Antibody based affinity capture LC-MS/MS in quantitative determination of proteins in biological matrices

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Abstract

Determination of proteins in complex biological matrices has massive attention and is exploited in many different scientific disciplines. Routinely, proteins are determined using antibody based immuno-metric assays. Although these assays are easy to perform and widely used, interpretation of the results is challenging: cross reactivity, high dose hook-effect, presence of heterophile- or auto-antibodies give rise to false results, sometimes with dramatic consequences. In the quest for more robust assays a combination of antibody sample clean-up, tryptic digestion and mass spectrometric determination is gaining more attention. This review discusses the advantages of antibody based affinity capture and subsequent LC-MS/MS in protein analysis like less false results and possibilities like multiplexing and isoform differentiation. It also considers the interplay between the analytical, biological and biochemical factors, which still give rise to false results, even with mass spectrometry as the ultimate selective detection step. The intention of this review is to point out both strengths and weaknesses of antibody based affinity capture LC-MS/MS in quantitative determination of proteins in biological matrices.

Keywords: immuno-capture; antibody; bottom-up proteomic strategy utilizing LC-MS/MS; protein quantification; false results.

Introduction

Historically the interest in, and importance of reliable protein quantification is shown by the attention method development by Lowry et al. and Bradford has gained. Both methods describe colourimetric measurements of the total protein content in the µg/mL scale and are within the top 3 of most cited research papers all times (more than 450 000 citations together [1] in 2014). The last paper in this top 3 was written by Laemmlli and descirbes protein separation using SDS-PAGE. Since the early 1950's the field of protein analysis evolved from total protein concentration determination and SDS-PAGE based separations (in the µg/mL scale) to complex multidimensional proteomic analyses allowing to separate thousands of proteins and to determine concentrations of certain proteins down to fg levels (and lower). These biochemical and analytical efforts revealed that there is an enormous variation in the type of proteins, amount of isoforms, post translational modifications, function and much more. Beside their biological function, many proteins have, by means of their concentration level in blood diagnostic value as well as they might, by means of their presence in certain samples, give insight in biochemical processes in the body.

Although there is an overwhelming variation in the biochemical and analytical approaches to proteins, targeted protein quantification using antibody based affinity materials together with a bottom-up proteomic strategy utilizing LC-MS/MS analysis is gaining much interest in clinical chemistry (proteins with diagnostic value), therapeutic drug monitoring, drug discovery and development of new drugs (protein biopharmaceuticals), medicine (explorative, revealing biochemical processes) and sports (doping analysis). This overview will mainly deal with the interplay between the analytical and biological aspects of antibody

based affinity materials combined with a bottom_up proteomic strategy utilizing LC-MS/MS analysis. It discusses the possibilities immunocapture LC-MS/MS gives for quantification and understanding biochemical processes as well as their limitations. It will deal with targeted applications for clinical diagnostics and protein drug analysis where triple quadrupole and iontrap due their robustness, cost and availability are the mass spectrometers of choice. It will not deal with the relative quantification (e.g. SILAC, iTRAQ, ^{18}O labelling) of the proteome, nor will it deal with DDA analyses to reveal protein modifications. Generic capture strategies for the enrichment of post translational modifications like ubiquitination, glycosylation and phosphorylation are not discussed.

Why combining antibody based affinity with bottom-up proteomic strategy utilizing LC-MS/MS?

Proteins are diverse in their biological function, and only 20 different amino acids make up for most of the protein variation. Additionally, proteins exhibit a chemical variability caused by, among others, their length, hydrophobicity, side chain pK_a values, pI values and post translational modifications. Proteins are not easy to separate from each other using conventional pre-analytical sample treatment. Mass spectrometry offers outstanding sensitivity, selectivity and ability to determine many substances simultaneously. These are some of the reasons why MS presently is frequently used in protein analysis. However, the broad range of which proteins occur in biological samples may give rise to analytical challenges.

Human plasma is mainly used as biological matrix for protein determination and contains many proteins in different concentrations: albumin and transferrin have

reference levels in the medium mg/mL, proteins like C-reactive protein in the low µg/mL range while interleukin-6 occurs in the low pg/mL range [2]. In a bottom-up approach (i.e. enzymatic cleavage of the protein prior to analysis) it is not possible for mass spectrometers to determine proteolytic peptides from for instance the low abundance interleukin-6 (low pg/mL level) with high precision and accuracy in the presence of proteolytic peptides from the high abundance protein albumin (medium mg/mL level). This is due to the limited dynamic range of the mass spectrometer [3], which can defined as the ratio between the highest and lowest reliable concentration measurement in the same analysis. In 2006, Anderson and Hunter [4] showed that the dynamic range of an MRM assay for more than 50 high and medium abundant plasma proteins with minimal pre-treatment of the plasma sample was 4.5 orders of magnitude allowing detection to sub µg/mL level. To allow determination of lower levels of a target protein in complex samples other approaches need to be applied. Among these multidimensional chromatographic separations (like MudPIT [5]) and depletion of the most abundant proteins (depending on the vendor one can deplete for up to more than ten abundant proteins) might be used. These techniques have a tremendous value in the field of exploratory proteomics they have the major setback of being time ineffective when proteins need to be determined for diagnostic purposes. In the latter case a high analytical throughput is of importance. Regardless the above-mentioned analytical approaches, the limit of detection (and quantification) for proteins in complex samples using LC-MS/MS remains at its best in the ng/mL level, and is thus with respect to sensitivity not competitive with immunological assays. A logical question would be why immunological assays need to be replaced by MS based methods.

Drawbacks in immunological methods for protein analysis

In routine determination of endogenous proteins in hospital laboratories, the methods of choice are often based on immunological principles, ranging from ELISA to RIA. After their introduction in the 1950-1960s, they gained popularity due to their relative simple, sensitive and robust analytical protocols. Although these methods are based on the use of specific antibodies, they have shown to have many drawbacks potentially giving rise to false diagnosis caused by factors like presence of auto-analyte antibodies, heterophile antibodies or human anti-reagent antibodies (like human anti-mouse), cross reacting reagents and high dose hook effect[6]. Another drawback is the lack of differentiating ability: multiplexing several diagnostic markers to enable more robust diagnosis mostly needs several analyses (one analysis per diagnostic marker), additionally, no subtyping or differentiation between isoforms or isovariants of a certain biomarker is not possible within one single analysis.

Combining immunoaffinity with mass spectrometry

As already mentioned, to be able to determine proteins of interest in complex biological samples by mass spectrometry at fg – pg levels clean-up and/or enrichment is needed. There are several generic ways to perform this, but the possibilities antibodies offer in term of sample clean-up are outstanding. A combination of the advantages of the mass spectrometer as well as the advantages of the use of antibodies in targeted protein determination is reported as early as 1996 by Liu and Bowers[7]. In this work human chorionic gonadotropin (hCG) is trapped by immunoaffinity from urine followed by tryptic digestion and mass

spectrometric detection of its proteotypic peptide. It took almost 10 years for a similar combination strategy was published (also on urinary hCG)[8] but after that, more target proteins were determined by combining immunoaffinity, proteolysis and LC-MS/MS for diagnostic purposes[9], doping related analyses[10] and bioanalysis of biopharmaceuticals[11]. The combined strategy not only allows to determine very low protein concentrations, it additionally allows to reveal and monitor protein isoforms and variations[12].

In general the workflows described here and in the following examples are divided in the protein capture approach and the peptide capture approach[13]. (see Figure 1)

In the following chapters a critical overview is given of the work done in the field of immunocapture LC-MS/MS for targeted determination of bioactive proteins.

Although work has been done on the top-down analysis of proteins like insulin, IGF1 and other hormones, this review is limited to protein determination after bottom-up analysis.

Antibodies: the essential ingredient

As mentioned earlier, the immuno-capture step can either be selective capture of intact proteins (placed prior to the digestion step) or selective capture of proteotypic peptides (placed after the digestion step. SISCAPA: see later). Either way, the antibody is essential for a well functioning method. In the circumstances were several antibodies against a target protein are available, selection can simply be carried out by evaluating their performance [14, 15] with respect to amount of antigen found in the bound fraction vs. antigen found in the unbound fraction.

Such evaluation using LC-MS/MS is much less time-consuming than an evaluation using conventional immunoassays.

Anti-protein antibodies

Many reports are on LC-MS methods with the immuno-capture step before the digestion where antibodies against intact proteins are used. Using this concept, the MS detection will avoid possible false positive results caused by cross-reactivity. However, the use of antibodies against intact proteins in immuno-capture LC-MS/MS still will have some of the potential drawbacks seen in immunoassays hampering reliable quantification of target proteins: Very high concentrations of antigen may not lead to a hook effect, but it might challenge the capture capacity thus lead to false low results. (this can be corrected for using a labelled protein standard which exhibits the same affinity as the target protein). Additionally, auto-analyte antibodies or anti-reagent antibodies present in the biological samples will to the same extent as in immunoassays lead to false low results.

Anti-peptide antibodies

Anti-peptide antibodies, known as the SISCAPA approach (Stable Isotope Standards and Capture by Anti-Peptide Antibodies) was firstly described by Anderson et al.[16] and thoroughly reviewed by Becker and Hoofnagle [17]. In this approach, polyclonal anti-peptide antibodies are raised against the already chosen proteotypic peptides of target proteins. Compared to antibodies raised against whole proteins the affinity of anti-peptide antibodies has been described to be somewhat lower[18]. The analytical advantage is that stable isotope peptides can correct for variations in the capture step as stable isotope peptides are captured to the same extent as proteotypic peptides from the endogeneous

proteins. Another advantage is the ability to reuse SISCAPAs antibodies as they are introduced after proteolysis[19]. Additionally compared to the use of anti-protein antibodies, presence of auto-analyte or anti-reagent antibodies will to a much lesser extent influence on the quantification of a target protein. A disadvantage is that isoform differentiation of the target protein is less, if at all, possible with the use of a SISCAPA.

Affinity formats

There are many formats and platforms used for the capture of the target protein or peptide. A comprehensive review discussing advantages and shortcoming of the different formats/platforms is published by Li et al.[20] in this journal and will thus not be covered here. No matter the choice of the format or support, materials used for the immobilisation should be inert. However, there will always be proteins and other compounds binding to them. This non-specific binding should preferably be eliminated as it might cause ion suppression of very low abundant target proteins thus hampering satisfactory low quantification limits. Removal can be carried out either before the affinity extraction or post-analysis by substracting the signal of the contaminant from the obtained spectra[21].

In choosing the right format of the support to attach the antibodies, one should bear in mind quantification limits needed as well as the labour intensiveness of the method. The 96-well plate format for extractions is well suited for integration in many analytical platforms, thus automatable and diminishing the labour intensity[22]. One of the disadvantages is the limited capacity of the wells: the active surface is low compared to beads. Another disadvantage, compared to the use of beads is the limited possibility for enrichment: The maximum amount of

sample (200 μL) might be reduced to 50 μL in wells (enabling up to 4 times enrichment). Using beads, the sample amount is not limiting, in other words, 1 mL of sample can easily be extracted and then enriched to 50 μL (enabling up to 20 times enrichment)[23].

Advantages and possibilities of immuno-capture LC-MS

Combining the immuno-capture step with mass spectrometric detection does not resolve all the limitations seen in the methods separately, however, it has his advantages as well as it opens for new possibilities.

Epitope mapping

The first description of immunocapture MS was the use in *epitope mapping* based on affinity capture of proteolytic peptides. As a first step proteolytic peptides were generated, these peptides were in the next step captured by immobilized antibodies (raised against the whole protein) followed by a washing step to remove unbound proteolytic peptides. After elution, the mass spectrometer was used to identify the bound peptides thus identifying the epitope. In this way epitope containing peptides for proteins like melittin and glucagon-like peptide[24] were identified. A slight different approach was chosen by Macht et al. who allowed coupling of the whole protein (cardiac troponin) to the antibody prior to proteolysis. Using limited proteolysis (trypsin:protein ratio below 1:100) the epitope was excised: the epitope containing part remains attached to the antibody during the proteolysis while the other parts of the protein were released. After elution, the epitope containing peptide was determined using mass spectrometry[25].

Multiplexing using one antibody

In a review of Kiernan, the main strength of immuno-capture followed by LC-MS was described to be that isoforms and protein modifications might be determined in a single step [26]. Since the (reagent) antibody will interact with only a relative small part of a protein, changes in the target protein, which are neither within nor close to the epitope region, will not affect the immune-reactivity of the reagent antibody. This allows capture of target protein isoforms and classes of proteins (figure 2a). An example, which supports this, is the immuno-capture LC-MS analysis of pro-gastric releasing peptide (ProGRP). This protein has been studied thoroughly in various studies since ProGRP is an acknowledged marker for Small Cell Lung Cancer. It is routinely determined by an immuno-metric assay and was demonstrated to consist of three isoforms when determined by immuno-capture LC-MS. Although only the total amount of ProGRP is used for diagnosis, it is clear that there is a diagnostic potential in differentiation of the ProGRP isoforms using immuno-capture LC-MS[27]. Other examples of simultaneous immuno-capture and analysis of related proteins/peptides exhibiting the same epitope are determination of 20 β-amyloid peptide signatures[28], three tau proteins in human cerebrospinal fluid (CSF) [29], and up to 8 variants of hCG in human urine and serum [30]. The quantification of 1-84 parathyroid hormone in patient samples at pM levels by immuno-capture LC-MS showed its superiority over conventional immunoassays with respect to the ability to distinguish and determine C-terminal metabolic peptide products[31]. In addition to these examples of endogenous proteins, seven different antibody therapeutics with immune reactivity for a single (reagent) antibody are described[12].

Multiplexing using several antibodies in the extraction step

Combinations of several (reagent) antibodies in a single analysis are also described. In this case several batches of immobilized (reagent) antibodies are mixed before the immuno-capture step. This mixture is then used to capture several target proteins in a single step (figure 2b). For multiple extractions at the protein level this is described up to capture of six proteins: In a study of Nicol et al. *five (reagent) antibodies* against carcinoembryonic antigen (CEA), secretory leukocyte peptidase inhibitor (SLPI), tissue factor pathway inhibitor (TFPI), tissue factor pathway inhibitor 2 (TFPI2) and metalloproteinase inhibitor 1 (TIMP1) were immobilized separately to beads and combined to allow a one-step extraction and determination of these lung cancer markers [32]. Lin et al. [33] combined *six different antibodies* to capture metalloproteinase inhibitor 1 (TIMP1), cartilage oligomeric matrix protein (COMP), thrombospondin-2 (THBS2), endoglin (ENG), mesothelin (MSLN) and matrix metalloproteinase-9 (MMP9) in a single extraction and analysis. These data was compared to data from ELISA experiments for each of the proteins and showed close correlation. A combination of both multiplexing and isoform differentiation was described for the isoforms of the lung cancer marker ProGRP and NSE where only *two (reagent) antibodies* were used [34]. This single analysis improves diagnostic robustness.

In all the above cases (reagent) antibodies against the target *protein* were used. This multiplex strategy can alo be applied by using the SISCAPA approach [35, 36]. Ippoliti et al. even showed automated multiplex extractions of more than 150 peptides[37].

The use of antibodies generated from libraries

The above mentioned approach is based on antibodies with proven specificity and applicability (for instance from immunoassays like ELISA). In the quest for

(reagent) antibodies multiplex immuno-capture LC-MS experiments can be carried using batches of antibodies generated from antibody libraries are used for capture purposes: motif-specific antibodies from the nCoDer library coupled to magnetic beads are mixed to capture classes of proteins. In such a manner Olsson et al. reduced the complexity of the yeast proteome down to 75 motif contain proteins, some of them expressed as low as only 50 copies per cell[38]. Whiteaker et al. used antibody fragments (Fabs) generated from the HuCAL PLATINUM library to capture only epitope containing tryptic peptides of the target proteins[39].

The advantages with the multiplexing approach mentioned in this section lead to the ability to determine several biomarkers simultaneously shorten the response time (instead of five separate analyses, only one needs to be performed), there is less room for analytical and operator errors, making the diagnosis more robust. Additionally, one can ad-hoc mix immobilized reagent antibodies to produce a new immuno-capture procedure allowing for personalized biomarker analysis
It must however be stressed that this only is the case when all these different variants of the target protein have sufficient immuno-reactivity.

Limitations and sources of variability in target protein quantification by immuno-capture LC-MS
Besides general pre-analytical variations like sample handling, sample collection and temperature as well as general analytical variations like injection reproducibility, mass spectrometric response and matrix effects reviewed by

Percy et al.[40] there are also biological and biochemical factors influencing the analysis of target proteins by the bottom-up approach.

Variability of digestion: impact on quantification

The choice and origin of the proteolytic enzyme affects the outcome of the quantitation of the target protein[40]. There are numerous of proteolytic enzymes which can be chosen for this purpose, all with their shortcomings (like challenging cleavage sites) and advantages (like degree of efficiency). This is reviewed by Giansanti et al.[41]

Variability in proteolysis of the target protein might be corrected for by using a suited internal standard. One should be aware that isotopically labelled proteotypic peptides will not correct for these variations, neither when anti-protein antibodies are used nor when anti-peptide antibodies are used. The 'ideal' internal standard which corrects for proteolytic variations (as well as the capture by the antibody) is the PSAQ approach (Protein Standard Absolute Quantification) which are isotopically labelled versions of the target proteins[42]. Another intermediate approach is shown by Faria et al.[43] who studied the quantification of human osteopontin by application of an extended stable isotope labelled internal standard (TYDR*GSDVV*YGLR*SKSKKF) containing cleavage sites on both N- and C-terminal of the chosen proteotypic peptide (*GSDVVYGLR*). The internal standard was added after the immuno-capture step (use of anti-protein antibody) but before the tryptic digestion. It showed that the extended internal standard improved the robustness of the quantification by both correcting for trypsin variability as well as peptide instability. Both PSAQ and the extended isotopically labelled internal standard would also work for immunocapture strategies using anti-peptide antibodies.

Modifications of the target protein affecting the immuno-reactivity (post translational/mutations)

Selection of the reagent antibody for capture during method development is essential. This was shown by Guy et al.[44] who studied the impact of antibodies directed against various epitopes of a target protein using mass spectrometric detection (this is of course also a challenge in immunometric assays). In the determination of cardiac troponin 1, post-translational phosphorylation to its mono- and di-phosphorylated form gave rise to a change in immunoreactivity of the target protein resulting in decreased signal intensities for all tested antibodies (see figure 3a/b). Changing one amino acid in the epitope even caused absence of signal for cardiac troponin 1 for one of the studied antibodies thus pointing out the importance of antibody selection in the method development. [44]

Modification of the target protein affecting the production of proteotypic peptides

Modifications (like post-translational modifications, mutations and degradation) in the proteotypic peptide leads to a change in *m/z* and often also in the retention time which makes it potential challenging to determine the target protein by means of LC-MS/MS.

One example is endogenous protease induced changes in the target protein that will affect the production of proteotypic peptides: Streng et al. [45, 46] showed the effect of in-sample proteolysis and methionine oxidation on the determination of cardiac troponin 1 by immunocapture followed by gel separation of the captured cardiac troponin 1 degradation products and the analysis of 18 peptides after in-gel digestion. As much as five different bands for cardiac troponin 1 were identified, but only one proteotypic peptide (located in a stable region) was able to reflect the original amount of cardiac troponin 1 in the sample.

Occurrence of proteotypic peptide modifications demonstrates that the choice of the proper proteotypic peptide for the quantitative analysis in some cases is crucial and exceptional demanding (see figure 3a/c). Software like Skyline (https://skyline.ms/project/home/begin.view?) can be a very helpful tool in this respect.

Considering the above it is recommended that, if possible, at least two proteotypic peptides should be used for the quantification of the target protein. This will in general lead to a more robust analysis method and reveal challenges or pitfalls in the method.

Auto-antibodies and anti-reagent / anti-drug antibodies

Another challenge in the affinity based extraction and following determination of proteins in biological matrices is the presence of auto-antibodies, anti-reagent antibodies or anti-drug antibodies (ADA) (see figure 3a/d). As with immunoassays presence of these antibodies might lead to false low or even false negative results as the segment of the protein important for the affinity extraction (epitope) might be shielded by these antibodies. To our knowledge there is only one report describing the impact of auto anti-reagent or auto anti-drug antibodies on the bottom-up proteomic strategy utilizing LC-MS/MS determination of a target. However, the efforts to circumvent this by using DMSO to prevent ADA´s effect on the determination of therapeutic mAbs. These efforts where without success[47].

Auto-antibodies also might shield the epitope which is used by the assay antibodies as in immuno-metric assays (see figure 3a/e). Thyroglobulin concentrations in plasma are used to monitor the success of thyroid cancer treatment but the assay outcome might be hampered by the possible presence of

auto-antibodies. In such a case, immuno-capture LC-MS using anti-thyroglobulin (reagent) antibodies will not be a better alternative. However, by using the anti-peptide antibody approach and an antibody towards the specific tryptic peptide (*VIFDANAPVAVR*) of thyroglobulin the challenge of auto-antibodies is circumvented as the capture step is placed, in time, after the digestion step [17, 48, 49].

Subunits

Target proteins consisting of subunits can be particularly challenging: in addition to variations occurring on the primary protein structure level (mutations, post translational modifications) also variations on the quaternary protein structure can occur through degree of association/dissociation of subunits. The much studied case of human chorionic gonadotropin (hCG) immuno-capture LC-MS gives insight in the challenges caused by this: hCG occurs as a hetero-dimer (α-β subunits) as well as a monomer (free β-subunit) and the clinical relevant variations in the hCG occur in the β-subunit (both in the hetero-dimer as in the monomer). Although the variations on the primary protein structure level can nicely be detected by determining their proteotypic peptides in the bottom-up approach, it will not be possible to determine if a particular proteotypic peptide originates from the β-subunit in the hetero-dimer or from the β-subunit in the monomer (variations in the quaternary protein structure). Sequential extractions for hCG were developed to enable determination of variations in the primary protein structure and quaternary protein structure in urine [50] and serum samples [30]. In the urine example first the monomers were extracted followed by the intact heterodimer, in the serum example the order of extraction was

reversed. In these studies, both extracts were then treated and analysed separately.

Conclusion

Immuno-metric assays are widely used but have several disadvantages. Many of these disadvantages are dealt with using antibody based affinity bottom-up proteomic LC-MS/MS.

The reviewed approach is either performed using peptide extractions (SISCAPA) or protein extractions. In both cases a proteolytic step is needed, both allow multiplexing and both can potentially rule-out false results caused by cross-reactivity. Protein extractions and peptide extractions have complementary advantages; a clear advantage is that anti-protein reagent antibodies capture isoforms of the target protein (if the epitope is situated in a constant region) allowing isoform differentiation through mass spectrometry. On the other hand, the presence of auto-antibodies which causes false negative results (in both immuno-assays and immuno-capture using anti-protein antibodies) is potentially circumvented using peptide extractions. Both extraction methods allow more than sufficient clean-up and enrichment compared to generic sample preparation techniques enabling protein quantification at ultra low protein concentrations.

Modifications of the protein in the epitope region or in the proteotypic peptide region might lead to false negative results either through limited capture or loss of signal through change in the mass of the proteotypic peptide, using both the protein and the peptide extraction approach. Although it is dependent on the protein of interest if antibody based affinity bottom-up LC-MS/MS is the best

alternative (compared to immunometric assays), for many proteins it has shown to improve both the quantitative and qualitative results.

Legend to the figures:

Figure 1:

a) Sample preparation order: protein capture – proteolysis - LC-MS: the target protein is captured by an antibody which is immobilized on a support. After removal of the unbound fraction by washing, the target protein is digested into proteolytic peptides. The amount of the target protein is then determined by means of one or several proteotypic peptides using LC-MS.

b) Sample preparation order: proteolysis - peptide capture – LC-MS: the sample is digested in its whole into proteolytic peptides. The proteotypic peptides of the target protein are captured by antibodies (SISCAPA) immobilized on a support. After removal of the unbound fraction, the proteotypic peptide is released and determined using LC-MS.
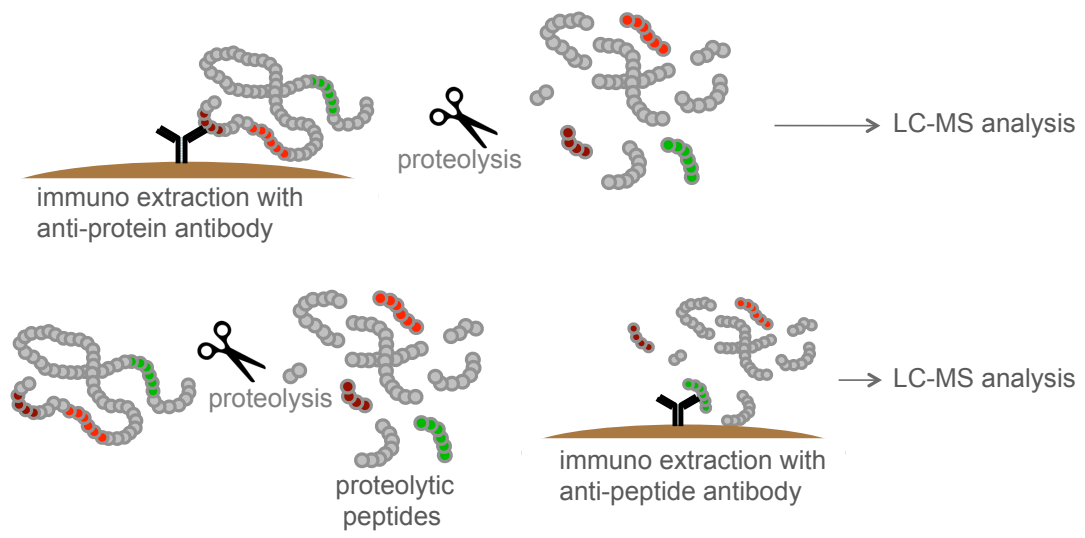
Figure 2:

a) Capture of isoforms or isovariants by anti-protein antibodies, which contain a common epitope. After proteolysis proteotypic peptides for each of the captured proteins are determined simultaneously by LC-MS/MS.

b) Multiplex capture of various target proteins using a cocktail of antibodies (either anti-protein or anti-peptide antibodies). The extraction occurs in one single step. After proteolysis proteotypic peptides for each of the target proteins are determined in a single LC-MS/MS analysis.
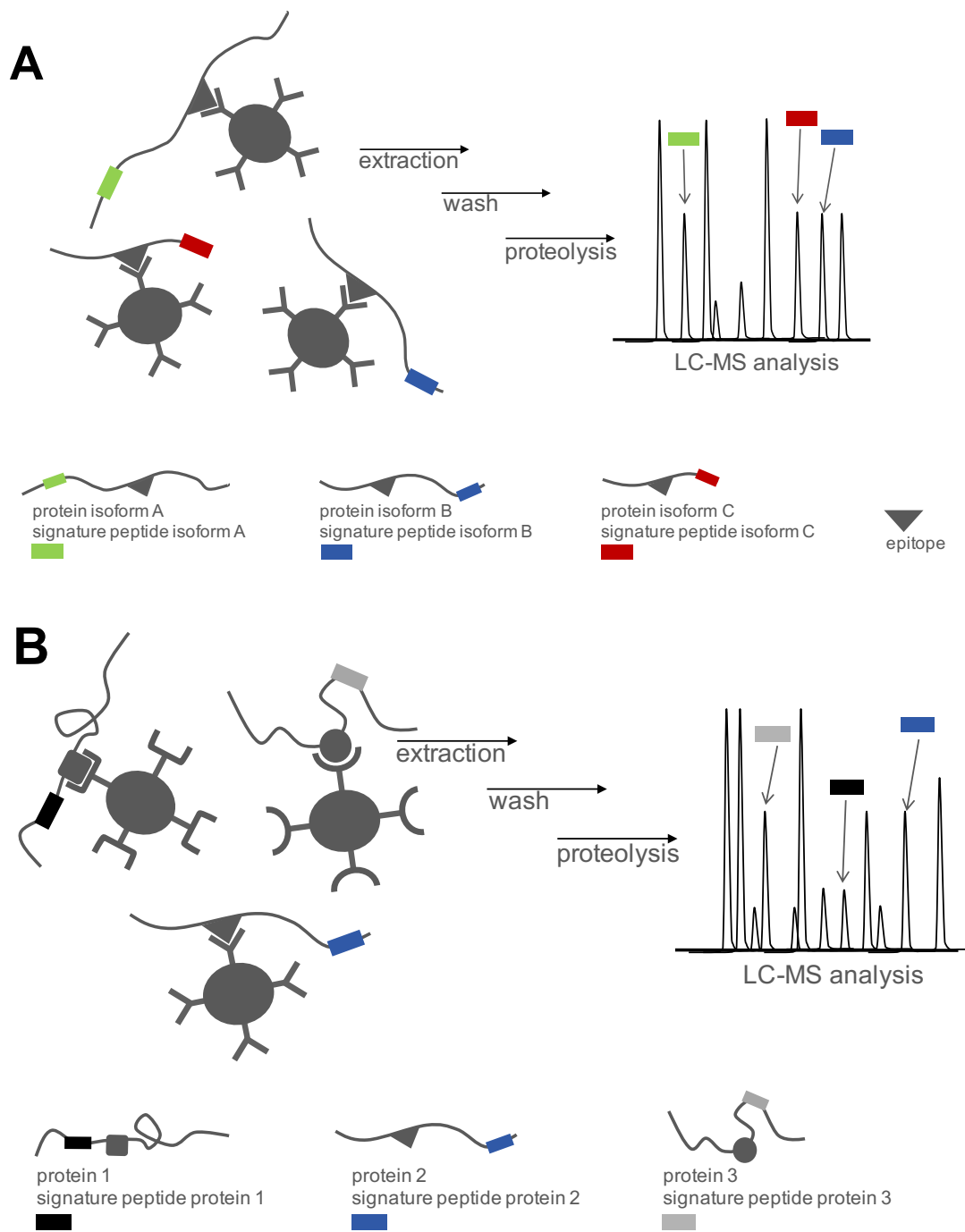
Figure 3:

Effect of target protein modification and presence of auto-antibodies on antibody-based protein determination using mass spectrometry.
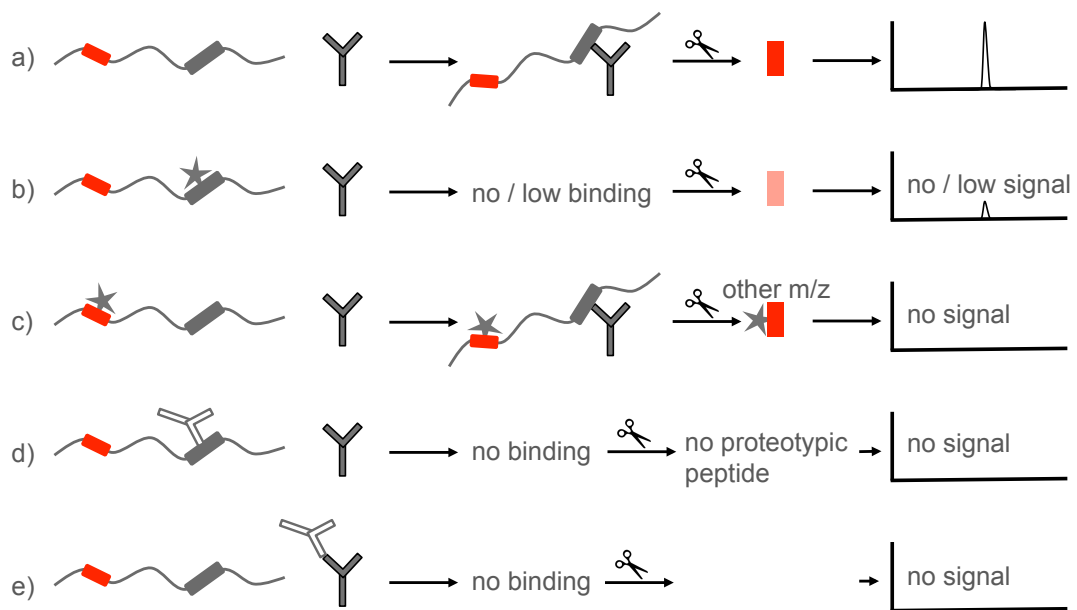
a) Common situation: target protein is captured by antibody and proteotypic peptide is determined by LC-MS/MS after proteolysis b) Modification in the epitope affecting the immunoreactivity of the target protein c) Modification in the proteotypic peptide amino acid sequence. Target protein exhibits the same immunoreactivity but does not yield the *m/z* of the proteotypic peptide after proteolysis d) Auto-antibodies/ anti-drug antibodies attached to the epitope or to the vicinity of the epitope will cause loss of or less immunoreactivity of the target protein e) Anti-reagent antibodies might interfere with the reagent antibody hampering the capture of target protein

immuno extraction with
anti-protein antibody

proteolysis

LC-MS analysis

proteolysis

proteolytic
peptides

immuno extraction with
anti-peptide antibody

LC-MS analysis

Figure 1

A

extraction
wash
proteolysis

LC-MS analysis

protein isoform A
signature peptide isoform A

protein isoform B
signature peptide isoform B

protein isoform C
signature peptide isoform C

epitope

B

extraction
wash
proteolysis

LC-MS analysis

protein 1
signature peptide protein 1

protein 2
signature peptide protein 2

protein 3
signature peptide protein 3

Figure 2

Figure 3

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