacodynamics. Clin Pharmacol Ther 84:548–558.

Wang H, Straubinger RM, Aletta JM, Cao J, Duan X, Yu H, Qu J. 2009. Accurate localization and relative quantification of arginine methylation using nanoflow liquid chromatography coupled to electron transfer dissociation and orbitrap mass spectrometry. J Am Soc Mass Spectrom 20:507–519.

Wang Y, Lu Q, Wu SL, Karger BL, Hancock WS. 2011. Characterization and comparison of disulfide linkages and scrambling patterns in therapeutic monoclonal antibodies: Using LC-MS with electron transfer dissociation. Anal Chem 83:3133–3140.

Wang D, Wynne C, Gu F, Becker C, Zhao J, Mueller HM, Li H, Shameem M, Liu YH. 2015. Characterization of drug-product-related impurities and variants of a therapeutic monoclonal antibody by higher energy C-trap dissociation mass spectrometry. Anal Chem 87:914–921.

Whiteaker JR, Zhao L, Anderson L, Paulovich AG. 2010. An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. Mol Cell Proteomics 9:184–196.

Whiteaker JR, Zhao L, Lin C, Yan P, Wang P, Paulovich AG. 2012. Sequential multiplexed analyte quantification using peptide immunoaffinity enrichment coupled to mass spectrometry. Mol Cell Proteomics 11:M111 015347.

Wiesner J, Resemann A, Evans C, Suckau D, Jabs W. 2015. Advanced mass spectrometry workflows for analyzing disulfide bonds in biologics. Expert Rev Proteomics 12:115–123.

Winther B, Nordlund M, Paus E, Reubsaet L, Halvorsen TG. 2009. Immunocapture as ultimate sample cleanup in LC-MS/MS determination of the early stage biomarker ProGRP. J Sep Sci 32:2937–2943.

Wisniewski JR, Zougman A, Nagaraj N, Mann M. 2009. Universal sample preparation method for proteome analysis. Nat Methods 6:359–362.

Witten J, Ruschak A, Poterba T, Jaramillo A, Miranker AD, Jaswal SS. 2015. Mapping protein conformational landscapes under strongly native conditions with hydrogen exchange mass spectrometry. J Phys Chem B 119:10016–10024.

Woods RJ, Xie MH, Von Kreudenstein TS, Ng GY, Dixit SB. 2013. LC-MS characterization and purity assessment of a prototype bispecific antibody. MAbs 5:711–722.

Wu SL, Jiang H, Lu Q, Dai S, Hancock WS, Karger BL. 2009. Mass spectrometric determination of disulfide linkages in recombinant therapeutic proteins using online LC-MS with electron-transfer dissociation. Anal Chem 81:112–122.

Wu S, Brown RN, Payne SH, Meng D, Zhao R, Tolic N, Cao L, Shukla A, Monroe ME, Moore RJ, Lipton MS, Pasa-Tolic L. 2013. Top-down characterization of the post-translationally modified intact periplasmic proteome from the bacterium novosphingobium aromaticivorans. Int J Proteomics 2013:279590.

Xie H, Chakraborty A, Ahn J, Yu YQ, Dakshinamoorthy DP, Gilar M, Chen W, Skilton SJ, Mazzeo JR. 2010. Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies. MAbs 2: 379–394.

Xiu L, Valeja SG, Alpert AJ, Jin S, Ge Y. 2014. Effective protein separation by coupling hydrophobic interaction and reverse phase chromatography for top-down proteomics. Anal Chem 86:7899–7906.

Xu K, Liu L, Saad OM, Baudys J, Williams L, Leipold D, Shen B, Raab H, Junutula JR, Kim A, Kaur S. 2011. Characterization of intact antibody-drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography-mass spectrometry. Anal Biochem 412:56–66.

Xu K, Liu L, Dere R, Mai E, Erickson R, Hendricks A, Lin K, Junutula JR, Kaur S. 2013. Characterization of the drug-to-antibody ratio distribution for antibody-drug conjugates in plasma/serum. Bioanalysis 5:1057–1071.

Xu K, Liu L, Maia M, Li J, Lowe J, Song A, Kaur S. 2014. A multiplexed hybrid LC-MS/MS pharmacokinetic assay to measure two co-administered monoclonal antibodies in a clinical study. Bioanalysis 6:1781–1794.

Yang Z, Hayes M, Fang X, Daley MP, Ettenberg S, Tse FL. 2007. LC-MS/MS approach for quantification of therapeutic proteins in plasma using a protein internal standard and 2D-solid-phase extraction cleanup. Anal Chem 79:9294–9301.

Yang HJ, Hong J, Lee S, Shin S, Kim J, Kim J. 2010. Pressure-assisted tryptic digestion using a syringe. Rapid Commun Mass Spectrom 24:901–908.

Yang F, Shen Y, Camp DG, II, Smith RD. 2012. High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis. Expert Rev Proteomics 9:129–134.

Yuan L, Arnold ME, Aubry AF, Ji QC. 2012. Simple and efficient digestion of a monoclonal antibody in serum using pellet digestion: Comparison with traditional digestion methods in LC-MS/MS bioanalysis. Bioanalysis 4:2887–2896.

Yuan L, Aubry AF, Arnold ME, Ji QC. 2013. Systematic investigation of orthogonal SPE sample preparation for the LC-MS/MS bioanalysis of a monoclonal antibody after pellet digestion. Bioanalysis 5: 2379–2391.

Zaia DAM, Verri WA, Zaia CTBV. 2000. Determination of total proteins in several tissues of rat: A comparative study among spectrophotometric methods. Microchem J 64:235–239.

Zaias J, Mineau M, Cray C, Yoon D, Altman NH. 2009. Reference values for serum proteins of common laboratory rodent strains. J Am Assoc Lab Anim Sci 48:387–390.

Zhang Z, Pan H, Chen X. 2009. Mass spectrometry for structural characterization of therapeutic antibodies. Mass Spectrom Rev 28:147–176.

Zhang YJ, Olah TV, Zeng J. 2014. The integration of ligand binding and LC-MS-based assays into bioanalytical strategies for protein analysis. Bioanalysis 6:1827–1841.

Zhang M, An B, Yu H, Qu J., ASMS, St. Louis, May 2015 2015; 49.

Zheng K, Bantog C, Bayer R. 2011. The impact of glycosylation on monoclonal antibody conformation and stability. MAbs 3: 568–576.

Zheng J, Mehl J, Zhu Y, Xin B, Olah T. 2014. Application and challenges in using LC-MS assays for absolute quantitative analysis of therapeutic proteins in drug discovery. Bioanalysis 6:859–879.