



## Review

# Internal standards in the quantitative determination of protein biopharmaceuticals using liquid chromatography coupled to mass spectrometry

Kees J. Bronsema<sup>a,b</sup>, Rainer Bischoff<sup>a</sup>, Nico C. van de Merbel<sup>a,b,\*</sup>

<sup>a</sup> Analytical Biochemistry, Department of Pharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

<sup>b</sup> Bioanalytical Laboratory, PRA International, Early Development Services, Westerbrink 3, 9405 BJ Assen, The Netherlands

## ARTICLE INFO

*Article history:*

Received 28 December 2011

Accepted 13 February 2012

Available online 21 February 2012

*Keywords:*

Internal standards

Bioanalysis

Biopharmaceuticals

Protein quantification

Quantification

LC–MS/MS

## ABSTRACT

Following the increase in development of protein biopharmaceuticals, there is a growing demand for the sensitive and reliable quantification of these proteins in complex biological matrices such as plasma and serum to support (pre)-clinical research. In this field, ligand binding assays (LBAs) are currently the standard analytical technique, but in recent years, there is a trend towards the use of liquid chromatography hyphenated with (tandem) mass spectrometry (LC–MS/MS). One of the reasons for this trend is the possibility to use internal standards to correct for analytical variability and thus improve the precision and accuracy of the results. In the LC–MS/MS bioanalysis of small molecules, internal standardization is quite straightforward: either a stable-isotope labeled (SIL) form of the analyte or a structural analogue is used. For the quantification of biopharmaceutical proteins, the situation is more complex. Since the protein of interest is digested to a mixture of peptides, one of which is subsequently used for quantification, there are more options for internal standardization. A SIL form or a structural analogue of either the intact protein or the signature peptide can be used. In addition, a modified form of the SIL-peptide internal standard, containing one or more cleavable groups is a possibility, and an internal standard can be generated during the analysis by using differential derivatization techniques. In this paper we provide an overview of the different options for internal standardization in the field of absolute targeted quantification of protein biopharmaceuticals using LC–MS/MS, based on literature from 2003 to 2011. The advantages and disadvantages of the different approaches are evaluated both with regard to the correction they provide for the variability of the different steps of the analysis and with regard to their generic availability. As most of the approaches used lead to acceptable results in terms of accuracy and precision, we conclude that there currently is no clear preferable method for internal standardization in the field of protein quantification by LC–MS/MS. It is essential, however, that any step in the analysis that is not covered by the internal standard chosen, should be carefully optimized and controlled.

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\* Corresponding author at: Bioanalytical Laboratory, PRA International, Early Development Services, Westerbrink 3, 9405 BJ, Assen, The Netherlands.  
Tel.: +31 592 303431.

E-mail address: [merbelnicovande@praintl.com](mailto:merbelnicovande@praintl.com) (N.C. van de Merbel).

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## 1. Introduction

In recent years, there has been a sharp increase in the development of macromolecular drugs, so-called biopharmaceuticals. In the period 2000–2009, 65 biopharmaceutical products received marketing approval from the US Food and Drug Administration (FDA), up from 39 in the 1990s and 13 in the 1980s [1]. Following this trend, analytical techniques to quantify biopharmaceuticals in complex biological matrices are continuously being developed and improved. The current standard method for quantifying proteins in biological matrices is based on ligand binding assays (LBAs). For years, no other analytical technique has been able to match the low detection limits of LBAs. Their excellent sensitivity and selectivity results from the use of an antibody raised against the protein of interest, or, in the case of the quantification of monoclonal antibodies (mAbs), the antigen, which very selectively extracts the analyte from the matrix, and significantly reduces the complexity of the sample.

LBAs require far lower investments in analytical equipment than chromatographic or mass spectrometric assays, have straightforward protocols and the 96- or 384-well plate they come in is truly a high-throughput format. However, when used for absolute quantitative determination of proteins some disadvantages arise [2]. Firstly, there is the time required to develop a new assay, typically some 4–6 months due to production and characterization of the antibodies and subsequent assay development and optimization. Secondly, there are analytical issues that can drastically influence results such as competition with endogenously generated anti-drug antibodies, non-specific binding and cross-reactivity which may remain undetected, since LBAs do not generate any chemical information about the analyte. Thirdly, most LBAs have a complex calibration model with a limited linear range. Finally, in LBAs, the use of an internal standard, correcting for these and other sources of variation, is technically not possible. Together with the fact that variation between different batches of antibodies is not uncommon, this may cause limited accuracy and precision, poor inter-laboratory reproducibility and significant discrepancies between products of different vendors.

Over the last few years it has been demonstrated that liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), the golden standard for quantitative determination of low-molecular-weight drugs, can be a viable alternative to LBAs for the quantification of proteins [3,4]. Ongoing improvements in chromatography and mass spectrometry instrumentation have led to a situation where the sensitivity of LBAs is sometimes already rivaled by LC–MS/MS, although the approach is not free of its own problems and pitfalls [5]. One of the main strengths of the technique lies in the possibility to use internal standards that correct for different sources of analytical variability. Furthermore, analytical methods using this technique can be set up and validated in a relative short period of time of typically a few weeks.

A disadvantage of using LC–MS/MS in protein quantification is that proteins are incompatible with LC–MS/MS because their high molecular mass and size result in poor ionization efficiency, a signal that is distributed over a large number of charge states and very inefficient or non-existent collision-induced dissociation (CID). To resolve these problems, the protein needs to be digested to a

mixture of smaller peptides, one of which is selected for quantification (the so-called signature or proteotypic peptide). The enzymatic digestion is, however, a potential source of variation and needs to be carefully controlled. In addition, selective extraction of the protein from the biological matrix is desirable to reach sufficient concentration sensitivity, which may be difficult to achieve without the use of immuno-affinity materials.

The transfer of protein analysis from the LBA to the LC–MS/MS platform is by no means straightforward. Compared to LBAs, the analytical approach is relatively complex, which makes the use of a proper internal standard essential. This paper describes and compares different approaches towards the use of internal standards in the field of quantitative bioanalysis of protein biopharmaceuticals. After a general discussion of the use of internal standards and the important step of protein digestion, an overview is given of different types of proteins and peptides as internal standards, the possibilities of differential derivatization to create internal standards and finally protein quantification without internal standards. Selected examples from the bioanalytical literature are used to compare and discuss the different approaches for internal standardization.

## 2. Protein quantification – general remarks

### 2.1. Internal standards

An internal standard is a compound that displays physical and chemical characteristics similar to that of the analyte of interest, but at the same time generates a response that can be distinguished from that of the analyte. Equal amounts of internal standard are added to all samples to be analyzed, and due to the similarity between the analyte and the internal standard, it is anticipated that their initial ratio does not change, because both suffer the same losses due to inefficiencies in extraction, digestion or ionization. Finally, both compounds are analyzed and the ratio of the measured signals is calculated. The internal standard thus corrects for variations in the analyte response caused by variability in the analytical procedure.

Mass spectrometric detectors for liquid chromatography have been widely used since the early 1990s. Before then, ultraviolet (UV) and fluorescence (FL) detectors were more common. An advantage of these detection systems over mass spectrometry is their stability. Good results in terms of accuracy and precision can be achieved, often even without the use of an internal standard, provided that sample handling steps are minimized and if needed, well optimized. When necessary, a compound displaying similar extraction and chromatographic characteristics as the analyte of interest can be used as internal standard to correct for instrumental variability. Due to the inability of these detectors to discriminate between the analyte and its internal standard, a chromatographic separation is required.

The reason why the much more expensive mass spectrometers rapidly became more popular is their enhanced sensitivity and selectivity compared to UV and FL detectors. Visible interferences from co-extracted matrix compounds or metabolites are much less common. However, when using mass spectrometry, the use

of an internal standard is an absolute necessity. In addition to the relatively large variability of the detector response itself, there are significant effects on signal intensity due to ion-suppression, in particular for the most widely used ionization technique – electrospray ionization – caused by zones of co-eluting matrix components that influence the efficiency of ion formation [5,6]. An important contributor to the rising popularity of mass-spectrometric detection has been the increasing commercial availability of stable-isotope labeled (SIL) internal standards, which differ from the analytes in molecular mass only. Since physicochemical properties are essentially identical, they generally co-elute with the analyte and correct for ion-suppression effects, as well as for other detection variability. Today, SIL-forms of a given analyte are the most commonly used internal standards in small-molecule bioanalysis using LC–MS/MS.

## 2.2. Protein digestion and signature peptide selection

As mentioned, LC–MS/MS is not the preferred analytical platform for direct protein quantification at low concentrations in biological matrices at present. To facilitate quantification using this technique, proteins are typically digested using a proteolytic enzyme, which cleaves certain peptide bonds. While trypsin is the most commonly used enzyme, since it is well characterized and can be obtained in high purity at a reasonable price, several alternative enzymes are available that will cleave the protein analyte at different positions, resulting in a different set of potential signature peptides. A recent comparison of proteolytic enzymes discusses their different cleavage site specificities, the average generated peptide length and the suitability of the resulting (signature) peptides for LC–MS quantification [7].

To allow trypsin or other enzyme access to all available cleavage sites, proteins are commonly subjected to a three-step pretreatment. First, a denaturing agent such as guanidinium hydrochloride is used to unfold the protein, secondly intramolecular disulfide bonds are reduced (e.g. with dithiothreitol) and finally reactive thiols are alkylated (e.g. with iodoacetamide) preventing uncontrolled reformation of disulfide bonds.

Digestion traditionally takes place overnight for ca 16 h, but when large numbers of samples need to be processed, this might not be practical. Reduction of the digestion time can also be desirable as it reduces the effect of unwanted side reactions such as the oxidation of methionine or deamidation of asparagine or glutamine. Recently, several alternative procedures have been reported that increase the speed of digestion. These include addition of denaturing agents like an organic solvent or a surfactant [8], increased temperature or pressure [9] and exposure to microwave radiation [10] or high-intensity focused ultrasound [11]. These procedures are reported to significantly decrease the time needed to digest a sample to a few hours and sometimes even to less than 1 h. An even more time-efficient approach uses immobilized enzyme reactors (IMERs). This procedure can achieve digestion efficiencies that are comparable to off-line approaches in several minutes and sometimes even seconds [12].

After the digestion step, a peptide needs to be selected for subsequent quantification, the so-called signature or proteotypic peptide. For this purpose, several selection criteria have been defined [13]. Firstly, the peptide has to uniquely identify the targeted protein. Several bioinformatics tools developed for this purpose are available, both commercially and free of charge (an extensive software list is available at [www.ms-utils.org](http://www.ms-utils.org)). Some of these tools use algorithms that match peptide sequences to a protein or translated nucleic acid sequence database that contains all known proteins (open reading frames) from a selected species. Candidate signature peptides should not match tryptic peptides of endogenous proteins to ensure selectivity of the method. Absence of interfering peptides arising from endogenous proteins should

always be confirmed experimentally by analysis of several matrix samples and the occurrence of any interfering peaks disqualifies this peptide from use. Secondly, to prevent chemical degradation of the signature peptide, candidates containing unstable amino acids such as methionine, cysteine, tryptophan, asparagine or glutamine should be avoided to prevent changes in concentration during sample handling or storage. Stability of an internal standard containing these amino acids may also be compromised. Careful consideration needs to be given when post-translational modifications (PTMs), such as carbohydrate chains, are present on a protein biopharmaceutical. PTMs and other modifications make that a protein is hardly ever present as one single species [14]. Quantitative information on a signature peptide obtained by LC–MS/MS may thus only reflect the amount of a given protein species and are not representative of the amount of all species derived from the biopharmaceutical. Whether this is relevant for the purpose of the quantitative analysis and whether peptides containing PTMs must be included in the analysis requires careful consideration. In the case of biopharmaceutical proteins, chemical entities such as polyethylene glycol groups are sometimes synthetically attached to the protein after production (PEGylation), in order to improve physicochemical properties, to increase in vivo half-life or to alter the immunological profile of the protein. This type of modification may affect digestion and ionization characteristics of the analyte and might thus impose additional limitations when selecting an internal standard. Thirdly, peptides resulting from proteolysis at adjacent cleavage sites should be avoided as this may give rise to missed cleavages. The final criterion when selecting a signature peptide is based on the analytical response that the peptide generates because sensitivity of the analytical method depends heavily on the ionization and fragmentation characteristics of the peptide. When sufficient information is present, these selection criteria can all be checked against the results of an *in silico* digestion prior to any experimental work. Software solutions designed specifically for this purpose are available [15].

## 2.3. Instrumental analysis

Until recently, only tandem mass spectrometric detectors coupled to liquid chromatography offered the robustness, selectivity and sensitivity necessary to quantify analytes in a complex biological matrix. Therefore, they have become the workhorse in this analytical field. Nevertheless, other types of mass analyzers, including several types of ion-traps and time-of-flight (TOF) instruments, can be used for this purpose. They enable significantly higher mass resolution and faster spectral acquisition compared to tandem MS, making them better suited for qualitative determinations, but they lack its sensitivity and usually display lower reproducibility. However, in the case of relatively abundant analytes, when a good internal standard is available, the choice for tandem mass spectrometry becomes less obvious, as is demonstrated in a recent comparison of the performance of a tandem quadrupole and a high-resolution (HR) ion trap instrument in the LC–MS(/MS) quantification of 17 drugs in patient plasma samples, using only close structural analogue or SIL internal standards where a very good correlation between the HR and SRM results was found [16].

To confirm that measured concentrations correspond uniquely to that of the protein of interest, it is common, in qualitative protein analysis using LC–MS/MS, to monitor multiple tryptic peptides from a protein simultaneously in a single analysis. Additionally, several selected reaction monitoring (SRM) transitions for each peptide can be used. The selectivity of analytical methods towards other proteins is established in a different manner in targeted quantitation of biopharmaceutical proteins. Typically, in this field, a single signature peptide is monitored using one SRM transition. The extensive method validation and quality control guidelines

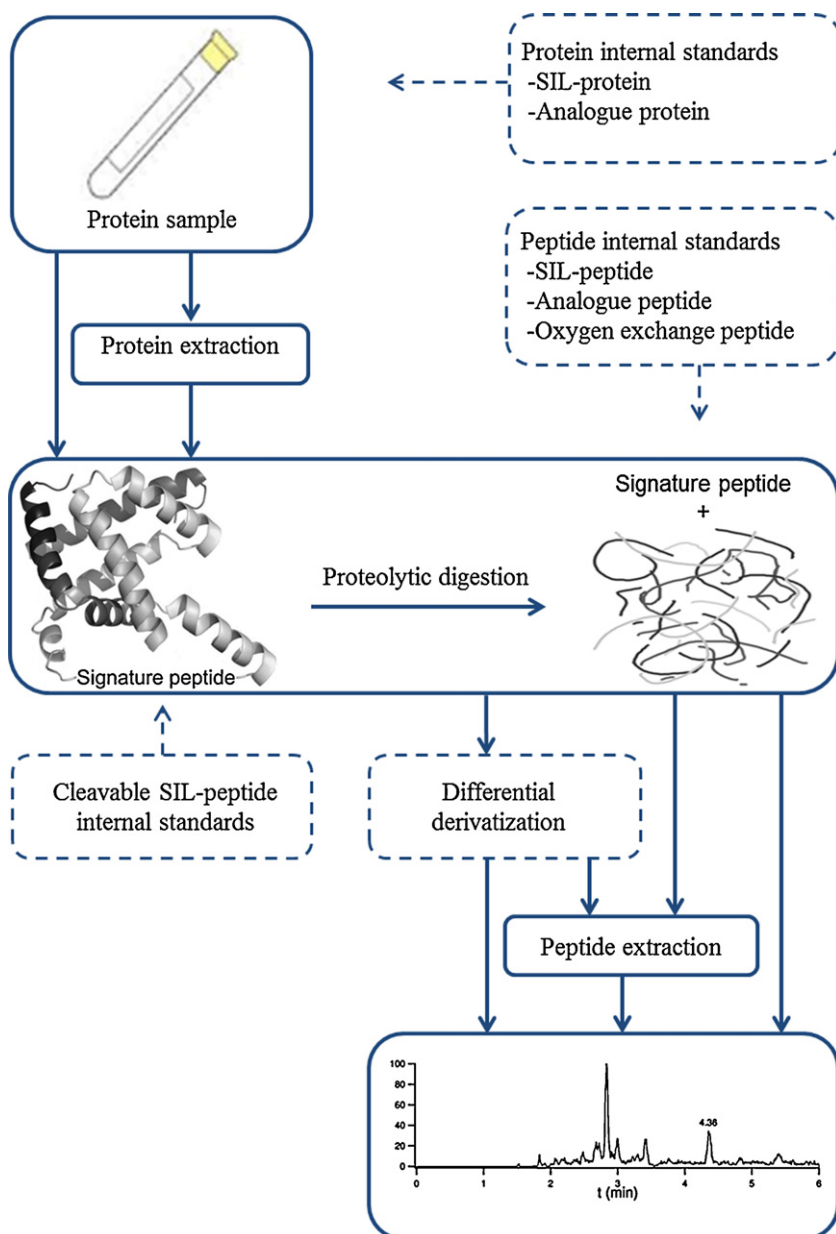


Fig. 1. Schematic representation of the different workflows and internal standardization possibilities.

combined with strict criteria provide the necessary confidence in the results.

#### 2.4. Workflow and possibilities for internal standardization

In comparison to bioanalysis of small molecules, the analytical workflow for protein quantification, shown in Fig. 1, is more complicated and several approaches for internal standardization in use in this field are not found in small-molecule bioanalysis.

Ideally, an internal standard is added to the sample at the beginning of the analytical procedure so that it may correct for all experimental variability. A first step in the analytical procedure is the extraction of the protein of interest from the biological matrix to reduce sample complexity and/or enrich the analyte. Due to the high complexity of the plasma and serum proteome [17], this is generally a difficult task. In the field of targeted absolute quantification with LC-MS/MS, only a few techniques are described: immuno-affinity extraction, albumin depletion and partial protein precipitation. In order to properly cover this initial step, the

internal standard needs to be an intact protein, either a SIL-form of the analyte or a structural analogue.

Digestion of an enriched protein or of an untreated complex sample is an enzymatic process, the efficiency of which depends on a multitude of factors. If an internal standard is to correct for variability resulting from this step, it has to be added to the sample before digestion, and should contain at least one cleavage site. When a protein internal standard is not available, a smaller peptide with a cleavable group can offer some correction for the digestion step.

For methods in which extraction of the protein analyte from the matrix is not performed, the sample after digestion is highly complex because of the presence of peptides released from endogenous proteins. To reduce this complexity, some form of extraction of the signature peptide can be applied, examples being reversed-phase or ion-exchange solid-phase extraction (SPE) as well as immuno-affinity extraction. A variety of peptides can be used as internal standards to correct for variation of the extraction step: a peptide resulting from digestion of a protein internal standard (SIL or

structural analogue) or of a peptide with a cleavable group, as well as a (SIL or structural analogue) peptide added to the sample prior to the extraction step.

The differential derivatization approach adds another possible step to the workflow. This step creates a SIL internal standard through chemical derivatization of a pure form of the peptide with a stable-isotope labeled reagent, which is performed in parallel to derivatization of the signature peptide, after extraction from the biological sample, with a non-labeled form of the derivatization reagent. The internal standard is added to the sample just before instrumental analysis and will thus not correct for any of the other steps.

LC–MS/MS itself will also introduce variability. Most internal standard approaches correct well for this step. A SIL-peptide internal standard added to the sample, released by digestion of a SIL-protein, a cleavable SIL-peptide or a SIL-peptide created by differential derivatization will have identical chromatographic and mass spectrometric characteristics as the signature peptide and will thus correct for instrumental variability. Analogue internal standards, both proteins and peptides, do not correct ideally for this final step of the analytical procedure.

In the following sections, an in depth review of the different approaches for using internal standards, illustrated with selected examples is presented, including their application, particular (dis)advantages and availability. Table 1 shows a summary of the relevant literature.

### 3. Protein quantification using protein internal standards

#### 3.1. Stable-isotope labeled proteins as internal standards

Considering the widespread use and the favorable performance of SIL internal standards in small-molecule bioanalysis, one would want to apply the principle of stable isotope labeling to the quantification of proteins as well. A SIL version of the protein biopharmaceutical added to the sample at the very beginning of the analytical procedure as internal standard corrects for all sources of variation throughout the entire analytical procedure. The extent and location of labeling should be chosen in such a manner that the signature peptide can be clearly distinguished from the analyte after digestion of the SIL-protein.

Small-molecule SIL internal standards are relatively easy to produce by organic synthesis. SIL-proteins, however, are more difficult to obtain because the specific folding of the linear amino acid sequence, the creation of intra-molecular disulfide linkages connecting multiple amino acid sequences and further PTMs are all processes that, in nature, occur only in living cells and are impossible to reproduce in a synthetic manner.

Biopharmaceuticals are produced by genetically modified cell lines, yeast or bacteria. The cell line is allowed to grow in a culture and at a certain point in time the biopharmaceutical protein is purified from the cell lysate or the cell culture supernatant. To produce a SIL-protein, cells are grown in a medium containing, for example, only the heavy isotope labeled form of one or several amino acids. The organism will incorporate the heavy isotope label in its proteins, thus creating the SIL-protein that is subsequently purified and used as an internal standard. This technique is also referred to as SILAC (Stable Isotope Labeling with Amino Acids in Culture) [18] and has been in use for years alongside other isotopic labeling techniques for proteins, for example, in structural magnetic resonance (NMR) analyses of proteins [19]. A limitation may be that the cell line and knowledge about the production process are typically only available to the company developing the biopharmaceutical, and is considered to be proprietary material. When the cell line is available, however, it is often possible to produce mg-amounts of a given SIL-protein in a commercially available protein production system.

An alternative approach is production of the SIL-protein in a cell-free system containing a lysate of *Escherichia coli* or wheat germ [20]. The supernatant of these cell lysates after centrifugation at  $30,000 \times g$ , also known as the S30 fraction, contains the molecular machinery for protein synthesis. After introduction of a suitable expression vector coding for the protein of interest, transcription and translation takes place in the presence of (labeled) amino acids and a number of additional reagents, resulting in the (labeled) protein of interest.

After the SIL-protein has been produced, it requires extensive purification and characterization before it can be used as internal standard. In the cell-free method, significantly less proteolytic enzymes are present compared to techniques that use living cells and this facilitates purification of the labeled target protein. The main disadvantage of the cell-free technique is that due to differences between the cell lines used in production of the biopharmaceutical protein and the cell-free transcription and translation of the internal standard, molecular differences between the two are likely, especially where PTMs are concerned. This makes an internal standard produced in this way less than ideal. For screening purposes, it does however represent a way to produce a close analogue of the biopharmaceutical protein that can be used as internal standard.

Heudi et al. [21] reported the use of a SIL-mAb as internal standard in an LC–MS/MS method for determination of the concentration of the mAb drug candidate in marmoset serum. The internal standard was produced in the same way as the analyte, with the exception that each threonine contained four  $^{13}\text{C}$  and one  $^{15}\text{N}$  atom. After digestion, a labeled version of the signature peptide was released from the internal standard and used to correct for analytical variability during the remainder of the procedure. The analytical method used SPE on a mixed-mode cation-exchanger to enrich the signature peptide and its labeled equivalent from the digested plasma sample. The SPE step was set up in a way that did not make use of the non-polar properties of the SPE material, effectively making it a cation-exchange extraction. Such a procedure will retain most tryptic peptides in the digest, because trypsin cleaves at lysine and arginine residues, resulting in the presence of a positive charge at the C-terminus. The resulting extract was evaporated to dryness and reconstituted prior to reversed-phase LC–MS/MS analysis. Reversed-phase LC provides a complementary selectivity dimension compared to cation-exchange SPE and is able to separate the signature peptide from interfering peptides.

Fig. 2 shows that the tryptic peptide of the mAb and its internal standard co-elute from the analytical column. Even though the recovery of analyte was quite low, about 14%, the internal standard suffers the same losses and, thus, the initial ratio of compound to internal standard is maintained throughout the procedure. The method was fully validated according to the FDA guidelines for small molecules. The method's intended use was to determine pharmacokinetics using marmoset serum. Due to the high doses given, a lower limit of quantitation (LLOQ) of  $5 \mu\text{g/mL}$  was sufficient. Incorporation of an immuno-affinity step to selectively enrich the mAb from serum would be an option, as the SIL-mAb internal standard is expected to correct for variability caused by the analytical procedure. This would probably boost the sensitivity of the assay, as can be deduced from Table 1, which shows that analytical procedures using enrichment of the intact protein generally result in more sensitive methods with LLOQs in the low nmol/L range.

#### 3.2. Structural analogue proteins as internal standards

A structural analogue of the protein of interest can also function as internal standard. This approach has the advantage that one can choose from many commercially available proteins, often of high

**Table 1**  
Concise summary of published methods for targeted protein quantification in biological matrices using LC–MS/MS.

First author	Year	Analyte	Analyte mass (kDa)	Internal standard	LLOQ (nmol/L) <sup>g</sup>	Matrix	Extraction technique	Ref. #
<i>Digestion of complete sample matrix, without subsequent peptide extraction</i>								
A. Lesur	2010	Human mAb	~150	SIL-protein	133	Human plasma	–	[10]
C. Seegmiller	2009	Human serum albumin	66	SIL-protein	47	Human urine	–	[41]
F. Li	2009	Somatropin	22	Analogue	91	Human plasma	–	[4]
M. Aguiar	2006	C-reactive protein	25	SIL-tryptic-peptide	2	Rat-urine	–	[42]
P. Bondar	2007	Zn- $\alpha$ -glycoprotein	41	SIL-tryptic-peptide	7.8	Human serum	–	[30]
D.R. Barnidge	2004	Prostate Specific Antigen	28	SIL-tryptic-peptide	160	Human serum	–	[43]
C. Ji	2009	Human mAb	~150	Dimethyl label	6.7	Human serum	–	[47]
<i>Digestion of complete sample matrix, with subsequent peptide extraction</i>								
O. Heudi	2008	Human mAb	~150	SIL-mAb	33	Marmoset serum	SPE (MCX)	[21]
Z. Yang	2007	Human mAb	~150	Analogue protein	6.7	Human plasma	SPE (2D)	[25]
A.N. Hoofnagle	2008	Human thyroglobulin	660	SIL-tryptic-peptide	0.004	Human serum	Immunocapture	[29]
<i>Digestion of extracted protein</i>								
V. Kumar	2010	1–84 parathyroid hormone	9	SIL-protein	0.004	Human serum	Immunocapture	[44]
M. Dubois	2008	Erbix	152	Analogue protein	0.13	Human serum	Immunocapture	[22]
S. Halquist	2011	Alefacept	91	Analogue protein	2.8	Human plasma	Partial protein precipitation	[45]
S.T. Wu	2011	Pegylated protein	52	Analogue protein and peptide	0.19	Monkey plasma	Partial protein precipitation	[24]
D.R. Barnidge	2003	Rhodopsin	39	SIL-tryptic-peptide	0.1	ROS membrane	Immunocapture	[46]
C. Hagman	2008	Human mAb	~150	SIL-tryptic-peptide	13	Human serum	Albumin depletion	[28]
E. Kuhn	2004	C-reactive Protein	25	SIL-tryptic-peptide	1	Human serum	depletion and SEC <sup>a</sup>	[48]
M. Ocaña	2010	Matrix metalloprotease-9	92	Cleavable SIL-tryptic-peptide	0.03	Mouse serum	Immunocapture and MC <sup>b</sup>	[35]
H. Jiang	2010	CFTR <sup>c</sup>	168	<sup>18</sup> O-tryptic peptide	0.25	Cell lysate	Immunocapture	[33]
F. Guan	2007	rhEPO <sup>d</sup> /DPO <sup>e</sup>	~35	None	0.003	Horse plasma	Immunocapture	[40]
N. Yu	2010	rhEPO/DPO/PEG-EPO <sup>f</sup>	~35/60	None	0.003	Horse plasma	Immunocapture	[49]

<sup>a</sup> Size exclusion chromatography.

<sup>b</sup> Multi-dimensional chromatography.

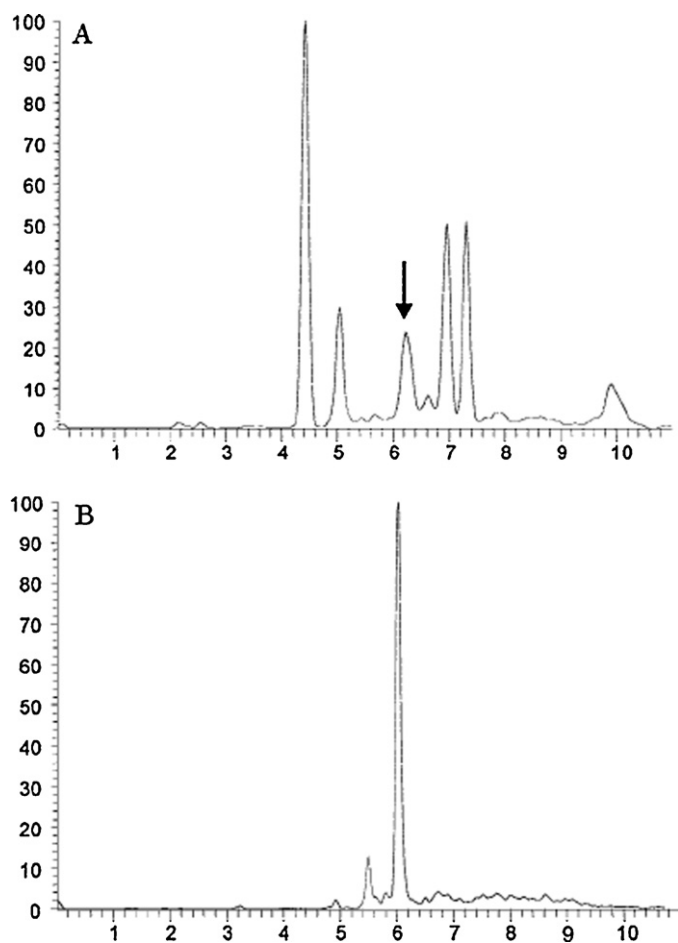
<sup>c</sup> Cystic fibrosis trans-membrane conductance regulator.

<sup>d</sup> Recombinant human erythropoietin.

<sup>e</sup> Darbopoietin.

<sup>f</sup> Polyethylene glycol erythropoietin.

<sup>g</sup> LLOQs have been converted to nmol/L to facilitate comparison of methods.



**Fig. 2.** LC-MS/MS chromatograms of the signature peptide TGPFDDY-WGQGTLLVTSSASTK after digestion of an untreated serum sample containing the mAb, spiked at the LLOQ of 5 µg/mL (A), and its  $^{13}\text{C}_4$ - $^{15}\text{N}$ -threonine labeled protein internal standard (B).

Adapted with permission from [21], copyright 2011 American Chemical Society.

purity and for a reasonable price, eliminating the need to synthesize a specific protein especially for this purpose. The selection of an analogue protein internal standard that sufficiently corrects during all sample handling steps is key to the success of such a method. Internal standard candidates should exhibit a close resemblance to the protein of interest. Factors to consider include molecular mass, isoelectric point, number of chargeable amino acids and the release of a suitable peptide upon digestion. With regards to the last issue, it is possible, using *in silico* digestion software, to screen peptides resulting from the digestion of the internal standard and evaluate the similarity to the signature peptide of the analyte.

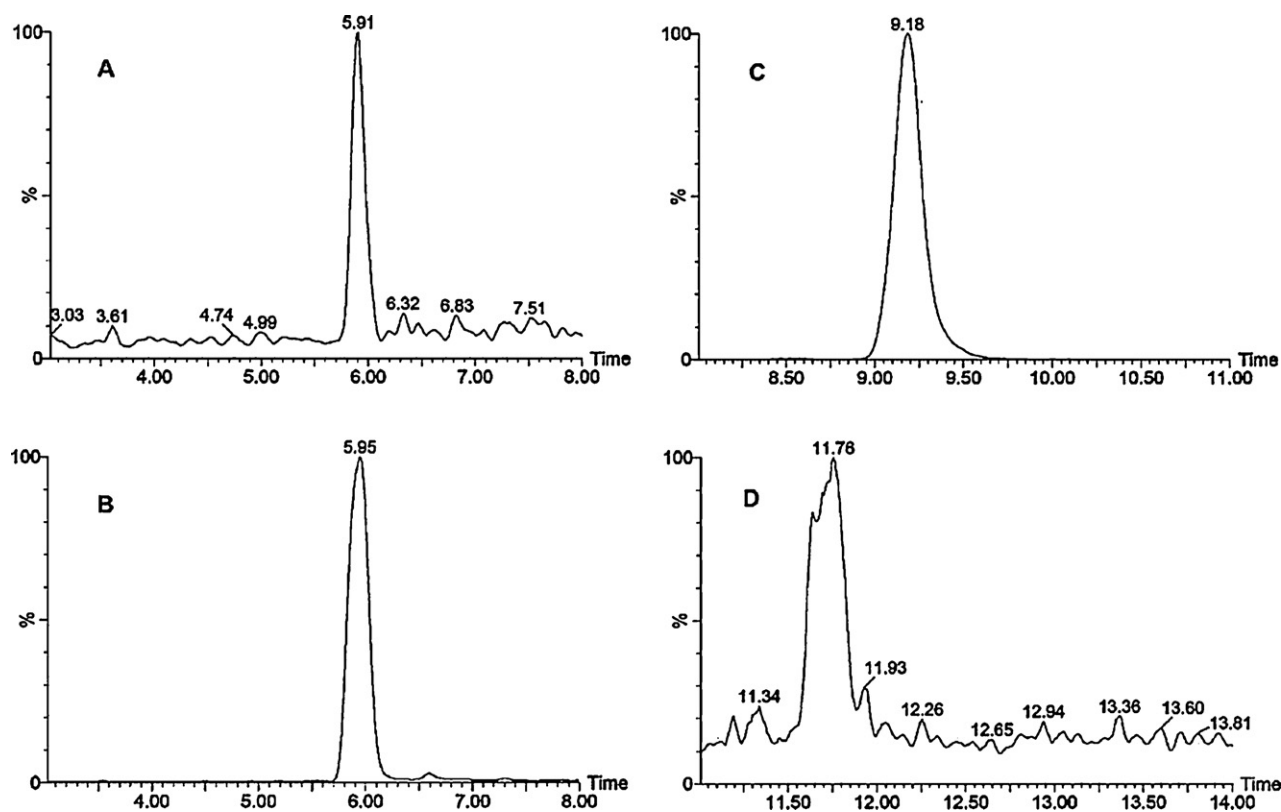
Unfortunately, such an internal standard will never correct completely for all of the sample preparation steps. The initial ratio of analyte to internal standard may be altered due to differences in e.g. extraction recovery, completeness of digestion, adsorption behavior and ion-suppression. When using an analogue protein as internal standard, protein extraction prior to digestion is rarely performed because the available options are rather limited. Immuno-affinity based extractions are highly specific and often fail to co-extract both analyte and the analogue internal standard.

One exception to this rule is the approach used in an analytical method reported by Dubois et al. [22]. In this method, a chimeric (containing both human and murine constituents) mAb drug candidate (erbitux) was quantified. As internal standard, a mouse mAb against the same antigen as the drug of interest was used. In this

way, an immuno-affinity extraction using the immobilized antigen could be used to selectively extract the drug as well as the internal standard from human serum. After protein extraction, the samples were denatured and digested using trypsin and the resulting peptide mixture was analyzed using reversed-phase chromatography and tandem mass spectrometry. The mouse mAb internal standard offered several tryptic peptides to select from (Fig. 3). Due to its non-human origin, a number of these peptides were not found in human proteins, and one of these (Fig. 3B) was selected because it showed similar chromatographic behavior as the signature peptide (Fig. 3A). A three-day partial validation showed that coefficient of variation (CV) was below 20%. The use of a suitable internal standard and optimization of the digestion step were instrumental in achieving this. The method has a LLOQ of 20 ng/mL, which is similar to the LBA for this mAb.

To reduce sample complexity, some reports describe the use of less selective methods, such as albumin depletion or partial protein precipitation techniques to remove highly abundant proteins [23,24]. These depletion techniques are losing popularity due to solubility issues, where analytes bound to albumin are at risk of being partially co-depleted. An alternative approach to reduce sample complexity is extraction of the signature peptides released from the analyte and its internal standard from a digest of the entire sample. Peptides are, in contrast to proteins, compatible with most extraction techniques from the small-molecule bioanalysis field and notably with the many options offered by SPE, which means that there is a wide range of possibilities for sample clean-up. In the method by Yang et al. [25], which uses this approach, bovine fetuin was used as internal standard in the quantification of a therapeutic mAb in human plasma. After addition of the internal standard, the complete sample was digested using trypsin and the resulting signature peptides of the analyte and the internal standard were extracted by two-dimensional SPE. A reversed-phase cartridge was used in the first, and a cation-exchange cartridge in the second dimension. The necessity for two-dimensional extraction is illustrated in Fig. 4. After pretreatment, the extracts were analyzed using LC-MS/MS. The signature tryptic peptides of the analyte and bovine fetuin eluted close to each other, but did not co-elute in the chosen chromatographic system. Despite the differences between the two proteins and their resulting tryptic peptides, the internal standard corrected quite well for the analytical variation. Results of quality control samples demonstrate that the method has acceptable performance. While the selection process of the internal standard is not described, the authors comment on the broad applicability of bovine fetuin as internal standard in protein analysis. Due to its bovine origin, many tryptic peptides that are formed during digestion are not commonly formed from human proteins. From these peptides, it is often possible to select one that is similar to the signature peptide of a large range of proteins of interest. This is an advantageous feature, because when using an analogue protein internal standard and extracting peptides from the digested plasma, one needs to optimize the extractions to quantitatively extract the signature peptides of both the analyte and the internal standard. The availability of several candidate internal standard peptides increases the chance of reaching a suitable compromise.

Furthermore, the publication offers a comparison between the performance of a SIL version of the signature peptide (see Section 4.1), and the protein bovine fetuin used as an analogue internal standard. In this comparison, the correlation coefficients of a set of calibration samples measured using both approaches are compared and it is concluded that the analogue protein internal standard outperforms the SIL signature peptide. This finding underlines the importance of having an internal standard that corrects for as many sample handling steps as possible and, in this example, the SIL-peptide does not correct for the digestion.



**Fig. 3.** LC-MS/MS chromatograms of three signature peptide candidates ASQSIGTNIHWYQQR (A), VVSLVTLVHQDWLNGK (C) and GLEWLGVWISGGNTDYNTPTFSR (D) of the humanized murine mAb erbitux spiked at 20 ng/mL into serum, and one peptide of the analogue mAb internal standard (B).

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## 4. Protein quantification using peptide internal standards

### 4.1. Stable-isotope labeled peptides as internal standards

When a SIL-protein is unavailable, a peptide added to the digestion mixture can be used as internal standard. The SIL version of the chosen signature peptide released from the protein can be created using solid-phase peptide synthesis [26]. Specialized synthetic labs offering the procedure as a service are becoming more common. For SIL-peptide internal standards, any one or a number of amino acids in the sequence can be replaced using the corresponding SIL amino acid.

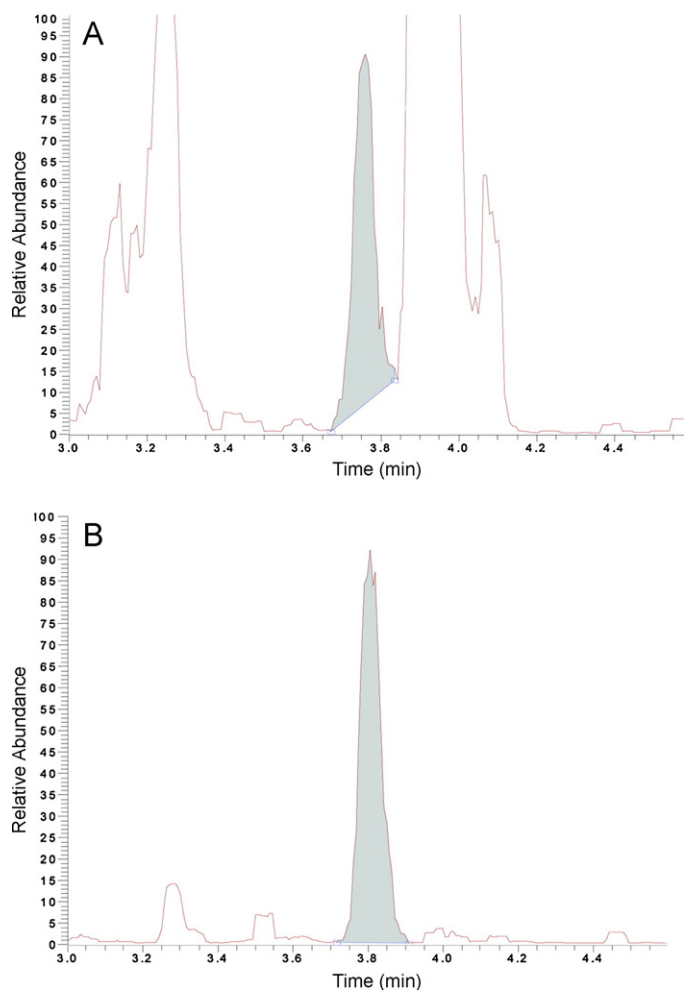
This internal standard will not correct for steps in the procedure preceding and including the actual release of the signature peptide from the protein during digestion. It will however correct for variations caused by the subsequent extraction steps and the final LC-MS/MS analysis. By optimizing all steps in the procedure that are not covered by the internal standard, acceptable results can be obtained, as is reported in several publications. Arsene et al. reported on the effects of digestion rate on the accuracy of an analytical method [27]. Using purified recombinant growth hormone as a model analyte, it was demonstrated that careful optimization of the digestion is crucial, as any variation in the completeness of proteolytic digestion will negatively affect the obtained results. Fig. 5 shows how the internal standard corrects for degradation of the tryptic peptides in the digestion mixture after release from the protein. In this case, the actual release of the tryptic peptides is relatively fast, and the reaction should be terminated after 1 h. Longer digestion times resulted in decreased sensitivity due to degradation or adsorption of the signature peptide. The SIL-peptide internal standard corrected for degradation when added prior to the digestion step, because it is degraded at the same rate as the

analyte, which will not typically be the case for analogue internal standards.

Hagman et al. described an analytical method for a therapeutic mAb using a SIL-signature peptide as internal standard [28]. The SIL-peptide internal standard is added to serum at the start of the sample preparation procedure. The method consists of an albumin depletion step, reduction and alkylation, overnight digestion using trypsin and analysis of the digest using LC-MS/MS. Even though the internal standard does not correct for any of the steps in the procedure, except for the final LC-MS/MS step, bias and precision (CV) results were acceptable (below 20%), probably because of the effort that was invested in choosing and optimizing the albumin depletion method, which is not covered by the internal standard.

When a SIL-peptide is used, it is preferable to use an approach that minimizes sample handling variability at steps for which the internal standard does not correct and to digest the entire sample matrix and perform extractions on the digest. A SIL-peptide internal standard will correct for the peptide extractions, because it is chemically identical to the signature peptide of the analyte. This approach was reported by Hoofnagle et al. [29] for thyroglobulin, a low abundance serum protein which is useful as a cancer biomarker. Quantification of thyroglobulin using LBAs is often problematic due to nonspecific binding and competing auto-antibodies. These issues are avoided when using the LC-MS/MS approach. After digestion of untreated serum, the SIL-peptide is added to the digest, an immuno-affinity extraction of the (SIL) peptide is performed, and the extract is analyzed using LC-MS/MS. This approach is known as Stable Isotope Standards and Capture by Anti Peptide Antibodies (SISCAPA). As both the non-specifically binding proteins and competing auto-antibodies are co-digested, these proteins are no longer able to negatively impact the results. While this approach allows the detection of tryptic peptides from



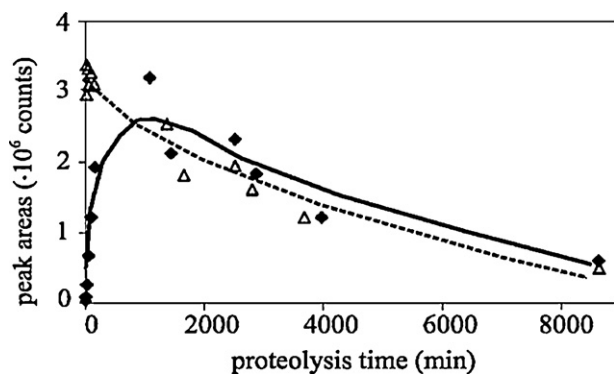


**Fig. 4.** LC-MS/MS chromatograms of the signature peptide (LFDNAMLR) after digestion of a plasma sample containing the therapeutic mAb somatropin spiked at 1 µg/mL. Sample clean-up with only reversed-phase SPE (A) or with a combination of reversed-phase SPE and ion-exchange SPE (B).

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low abundance proteins, there still is the undesirable need for the time-consuming production and characterization of antibodies.

Several methods have been published, reporting the SIL-peptide internal standard approach, where the untreated sample is digested



**Fig. 5.** Time profile of the abundance of the unstable peptide YSFLQNPQTSLCF-SESIIPTPSNR (filled diamonds, solid line) during trypsin digestion of purified recombinant growth hormone and of the SIL-peptide internal standard containing a  $^{13}\text{C}_6$ - $^{15}\text{N}$ -leucine in position 11 (triangles, dashed line) added prior to digestion. Adapted with permission from [27], copyright 2007 American Chemical Society.

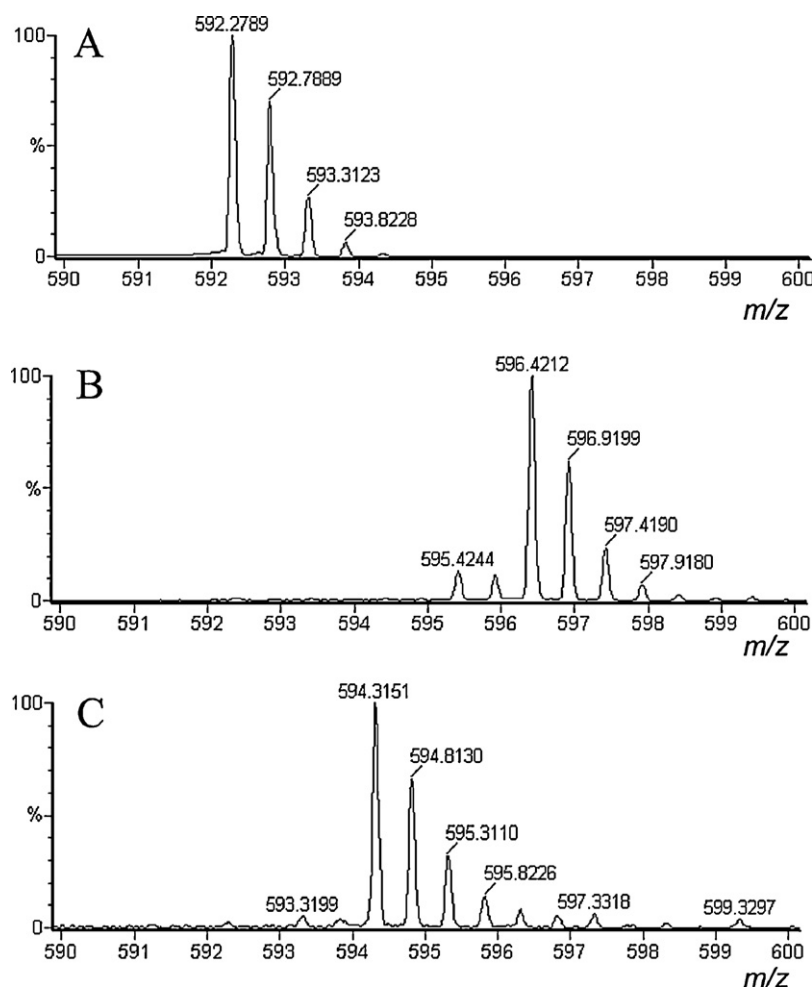
and analyzed without further pretreatment using LC-MS/MS, making this a relatively straightforward approach. Selectivity results from the chromatographic separation and the mass spectrometric detection. As the SIL-peptide internal standard corrects ideally for this part of the method, this approach is expected to perform well. However, sensitivity is often limited, because the high abundance of other peptides in the digested sample restricts the injection volume (to avoid overloading of the chromatographic column) and may also cause ion suppression of the analyte. An example of such an approach was reported by Bondar et al. [30]. The analyte was the abundant serum protein Zn- $\alpha$ -glycoprotein which is a potential prostate cancer biomarker. In the reported method, the untreated serum sample is subjected to trypsin digestion, after which the internal standard is added and finally the digest is analyzed using LC-MS/MS. For this application, an LLOQ of 0.32 mg/mL was sufficient and, therefore, the method did not require incorporation of sophisticated extraction techniques. For low-abundance proteins that need to be quantified at much lower concentrations, this approach will usually not provide sufficient sensitivity.

In a special type of SIL-peptide internal standard, the  $^{16}\text{O}$  atoms of carbonyl groups present on a peptide are exchanged with  $^{18}\text{O}$  from  $\text{H}_2^{18}\text{O}$  under acidic conditions. This reaction uses available chemicals in a straightforward procedure, and potentially offers a generic and relatively inexpensive platform to produce internal standards [31]. The C-terminal carboxylic acid group as well as aspartic and glutamic acid residues are all locations for this type of acid-catalyzed oxygen exchange. Each carboxylic acid group in the peptide will receive an additional mass of 4 Da. Any peptide can be treated in this way and subsequently used as a SIL internal standard, provided that the mass difference between the treated and untreated peptide is large enough. Although this sounds promising, the procedure has some disadvantages. Firstly, back exchange with naturally abundant  $^{16}\text{O}$  in water can occur during sample processing or during storage in for example an autosampler, when pH and storage conditions are not optimal [32]. Secondly, complete exchange of all susceptible oxygen atoms in a molecule is difficult, often resulting in a mixture of several partially labeled molecules. Finally, addition of the  $^{18}\text{O}$ -SIL-peptide to the sample prior to digestion will result in enzyme-catalyzed back-exchange of the C-terminal oxygen atoms, resulting in a reduction of the mass difference. Therefore, the use of this type of internal standard in quantitative bioanalysis is rarely reported.

Jiang et al. [33] reported the use of this approach for the absolute quantification of the cystic fibrosis transmembrane conductance regulator (CFTR) in cell lysate using both Lys-C and trypsin for digestion. A SIL-peptide containing four  $^{18}\text{O}$  atoms, created by acid-catalyzed oxygen exchange, was added to the sample. To compare differences in performance of the method when adding the internal standard at different points in the procedure, it was added before and after trypsin digestion. The  $^{18}\text{O}_4$  SIL-peptide added to the sample before digestion was converted to an  $^{18}\text{O}_2$  SIL-peptide by enzyme catalyzed oxygen exchange. The  $^{18}\text{O}_4$  and the  $^{18}\text{O}_2$  SIL-peptides were found to perform equally during quantification. In Fig. 6, high resolution mass spectra of the peptide and its two internal standards are shown. Enzyme-catalyzed  $^{18}\text{O}$  incorporation into the C-terminal carboxylic acid group during trypsin digestion is used in quantitative proteomics, and has recently been applied to targeted protein quantification. Other carboxylic groups are unaffected and the mass difference is therefore smaller than in acid catalyzed exchange.

#### 4.2. Stable-isotope labeled peptides containing a cleavable group as internal standards

Recently, SIL-peptide internal standards containing a cleavable group have received increased attention. This approach for internal



**Fig. 6.** High-resolution mass spectra of the doubly charged signature peptide NSILTELHR released by digestion from the protein CFTR; native form containing solely  $^{16}\text{O}$  (A), after acid-catalyzed  $^{18}\text{O}$ -exchange of the digest, resulting in NSILTE( $^{18}\text{O}_2$ )TLHR( $^{18}\text{O}_2$ ) (B) and after digestion of the  $^{18}\text{O}$ -containing peptide, resulting in NSILTE( $^{18}\text{O}_2$ )TLHR because of partial  $^{16}\text{O}$ -back exchange (C).

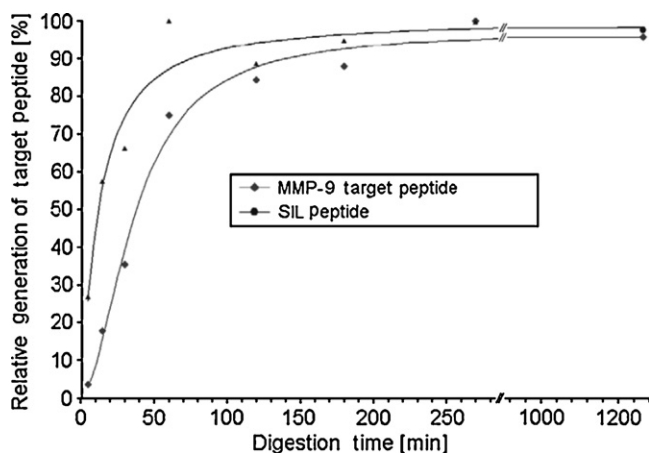
Adapted with permission from [33], copyright 2010 American Chemical Society.

standardization offers partial correction for variations in trypsin digestion efficiency in addition to correcting for any subsequent sample handling and instrumental analysis steps. The internal standard molecule consists of a SIL version of the signature peptide to which a cleavable sequence tag has been attached. This tag is cleaved during trypsin digestion releasing the SIL-peptide. Different cleavable tags can be connected to the SIL-peptide, so one might choose to use one that also improves the physicochemical properties of the internal standard. For example, using a polar group will improve solubility of the internal standard in an aqueous environment while using an extended amino acid chain might alter the digestion characteristics of the internal standard to better mimic those of the analyte protein.

Depending on the location of the signature peptide in the protein it is released either in the early phase of digestion, which is the case when it is located on the surface of the protein, or not until the digestion is reaching completeness in case of less accessible internal peptides. As a rule, the cleavage site in this type of internal standard is rather accessible compared to the protein analyte, which means that it will provide the best correction when the corresponding signature peptide is located on the surface of the target protein. A comparison between a SIL-peptide internal standard with and without a cleavable tag was reported by Barnidge et al. [34]. The model protein human serum albumin (HSA) was subjected to a number of digestion procedures in the presence of these two types

of internal standards. In order to be able to distinguish between the two SIL internal standards after digestion, a different amount of labeling was used in each of them. The cleavable internal standard contained two separate cleavage sites. Release of the HSA-signature peptide was complete in 20 min, while the release of the labeled HSA-signature peptide from the cleavable internal standard was complete in 1 min. Linear regression using the signature peptide and the two different internal standards demonstrated no significant differences in performance between the two approaches of internal standardization. The different digestion kinetics between the protein and the cleavable signature peptide internal standard explains the lack of difference in performance between a cleavable and a regular signature peptide. It was concluded that, in this case, a cleavable SIL-peptide cannot ideally correct for the digestion, and thus offers little advantage over a regular SIL-peptide internal standard.

An application of the approach is reported by Ocaña and Neubert [35], who describe an analytical method to quantify matrix metalloproteinase 9 (MMP-9) in mouse serum. MMP-9 was enriched from serum by immuno-affinity extraction. After desorption from the beads, the cleavable SIL-peptide was added and the sample digested using trypsin followed by multi-dimensional chromatography comprising three trap columns (reversed-phase, strong cation-exchange and reversed-phase), prior to nano-flow HPLC and tandem mass spectrometry. The release of the SIL-signature



**Fig. 7.** Time profile showing the differences in speed of release of the (SIL) peptide GSPLQGPFLTAR from MMP-9 and the cleavable SIL-peptide internal standard KFLNHR-GSPLQG[ $^{13}\text{C}_5$ - $^{15}\text{N}$ -P]F[ $^{13}\text{C}_6$ - $^{15}\text{N}$ -L]LTAR-TWPALP during trypsin digestion.

Adapted with permission from [35], copyright 2010 American Chemical Society.

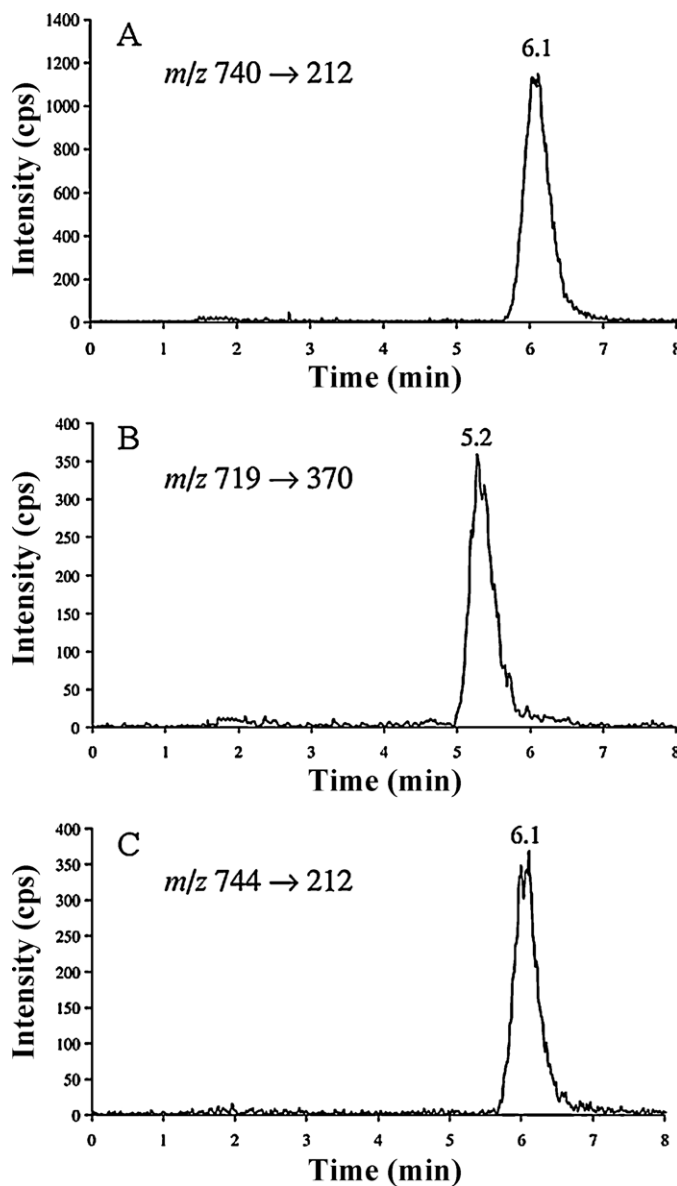
peptide from the cleavable SIL internal standard was significantly faster than the release of the signature peptide from the protein. A plateau was reached for both digestions after about 4.5 h indicating that both reactions had reached completeness resulting in a stable analyte/internal standard ratio, as is depicted in Fig. 7. Excellent stability of the signature peptide was observed under digestion conditions. Therefore an overnight digestion time of 21 h was used.

#### 4.3. Structural analogue peptides as internal standards

An analogue peptide may also be used as internal standard. Selection of a close analogue is required to sufficiently correct for the LC-MS/MS analysis as well as the preceding sample treatments. Of all possible internal standard approaches, this is the least attractive because it provides no correction for protein extraction and digestion and only limited correction for peptide extraction and instrumental analysis. Due to the increasing availability of SIL-peptide internal standards, this approach is now rarely used. The difference in performance of an analogue and a SIL-peptide internal standard was demonstrated by Stokvis et al. [36]. In the quantitative determination of the synthetic cyclic depsipeptide kahalalide F (1464 Da), these two approaches of internal standardization were compared. For this analyte, a digestion is not necessary and both internal standards could be added to the sample at the start of the procedure. The difference in assay performance when using these two different internal standards can therefore be directly attributed to the correction that each offers. It was concluded that the SIL-peptide internal standard offered a significantly better assay performance compared to the analogue internal standard. This difference in performance is explained by a difference in physicochemical properties of the analogue internal standard and the analyte, which, for example, results in a difference in retention time between the analyte and the analogue internal standard peptide while no such difference is observed for the SIL-peptide (Fig. 8).

### 5. Protein quantification using differential derivatization of peptides

Differential derivatization (labeling) of tryptic peptides is a common approach in the field of quantitative proteomics. Several methodologies have been developed and are commercially



**Fig. 8.** LC-MS/MS chromatograms of the doubly charged peptide kahalalide F (A) and two different types of internal standards: an analogue peptide (B) and a SIL-form of the analyte (C).

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available [37]. Due to the non-targeted nature of the analytical approach, a great advantage of differential labeling lies in the simultaneous creation of SIL-peptide internal standards for all tryptic peptides in a sample in one single step (global internal standard). The broad applicability of this approach has resulted in a number of new, innovative strategies. Because the field has been adequately reviewed [38,39], only the approaches applicable to targeted protein quantification are covered in this review. Due to the targeted nature of absolute biopharmaceutical quantification, creation of multiple SIL-peptide internal standards is not as advantageous as in proteomics, making the approach less popular in this field.

Dimethyl labeling using formaldehyde was the first differential derivatization technique to be used in the field of quantification of biopharmaceuticals. In dimethyl labeling, the biological sample containing the protein of interest is digested and the resulting peptides, including the signature peptide, are derivatized using formaldehyde. In parallel, a fixed amount of a standard solution

**Table 2**  
Comparison of different approaches for internal standardization for targeted protein quantification by LC–MS/MS.

Approach	Offers correction for				Availability
	Protein extraction	Digestion	Peptide extraction	LC–MS/MS	
SIL-protein	+	+	+	+	–
Analogue protein	–	±	±	±	+
SIL-peptide	–	–	+	+	+
Cleavable SIL-peptide	–	±	+	+	+
Analogue peptide	–	–	–	–	+
Differential labeling	±	±	+	+	+
No internal standard	–	–	–	–	Not applicable

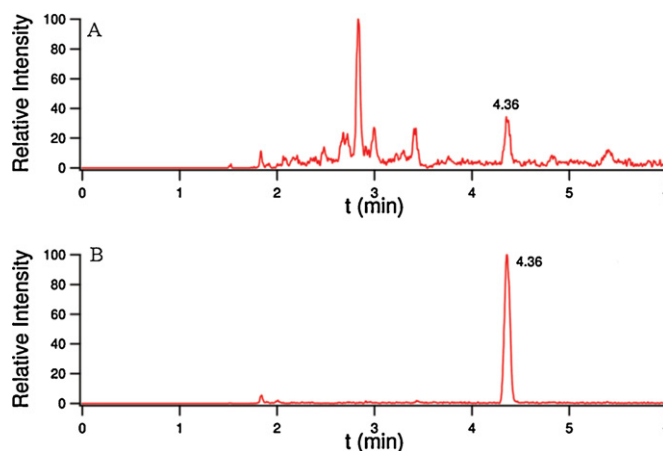
of the protein is digested and the signature peptide derivatized with formaldehyde- $d_2$ , thus creating a SIL internal standard for the derivatized sample. The samples are mixed and subsequently analyzed using LC–MS/MS. The mass difference between the analyte and the internal standard peptides after derivatization depends on the number of free amines in the signature peptide. Free primary amines are present at the N-terminus and in lysine side chains. After derivatization, each amine is methylated twice with labeled or unlabeled formaldehyde, resulting in a mass difference of 4Da. An advantageous characteristic of the approach is its broad applicability, as every tryptic peptide contains at least one free amine at the N-terminus. Furthermore, chemicals used in the procedure are inexpensive and readily available. During the sample handling steps preceding instrumental analysis, both the analyte and its internal standard undergo the same treatment. However, the internal standard and the analyte are derivatized in a different tube and, therefore, any between-sample variability during this step will negatively impact the final results. Hence, it is advised to limit sample handling prior to the derivatization step. Once the labeled internal standard has been added to the sample, it is expected to correct for instrumental variability.

Ji et al. [47] reported on the applicability of this approach for the absolute targeted quantification of a therapeutic monoclonal antibody as a model compound. After digestion of the samples in a 96-well format, formaldehyde- $d_2$  was added to a single well, which contained a high concentration of the digested protein of interest. To the rest of the wells, undeuterated formaldehyde was added. After completion of the reaction, the content of the well containing the SIL internal standard was diluted and an equal amount was added to all other wells. In this approach, a borane–pyridine complex was used as reductive agent. For this specific application, sensitivity and selectivity were sufficient, even though no extractions were performed. This is demonstrated in Fig. 9, which shows representative chromatograms obtained using this method. A partial method validation in human plasma demonstrated acceptable method performance.

## 6. Protein quantification without internal standards

To the best of our knowledge, no targeted quantitative methods for proteins have been described which use LC–MS/MS without internal standards. The cause for this lies in the complexity of the required analytical procedure and the necessity of correcting for the inherent experimental variability caused by the detection system.

For methods used in screening, however, this approach has been described. To discuss the performance of the technique in this review, we refer to an example from the area of doping control [40]. LC–MS/MS is used to determine the presence of the performance enhancing drugs recombinant human erythropoietin (rhEPO) and darbepoetin (DPO) in equine plasma



**Fig. 9.** LC–MS/MS chromatograms showing the triply charged derivatized signature peptide  $(CH_3)_2ASQSVINYLA WYQQK(CH_3)_2PGQAPR$ , released from a therapeutic mAb spiked at the LLOQ of  $1 \mu\text{g/mL}$  in monkey plasma, after dimethyl labeling with formaldehyde (A) and the corresponding internal standard, created by dimethyl labeling using formaldehyde- $d_2$ , resulting in triply charged  $(CHD_2)_2ASQSVINYLA WYQQK(CHD_2)_2PGQAPR$  (B).

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samples from the horse racing industry. The two proteins are simultaneously extracted from equine plasma using anti-EPO antibodies in a magnetic bead format. After elution from the antibodies, the samples are buffer-exchanged using a centrifugal filtration device to a buffer suited for trypsin digestion. Following trypsin digestion the samples are stored at  $-70^\circ\text{C}$  before analysis by LC–MS/MS. One signature tryptic peptide present in both proteins was selected and quantified by LC–MS/MS using reversed phase gradient elution coupled to a linear ion-trap mass spectrometer.

The lack of internal standards and the complex sample treatment procedures affect the performance of the method but apparently no formal validation was required. For this reason, the method can only be considered suitable for semi-quantitative estimation of the concentrations of hrEPO and DPO. The range of the method is 0.1–2.5 ng/mL and the limit of confirmation was set at 0.2 ng/mL. Any sample having rhEPO or DPO concentrations exceeding this value was considered positive for these controlled substances. Reanalysis of some samples previously analyzed using a LBA showed that both methods have comparable performance in terms of providing confirmatory evidence.

## 7. Conclusion and perspectives

The LC–MS/MS methods for quantitative determination of biopharmaceuticals, shown in Table 1, have been categorized according to three main workflows: (1) digestion of proteins in the untreated sample (e.g. plasma) with no further peptide extraction,

(2) idem with subsequent peptide extraction and (3) digestion of the protein after its extraction from the matrix. To enable a relatively unbiased comparison, the reported LLOQs have all been converted to the same concentration unit, nmol/L. Some interesting conclusions can be drawn from this summary.

In procedures where the protein is not extracted (approaches 1 and 2), digestion is performed on all proteins in the sample, resulting in a digest containing an extremely large number of peptides with a very high total concentration. The complexity of such a digest is significantly greater than that of the sample before digestion because the digestion step multiplies the number of (possibly interfering) compounds in the sample. Furthermore, proteins are cleaved at specific locations, making the resulting peptides chemically more similar than the original proteins, rendering selective extraction of peptides from the digested sample more difficult.

In approach (1) selectivity results entirely from the LC–MS/MS analysis, as no extractions are performed. As this approach often suffers from significant ionization suppression resulting from tryptic peptides that co-elute with the signature peptide, this approach typically results in relatively poor sensitivity. The application of this approach is, therefore, limited to more abundant proteins or high-dose biopharmaceuticals such as mAbs. On the other hand, the absence of extraction-related variability when using this approach is expected to positively impact accuracy and precision.

In approach (2), the signature peptide is extracted from the digested untreated sample. Several extraction techniques with different selectivity have been used. Even though SPE is considered to be quite selective, one or two-dimensional SPE applied to a digested sample did not result in a significant improvement of sensitivity compared to approaches where no post-digestion extractions were performed (see Table 1). The selectivity of the applied extractions was apparently not sufficient to reduce matrix interferences. A poor recovery of the peptide from the digest, adsorption of the peptide to surfaces or reduced solubility in specific solvent combinations might have also lowered sensitivity. The optimization approaches taken when using SPE for extracting a signature peptide from a digest should perhaps be different from the ones taken for extraction of a small molecule in a manner that provides a higher resolution for this specific class of analyte.

The use of immuno-affinity extraction of the signature peptide from a digested sample has been reported. The sensitivity of this method exceeds that of any other method where the protein itself is not extracted before digestion and is comparable to that of the most sensitive methods using protein extractions before digestion. A SIL-peptide internal standard will correct for losses during immuno-affinity extraction resulting in acceptable accuracy and precision.

The highest sensitivity is reported for methods that use approach (3), in which the protein of interest is extracted from the biological matrix before the sample is digested. Compared to the other approaches, it results in the cleanest extracts because, ideally, only the protein of interest and its internal standard are digested which results in a small number of tryptic peptides. Since a generic platform for protein extraction from plasma has not been described, immuno-affinity extractions are typically used for this purpose. The use of an internal standard that corrects for the variability caused by this step of the procedure is uncommon, since the SIL-protein is difficult to obtain without access to the production cell line. The use of an immuno-affinity extraction introduces some problems typically associated with LBAs into the LC–MS/MS method. Firstly, there is a need to produce and characterize antibodies, which increases the time needed to set up the method. Secondly, without a SIL-protein internal standard, variability in

protein extraction is not corrected for which negatively impacts accuracy and precision. At the moment, high-sensitivity LC–MS/MS in extremely complex samples such as plasma requires the need for immuno-affinity enrichment either at the protein or the peptide level.

There is currently no single approach for internal standardization in the field of the quantitative determination of biopharmaceuticals. Table 2 compares the different internal standard approaches with respect to their ability to correct for variability of the different analytical steps and their generic availability. While most of the discussed approaches do correct for LC–MS/MS analysis and peptide extraction, proper correction for protein extraction and digestion is more difficult to achieve. For reliable analytical results it is thus essential that any step which is not covered by the internal standard be thoroughly optimized and well controlled during sample analysis.

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