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Review

Quantitative mass spectrometry-based techniques for clinical use: Biomarker identification and quantification[☆]

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ABSTRACT

The potential for development of personalised medicine through the characterisation of novel biomarkers is an exciting prospect for improved patient care. Recent advances in mass spectrometric (MS) techniques, liquid phase analyte separation and bioinformatic tools for high throughput now mean that this goal may soon become a reality. However, there are challenges to be overcome for the identification and validation of robust biomarkers. Bio-fluids such as plasma and serum are a rich source of protein, many of which may reflect disease status, and due to the ease of sampling and handling, novel blood borne biomarkers are very much sought after. MS-based methods for high throughput protein identification and quantification are now available such that the issues arising from the huge dynamic range of proteins present in plasma may be overcome, allowing deep mining of the blood proteome to reveal novel biomarker signatures for clinical use. In addition, the development of sensitive MS-based methods for biomarker validation may bypass the bottleneck created by the need for generation and usage of reliable antibodies prior to large scale screening. In this review, we discuss the MS-based methods that are available for clinical proteomic analysis and highlight the progress made and future challenges faced in this cutting edge area of research.

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1. Introduction

1.1. What is clinical proteomics?

The discovery of novel, disease-related biomarkers by proteomic analyses of readily accessible bio-fluids such as plasma using liquid chromatography (LC)-coupled mass spectrometry (MS)-based methods, is an exciting prospect for improved patient care. The major goal of clinical proteomics is to use these highly specific disease/pathology-related signatures to enhance current clinical practice by enabling accurate early diagnosis, selection of appropriate therapeutic strategy and to monitor disease progression and/or possible side effects on a patient by patient basis [1]. It is only with the use of recent advances in analytical biochemistry such as MS technologies and high resolution liquid phase separations that personalised medicine may become a reality [2].

In order for MS-based proteomics to be successful, clinically effective novel biomarkers must have high sensitivity (indicate a positive test for patients who are positive for the disease), high specificity (negative for patients who do not have the disease) and be sufficiently robust to operate in many different centres [3]. This demands rigorous biomarker identification and qualification strategies and the need for well designed, large scale clinical trials to validate the use of novel proteomic signatures [1]. It is also essential that the translation from pre-clinical findings to regulatory-approved biomarkers is undertaken with maximum possible efficiency and realism, with appreciation for the many challenges that this entails.

1.2. Why use serum/plasma?

Much emphasis has been placed on the identification of novel blood borne biomarkers due to the ethical situation pertaining to biopsies, plus the ease and cost of patient sampling when compared to standard methods such as biopsy. Serum and/or plasma also offers the option of longitudinal sampling and monitoring of individuals which may also lead to the detection of disease in patients who are asymptomatic or have early stage disease. Serum represents the soluble fraction of blood that remains following the clotting process, and thus is considered to be a more simplified matrix than plasma, which contains all soluble blood borne factors, including clotting factors. While serum is thought to be less complex than plasma, and thus the probability of identifying novel proteins may be increased, the clotting process is not uniform (unlike the preparation of plasma), and may also lead to the loss of novel factors which remain bound to the insoluble protein clot [4].

Diseases that have received the most attention for blood borne biomarker discovery include cancer and cardiovascular disease [5,6]. Bio-fluids that have been analysed also include urine (reviewed in [7]), cerebro spinal fluid (CSF) [8], nipple aspirate fluid (NSF) [9–12] and tumour ascites fluid have also received attention as potential sources of novel clinically relevant biomarkers. Indeed, in the latter three examples it is thought that these fluids may contain higher concentrations of disease-specific proteins due to their proximity to the primary lesion. Aside from the obvious benefits of blood sampling rather than procedures such as biopsy and CSF withdrawal, it is thought that blood, unlike urine (unless for specific diseases of the urological system), may directly contact the disease site and thus is more likely to contain primary biomarker information.

1.3. Mass spectrometry

Biomarker discovery using mass spectrometry to identify and quantify the protein components of bio-fluids such as plasma is

based upon the measurement of the mass of proteins and peptides as determined by their mass:charge ratio (m/z). This can be determined by ionisation of a sample to generate charged species which depending on their m/z value will reach the detector at a specific time (time of flight, ToF mass analyser), or by 'trapping' the ions in an electric field and then sequentially filtering them out based on size (smallest first) and measuring the time at which they arrive at the detector. In order to generate peptides that are small enough to be efficiently ionised the sample must be digested with trypsin prior to MS analysis. Because trypsin cleaves proteins at specific amino acid residues these m/z values can then be searched against a database of cleavage products (of known m/z values) in order to generate a 'peptide mass fingerprint'. Protein sequence information can be more accurately achieved by MS/MS analysis. As previously described, ionised peptides are selected based upon their m/z value, however, once all the other ions have been filtered out, they are then induced to fragment by collision with an inert gas, such as argon or nitrogen. This causes fragmentation of the peptide along the peptide backbone in a highly predictable manner, and thus the time at which these fragments (with particular m/z values) reach the detector allows identification of the peptide/protein sequence.

1.4. Challenges for biomarker identification using MS

There are several practical challenges for the use of MS methods in the discovery and usage of robust clinical biomarkers, the details of which will be discussed below. Foremost is the need for accurate quantification from MS or MS/MS spectra coupled with protein/peptide identification. Biomarker information must be strictly quality controlled and validated and the appropriate statistical methods must be applied. Ultimately this may mean that individual steps be combined into a workflow that ideally allows for the automation of most of the tasks to minimise external sources of error.

1.5. Variation

Recent advances in MS technology have led to the development of equipment with superior sensitivity and specificity that has made the detailed study of complex biological fluids such as plasma possible. Although the blood has been described as the most comprehensive human proteome, a circulating representation of all body tissues and thus reflective of disease status [4], the question still remains as to whether changes in plasma or serum form a linear relationship with events that occur at the site of disease or injury [1]. However, blood sampling is a routine diagnostic tool in the clinic and therefore requires extensive investigation to achieve identification of novel biomarkers [13].

The proteome is a constantly changing entity, with complexity generated at many levels. It is essential that signatures are verified as being disease related, rather than as a result of the background noise inherent in any complex system, further adding to the challenge of biomarker identification. Indeed, due to the heterogeneous nature of human beings and their diseases, a panel of biomarkers rather than a single marker may be required to achieve the high sensitivity and specificity required for clinical applications [14]. With particular regard to oncology, most studies to date have involved patients with advanced disease, and given that genomic studies indicate that the molecular composition of early and late stage tumours can be different, the hope that these signatures will translate to early stage pre-invasive lesions where there are no reliable diagnostic tools may prove to be too simplistic [1].

1.6. Dynamic range can be addressed by fractionation

When using MS-based methods to obtain clinically relevant information from biological samples, the quantity and quality of identification and quantification are direct functions of sample complexity. In the clinical proteomics setting where serum/plasma is the source material, extensive pre-fractionation steps are essential due to the huge dynamic range of protein found in the blood. In human plasma the 22 most abundant proteins represent ~99% of total protein mass in plasma with extraordinary dynamic range (>10 orders of magnitude) from serum albumin at ~45 mg/mL to cytokines at around 1–10 pmol/mL or lower [4]. In addition, the necessity for tryptic peptides to be generated for direct identification of proteins by MS leads to a concomitant increase in the level of complexity of a given sample, thus the need for pre-fractionation methods becomes essential if the (relative) quantity of low abundance proteins is to be determined with accuracy and precision.

These methods generally involve the use of liquid chromatography, including reversed phase (RP) systems and affinity elution to deplete the major abundant proteins, of which several columns are commercially available. These have been designed to deplete the high abundance proteins, including the top 20 (Sigma ProteoPrep20™, Sigma–Aldrich, St. Louis, MO), the top 12 (ProteomeLab IgY12, Beckman Coulter, Fullerton, CA) and the top 14, 7 and 6 human proteins (High-Capacity Human-14 (-7, -6) MARS columns, Agilent Technologies Inc., Santa Clara, CA). As an additional pre-fractionation step it is also possible to use strong cation exchange (SCX) chromatography prior to the RP step. Both SCX and RP chromatography are used for fractionation of the sample post-trypsin digestion, prior to MS analysis. This peptide level fractionation enables low abundance peptides to be detected by the mass spectrometer, thus increasing the likelihood of identifying low abundance biomarkers from complex matrices such as plasma.

1.7. Quantitative techniques for biomarker ID using MS

Quantification is at the centre of clinical proteomics, without reliable methods to accurately quantify differentially expressed proteins it would not be possible to identify disease biomarkers, and as such, clinical proteomics would fail. Many advances have been made in the field of LC–MS/MS towards this end, and these will be discussed below.

Broadly speaking, quantification techniques have been developed based upon two methods, the incorporation of labels into peptides and proteins prior to MS analysis, or label-free methods. The use of labels is based on the principle of stable isotope dilution theory which states that a stable isotope labelled peptide will behave in a chemically identical manner to its unlabelled counterpart, and thus the two peptides will have identical chromatographic and/or MS properties. Provided that the label imparts a sufficient mass difference between these two peptide forms, their relative abundance may be calculated by comparing their respective signal intensities in the same MS run [15]. Mass tags can be incorporated in a variety of ways, either metabolically, chemically or enzymically. In addition, if the identity of the protein(s) of interest is known, quantification can be achieved by spiking the test sample with labelled synthetic peptides for direct comparison with levels of the corresponding endogenous peptide(s).

Label free methods generate quantification information by directly correlating the MS signal with the relative or absolute protein quantity. This can be achieved by several different methods. One of these uses the integrated ion intensities in MS mode where the number and intensity of precursor ions at selected m/z ratios are counted and peak areas from the extracted ion chromatogram (XIC) are calculated. Systematic errors (sample loading, HPLC reten-

tion times and MS instrument performance) are minimised by normalisation of peak intensities over the entire run [16] and ion suppression effects are countered for by the use of internal standard peptides included in each run at equal concentrations [17]. Alternatively, the spectral counting approach [18–20] uses data acquired in MS/MS mode to count and identify the number of fragment spectra that identify peptides of a given protein. These are then used to compare abundances between samples based on the number of MS/MS spectra identified for each protein corrected for protein length or expected number of tryptic peptides [21].

However, these methods assume that the linearity of response is the same for every protein, when in fact the spectrum count response is different for every peptide, for example, because the chromatographic behaviour of each peptide will vary. This then necessitates the acquisition of many spectra in order to accurately quantitate levels of any given protein, and as a result low abundance proteins can be difficult to identify and accurately quantify [16]. Meanwhile, saturation of the detector will occur at higher spectral counts, again with different levels for different proteins, in turn leading to potential problems with dynamic range [15]. As such, the performance of both methods is hampered by a need for large sampling numbers.

However, label free approaches to biomarker discovery by MS are continually being developed and refined. One approach is to combine spectral counting to give accurate fold changes with peptide ion intensity measurements using standard peptides to correct for variations in signal (ion abundance) [16,22]. An additional method, known as spectral feature analysis has recently been developed whereby quantitative and qualitative information can be gathered by aligning and comparing LC–MS datasets without the initial need for MS/MS analysis [23]. The increased costs and processing times associated with labelling have meant that label free techniques have generally been considered advantageous in their application to large scale clinical proteomics. However, the introduction of 8-plex iTRAQ reagents may enhance throughput for this technique compared to other methods. Furthermore, label free techniques are generally considered inferior in their quantification accuracy when compared to methods relying upon stable isotopes [15]. In particular, an early study conducted by Petricoin et al. [24] used surface enhanced laser desorption ionisation (SELDI) (a label free approach) to identify a proteomic signature associated with ovarian cancer, however, this study was later disregarded as the results were not reproducible and found to be most likely due to variables introduced during sample processing [25,26].

In all cases the properties of the mass spectrometer will affect the quantification in MS survey. For example, detection of low abundance ions will be obscured by the background noise, or quantification may be prevented by saturation of the detector. Although for quantification in MS/MS mode saturation is rarely a problem, however, in all cases true low abundance peptides may lead to poor quantification due to poor ion statistics (which define the sensitivity of detection; at high data acquisition rates fewer ions entering the mass spectrometer are allocated to the generation of each spectrum, leading to increased signal:noise ratios and low abundance peptides may not be identified) [15]. These factors coupled with the qualities of the label (if a label is used) mean that optimisation of peptide/protein ID and quantification must be achieved by decreasing the sample complexity prior to MS. Decreasing the sample complexity by fractionation (although overall analysis time will be increased) means that a greater number of peptides will be potentially analysed as more MS time will be committed to each one.

The most commonly used MS-based methods for clinical biomarker discovery and their advantages and limitations are summarised in Table 1 and will be discussed in detail below.

Table 1
Characteristics of quantitative mass spectrometry methods (adapted from [15,17]).

Labelling technique	Methodology overview	Application	Linear dynamic range*	Advantages	Limitations
Metabolic protein labelling (e.g. SILAC)	Growth of cells on general or specific isotope source	Cell culture systems	1–2 logs	Incorporation of label at earliest possible step	Cannot be used for clinical proteomics or primary tissue Expensive growth media Tag leads to increased complexity of MS analysis
		Complex biochemical workflows Comparison of 2–3 states		Can be tailored for specific residues	
Chemical labelling of thiol groups (e.g. ICAT)	Modification of cysteine followed by avidin-based enrichment	Comparison of 2 states	2 logs	Less complex samples	Loss of non-cysteine-containing proteins Tag leads to increased complexity of MS analysis
		Clinical proteomics and cell culture			
Chemical labelling of N-terminus and lysine residues (e.g. iTRAQ and TMTs)	N-hydroxysuccinimide (NHS) modification of N-termini and epsilon amino groups with isobaric tags	Comparison of up to 8 states	2 logs*	Complex samples	Increased duty cycle
		Clinical proteomics, primary tissue and cell culture			
Enzymatic labelling	C-terminal modification during proteolytic cleavage	Comparison of 2 states	1–2 logs	Versatile	Small isotope shift Late-stage incorporation of isotope
		Clinical proteomics and cell culture systems		Relatively cheap	
Spiked peptides	Isotope-labelled standards spiked into reaction	Targeted analysis of few proteins	2 logs	Targeted analysis	Identifies known peptides/proteins
Label free differential mass mapping	Comparison of mass maps of chromatographically separated proteins	Comparison of multiple states	2–3 logs	Simple workflow	Reproducible high resolution separation required
		Clinical samples and cell culture systems		Multiple comparisons	
Label free ion intensity measurements (e.g. SELDI)	Affinity-based enrichment of proteins from biological samples followed by MS	Comparison of multiple states	2–3 logs	Simple workflow	No identification of proteins
		Clinical proteomics and cell culture systems		Multiple comparisons	

* In MRM mode, dynamic range may be extended to 4–5 logs [104].

1.8. Protein identification and quantification using two-dimensional gels and MS

The first method developed for identification of differentially expressed proteins from complex proteomic samples was a combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) fractionation and MS analysis [27,28]. Samples are first separated by 2D-PAGE, which employs a two-step separation technique whereby denatured proteins are separated based upon their isoelectric point (the pH at which the net charge on the protein is zero) followed by separation based upon molecular size. The gels are stained and spots that appear to be differentially expressed are excised and in-gel digested with trypsin prior to MS analysis in order to determine the identity of the protein.

The development of ultrasensitive fluorescent tags with broad dynamic range and linearity of quantification, high performance digital imaging and analysis software, faster identification of spots

by MS, large scale application of these techniques and major progress in genomics and bioinformatics has accelerated the development of 2D gel based proteomics. Technical issues such as poor gel to gel variability and low sensitivity of detection have been minimised. As 2D-PAGE can only separate proteins in the mass range of 10–300 kDa it can be considered complimentary to other techniques, such as SELDI-ToF (discussed later) as this proteomic method can only be used to identify proteins below 20 kDa in size [29]. However, the presence of highly abundant serum/plasma proteins such as albumin and immunoglobulins are a major challenge for the success of 2D-PAGE-MS in the identification of differentially regulated proteins from clinically relevant bio-fluids such as these [30]. These abundant proteins result in large smears that mask lower abundance proteins [31], and thus depletion of these highly abundant proteins prior to 2D-PAGE is essential. The issue of multiple proteins present in a single spot still however remains problematic.

A variation on gel-based 2D separation has been developed, known as the ProteomeLab™ PF 2D system (Beckman Coulter, Fullerton, CA, USA). This is a liquid-phase 2D HPLC fractionation system that separates complex protein mixtures in liquid phase by chromatofocusing in the first dimension followed by high resolution non-porous silica reversed-phase chromatography (RPLC) in the second dimension thereby separating proteins based first upon pI, followed by hydrophobicity [32,33]. In addition, combining this method with iTRAQ tagging offers the potential for reducing sample complexity and identifying proteins that co-elute [34].

Although 2D-PAGE/MS is a relatively low throughput method, it does have the advantage that mass spectrometer analysis time is relatively short, as it is only used to compare differentially expressed proteins. In addition, this technique involves the study of intact proteins, rather than peptides and can therefore distinguish between protein isoforms as well as different post-translationally modified forms of the same protein. Several studies have demonstrated the clinical utility of this method, for example in the identification of differentially expressed proteins associated with neurodegenerative diseases including Alzheimer's and Parkinson's disease (reviewed in [29]). Several studies have also reported identification of urinary biomarkers using 2D gel-based approaches, which also demonstrated a positive correlation between protein abundance and disease stage (reviewed in [7]).

1.9. Quantification using stable isotope labelling

For the purposes of this review we will only consider chemical stable isotope labelling and live cell labelling techniques will not be discussed as these methods are not amenable to clinical proteomics. Chemical labels include isotope coded affinity tags (ICAT) [35], or isobaric tags such as iTRAQ [36], which rely on the use of a derivatisation reagent for chemical modification of proteins in a site specific manner. Because these labels are chemically identical the same peptide from two (or more) samples will behave identically in terms of chromatographic retention and ionisation efficiency, allowing samples to be analysed and compared simultaneously [37] (Fig. 1).

In the case of iTRAQ experiments throughput can be improved compared to other labelling methods, because 6 or 7 experimental samples can be analysed simultaneously compared to 1, but this is at the cost of a poor duty cycle due to the need to carry out MS/MS on all peptides. On the other hand, because iTRAQ is one of only two tagging technologies (the other being Tandem Mass Tags (TMTs) [44]) where quantification is carried out in MS/MS mode, this leads to increased accuracy and more reliable quantification. Indeed, stable isotope labelling should not affect the physiochemical properties of the peptide.

Chemical labelling techniques currently employed in clinical proteomics research will be discussed below.

1.10. ICAT

This method, developed by Gygi et al. [35], specifically targets cysteine residues and allows differentially labelled samples to be individually resolved during MS analysis. The original ICAT reagent contained either zero or eight deuterium atoms, second generation reagents, in particular, cleavable ICAT (cICAT) has since been developed and contains nine ^{13}C atoms as the heavy isotope which imparts a mass difference of 9 Da between labels [38] (Fig. 1). ICAT reagents also contain a biotin group for affinity purification of derivatised peptides prior to MS. This can cause problems during MS due to its bulky nature; however, cICAT reagents circumvent this issue as they contain an acid-cleavable linker between the reactive sulfhydryl tag and the biotin moiety, which allows for its removal following affinity purification.

In addition to ICAT, other thiol specific reagents have been developed (see [15] for review), including a metal coded affinity tagging method, which also targets cysteine residues [39]. Perhaps due to the issues associated with targeting cysteine residues (one in seven proteins in the vertebrate proteome do not contain cysteine [40]) the use of ICAT in a clinical setting has been limited, however, it has been used to investigate age-related [41] and Alzheimer's disease-associated changes in cerebrospinal fluid proteins [42].

1.11. iTRAQ

A further group of labelling reagents are those that have been synthesised to target the peptide amino group and the epsilon-amino group of lysine residues. These tags are considered favourable over methods such as ICAT as they target all tryptic peptides in a sample digest, and thus the depth of coverage is greatly enhanced. In most cases these types of tag utilise *N*-hydroxysuccinimide (NHS) chemistry or other active esters and anhydrides, for example in the isotope-coded protein label (ICPL) [43], isotope tags for relative and absolute quantification (iTRAQ) [36], tandem mass tags (TMTs) [44] and acetic/succinic anhydride-based methods [45–48]. Other less used methods include the use of isocyanates, or isothiocyanates [49,50], and methylation of lysine residues using formaldehyde [51–53].

With the exception of iTRAQ and new 6-plex TMTs, relative quantification is achieved by integration of the MS signal over isotopomers of 'heavy' and 'light' labelled peptides. iTRAQ differs from these approaches in that it is based upon the use of isobaric tags which are fragmented in tandem MS/MS mode to produce a 'reporter' ion signature in a quiet region of the MS/MS spectrum to allow relative quantification [44] (Fig. 1). Because of the isobaric nature of iTRAQ-labelled peptides this allows the signal from all peptides to be summed in both MS and MS/MS modes thus enhancing the sensitivity of detection. This potential benefit to identify and quantify low abundance proteins in complex samples, coupled with the ability to multiplex up to eight samples in parallel [54] (unlike ICAT, which is limited to two labelled samples per run) suggests that iTRAQ holds the most promise for quantitative biomarker discovery.

Because iTRAQ results in fragmentation of all precursors this necessitates the use of inclusion lists to ensure that the same peptides are being fragmented each time. For example, two runs may have independently identified 200 proteins each, of which there may only be a 50% overlap. Repeated experimental iterations should increase this overlap.

Although the iTRAQ reporter ions have *m/z* values in the "quiet" region of the mass spectrum if there are any additional peptide ions present in this selection window these will adversely affect quantification [15]. Other methods, such as enzymatic labelling of samples such as the use of trypsin or Glu-C catalysed incorporation of ^{18}O during digestion avoids side reaction artefacts, however, different peptides incorporate at different rates, and full labelling is rarely achieved [55,56]. Furthermore, enzymatic labelling requires at least a 4 Da mass shift in order to distinguish isotopomer clusters of labelled and unlabelled peptide forms, and as these clusters increase with peptide mass thus enzymatic labelling has limited use for larger peptides [57].

Several papers have been published which highlight the promise of iTRAQ coupled with LC-MS/MS as a tool for identifying potential biomarker signatures indicative of disease from a variety of sources such as serum, CSF and tissue. For example, studies with serum using iTRAQ coupled with LC-MS/MS identified 160 proteins, of which 31 were differentially expressed following traumatic brain injury; three of which (serum amyloid A, C-reactive protein and retinol binding protein 4) were verified independently and shown

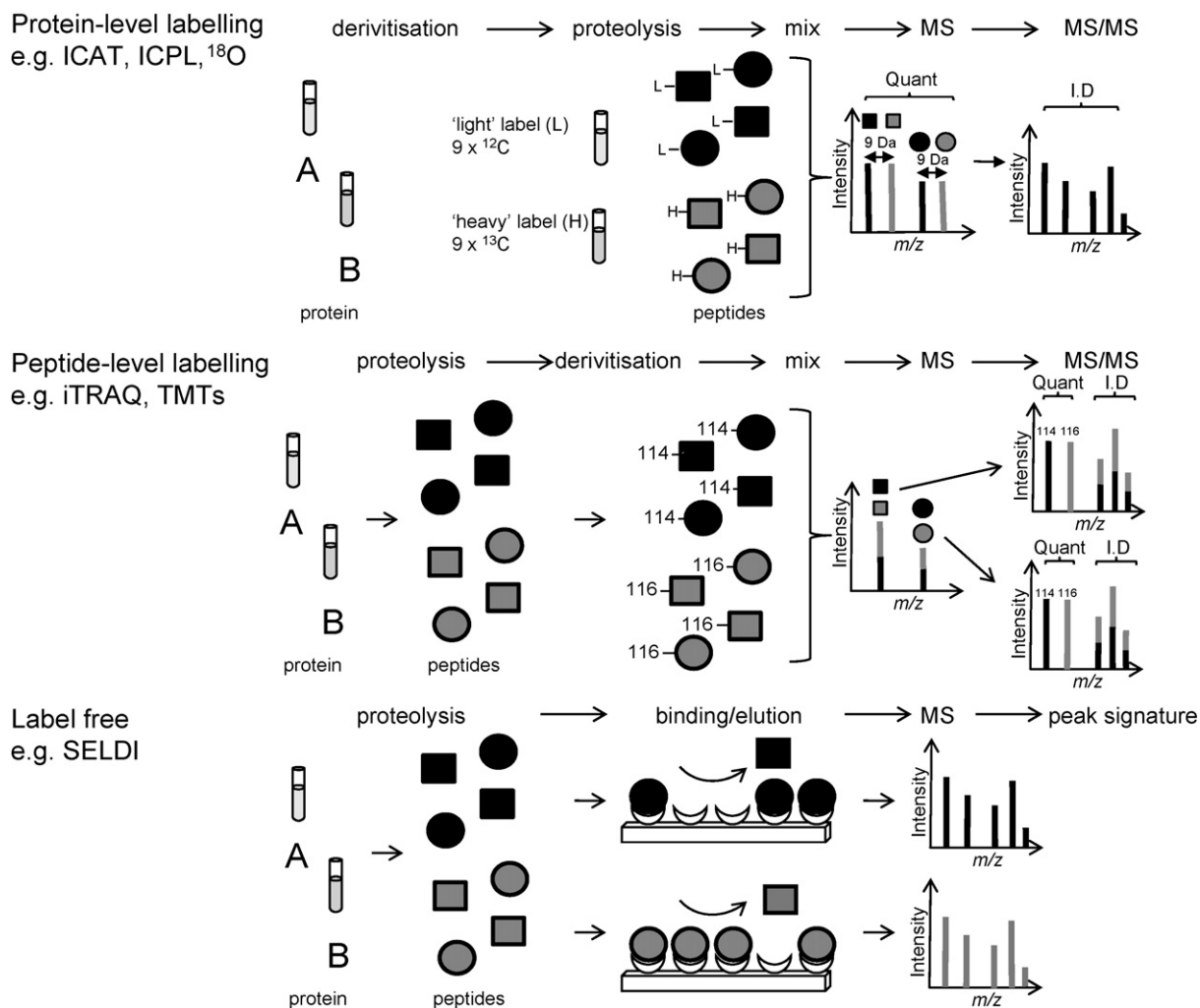


Fig. 1. Schematic representation of three methods for relative quantification by mass spectrometry (adapted from [30,37]). (a) Protein-level labelling either by culturing cells in the presence or absence of a 'heavy' isotope amino acid (e.g. stable isotope labelling with amino acids in cell culture (SILAC) [33], not amenable to clinical proteomics) or using chemical derivitisation, by methods such as ICAT (as shown) allows two conditions to be tested simultaneously. In the case of ICAT, the 'heavy' and 'light' labels impart a mass difference of 9 Da without affecting the chromatographic properties of the labelled peptides, thus allowing relative quantification in MS. Subsequent MS/MS analysis must be conducted on targeted ion pairs to enable identification of differentially expressed proteins. (b) Peptide level labelling with isobaric tags such as iTRAQ (shown here) which allows multiplexing of up to eight samples in one run (two are shown for clarity). The different masses of each 'reporter' group are counteracted by a 'balance' group which confers isobaric properties on each tag in MS mode. Subsequently, multiplexed samples containing the same mix of peptides labelled with different iTRAQ tags will behave identically until they are fragmented during MS/MS. This provides several advantageous properties, as all equivalent peptides will behave identically in LC separation steps, and in MS and MS/MS mode the signal from all peptides may be summed (as they have the same mass), thus enhancing the sensitivity of detection. (c) Label-free methods such as SELDI (shown here) enrich for specific peptides by binding and eluting them from a 'chip' with a particular chromatographic surface prior to MS analysis. Proteins are not identified by this method, instead, peak patterns are derived in order to generate a proteomic profile which is used to compare multiple samples processed via the same method.

to have good sensitivity for the early detection of increased intracranial pressure indicative of traumatic brain injury [58].

iTRAQ-MS/MS has been used to identify 219 proteins in human CSF, with 12 proteins differentially expressed between male and female subjects. This represents a comparable, and in most cases, slightly better penetration of the CSF proteome than previously reported using 2D gel-based methods, and indicates that this is a robust method to use in clinical analysis of the CSF proteome during diseases, such as Alzheimer's or Parkinson's [8].

Studies using iTRAQ tagged tissue samples from patients with head and neck squamous cell carcinoma (HNSCC) followed by multidimensional LC-MS/MS identified a panel of differentially regulated proteins when comparing HNSCC samples with pooled normal controls. Three of these proteins (YWHAZ, stratifin and S100A7) were shown to have high sensitivity and specificity for differentiating normal versus cancerous tissue in an independent

HNSCC set and show potential for development as clinically relevant biomarkers for diagnosis of this disease [59]. Studies using endometrial tissue also show promise using iTRAQ to determine differential expression profiles in patients with type I and type II endometrial cancer [60–62]. Indeed, there is a growing interest in the use of tagging technology in combination with sensitive MS/MS techniques for use in cancer diagnosis, prognosis or monitoring of treatment and relapse.

1.12. Benefits and caveats of label free approaches

The clinical use of MS-based methods for the proteomic profiling of bio-fluids for diagnostic and/or prognostic information presents many challenges. It is imperative that sample processing should not affect the outcome of any analyses and that the chosen platform should be robust and reliable, thus reducing

detrimental effects introduced by experimental variables. Label free quantification methods are favourable in practical terms as they are relatively high-throughput, requiring no time-consuming and expensive labelling step. Consequently there is no theoretical limit to the number of samples that can be analysed in any given experiment, as it is not restricted by the number of labels available. However, unlike isotope labelling methods, label free approaches do not allow for sample multiplexing, and thus may not be faster. In addition, lack of topoisomeric peptides reduces the spectral complexity at any given chromatographic time, potentially increasing the number of peptides identified, although again this is not true of isobaric tagging reagents such as iTRAQ. Although there is evidence that label free methods show increased dynamic range of quantification over stable isotope labelling, label free methods are particularly susceptible to error, and there is inconclusive data regarding the accuracy and linearity of label free techniques [16].

1.13. SELDI-ToF MS

Several label-free proteomic profiling techniques have been developed which are based upon the application of an unprocessed bio-fluid to a “chip” with a specific chemistry, i.e. a particular chromatographic surface. Unbound proteins are washed off and bound proteins are analysed in a simple time-of-flight mass spectrometer. In this method, proteins are not identified but signature peak patterns are derived and compared between test groups to generate a proteomic profile. The primary example of this type of method is SELDI-ToF whereby samples such as serum, plasma and urine can be applied to chromatographic chips designed to enrich for different populations of protein/peptide analyte. Consequently, the main advantage of this technique is ease of use and apparent throughput, a possible reason as to why this method is so heavily used in clinical proteomics, particularly in comparison to other MS-based proteomics approaches [63].

Although there is evidence that label free approaches have an enhanced dynamic range for detection compared with stable isotope labelling, these techniques have been shown to be the least accurate for quantification purposes and are extremely sensitive to experimental variation. Indeed controversy surrounds the long term viability of SELDI as a platform for wide-spread, large scale clinical use, as concern still remains regarding the semi-quantitative nature of the method, and its reproducibility [15]. A classic example of this is an early study by Petricoin et al. [24], whereby a biomarker signature for ovarian cancer determined by SELDI was subsequently found to be not reproducible and the differences were proposed to be due to variables introduced during sample processing [64,65]. In addition, the reproducibility and inter-lab variability of SELDI to detect a three peak signature identified to detect prostate cancer was tested by six independent laboratories, and the inter-lab coefficients of variation of the normalised peak intensities were found to be between 15–36% [66].

Nonetheless, SELDI has been used in several disease areas, for example, to identify diagnostic markers of tuberculosis [67], severe acute respiratory syndrome (SARS) [68,69] and intra-amniotic infection [70,71]. SELDI has also been used to identify biomarkers of neurologic disorders, such as Alzheimer's [72,73]. However, this methodology has been utilised most heavily in oncology, and various signatures have been described as diagnostic indicators of various types of cancer. For example, in several studies of patients with hepatocellular carcinoma (HCC) SELDI has been used to identify patients with HCC [74] and to distinguish between patients with HCC and hepatitis C virus [75–77]. The most recent study by Zinkin et al. [77], found that SELDI-ToF was more accurate than traditional biomarkers at detecting small tumours and although the authors recognised that the sample set was relatively small, this

highlights the potential for this technique in clinical applications such as diagnosis of HCC.

In a recent study by Taguchi et al. [78], SELDI was used on samples from patients with small cell lung cancer (SCLC) and an 8 peak feature map was generated that was able to predict good or poor prognosis groups in response to treatment with epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI's). In addition, the results of this study were reproducible between two independent laboratories. In another study involving SCLC, biomarker profiles were identified that could distinguish between SCLC and healthy controls, SCLC from pneumonia and SCLC from non-small cell lung cancer (NSCLC) [79]. However, a note of caution must be introduced as the sample sets were relatively small, and the results require further validation with additional patient samples. In addition to lung cancer, SELDI has been reported in the identification of biomarker signatures able to distinguish between prostate cancer, benign prostate hyperplasia and healthy men [80]. SELDI has also been reported in the detection of colorectal cancer signatures [81], early stage ovarian cancer [82] and to distinguish patients with Transitional Cell Carcinoma (TCC) of the bladder [83,84]. Furthermore, in several cancer types SELDI has been applied to the derivation of prognostic signatures, for example in the predication of relapse in breast cancer patients [85].

Several studies have described the use of SELDI in identification of peaks that are indicative of breast cancer from nipple aspirate fluid [9–12]. NAF is an attractive source for proteomic information in breast cancer, primarily due to its proximity to the primary tumour and the relative ease of sample collection compared to biopsy. However, caution must be employed when analysing these data, as these studies show minimal, if any overlap in the peaks/proteins that were identified as associated with breast cancer. This highlights a major challenge for the multi-centre use of SELDI in routine clinical use, as variables such as sample handling and processing, loading onto the target, washing, matrix type and the method of data acquisition and processing can all significantly affect the final data output. In addition, SELDI has thus far been unable to identify conventional tumour markers, such as α -fetoprotein [63].

Although screening is rapid, because potential biomarkers are not identified this is likely to produce a bottleneck in the biomarker validation step. Theoretically, the biomarker(s) does not have to be identified in order to provide diagnostic/prognostic information. However, as questions still remain regarding the reliability of this method, it would seem that the logical step in moving forward and validating SELDI as a useful tool for clinical proteomics would be to identify the protein(s) responsible for the characteristic m/z peak in order to develop more robust methods for high throughput use, such as ELISA assays.

Other proteomic profiling methods have been developed based upon the principles of SELDI, such as ClinProt (Bruker Daltonics, Billerica, MA), which is bead rather than chip-based and both bound and eluted proteins can be identified [86].

2. Spiked synthetic peptide standards

2.1. AQUA (absolute quantification of proteins)

The substantial resolving power of modern mass spectrometers can only be fully realised in the clinical arena by the use of accurate methods for absolute quantification. Unless a standardised reference sample is used, coded isotope labelling can only provide relative quantification, which can lead to difficulties when interpreting inter-study comparisons. The use of internal standards has long been a tool for absolute quantification of small molecules in isotope dilution experiments. Quantification is achieved by spiking

known amounts of an isotopically labelled form of a known analyte into the sample prior to MS, and the relative levels of labelled and endogenous forms can be calculated. It must be noted that in this case the identity of the analyte is known prior to analysis.

Variations on the internal standard method have been developed for use in proteomics, including AQUA (absolute quantification of proteins) [87], PC-IDMS (protein cleavage-isotope dilution mass spectrometry) [88], SISCAPA (stable isotope standards and capture by anti-peptide antibodies) [89] and VICAT (visual isotope coded affinity tags) [90] and these methods are able to measure absolute protein amounts and post-translational levels of proteins, ultimately essential for the validation of any novel protein biomarker. A critical difference between the isotope dilution approach and AQUA is that while isotope dilution experiments conducted on small molecules involve direct measurement of the analyte, quantification of proteins by AQUA is carried out at the peptide level [91].

The specificity of the spiked standard may also lead to inaccurate quantification if it has the same m/z value of other peptides in the sample. However, combining AQUA with use of multiple reaction monitoring (MRM) [91], a highly sensitive method routinely used to measure drug metabolites, hormones, protein degradation products and pesticides with high precision and, known, reproducible LC retention time can reduce these effects. MRM involves two stages of mass selection, in the first instance a parent ion is selected and isolated at a particular m/z ratio. The parent ion is then fragmented and a second selection step is then used, whereby a specific product ion is accumulated and monitored, making this a highly specific and sensitive quantitative technique when combined with the appropriate isotope labelled protein standards [92] (Fig. 2).

Because MRM focuses on a handful of proteins of interest rather than a global proteomics approach this technique is attractive dur-

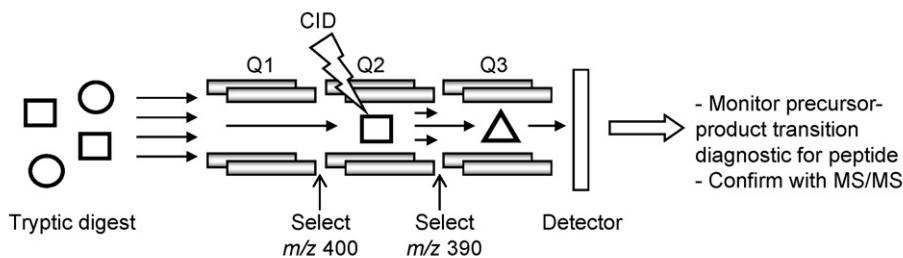
ing the validation and assay development phases of biomarker discovery. In the clinical proteomics setting, MRM has been successfully used to isolate and quantitate tryptic peptides in plasma which are indicative of disease, including C-reactive protein [26], apolipoprotein A-1 [25] and prostate-specific antigen [93]. Traditional diagnostics based upon 1 or several protein biomarkers involve the use of antibodies, typically requiring the development of an ELISA method. Antibody microarrays have been shown to have sensitivity ranging from 1 to 1000 pg/mL for cytokines in plasma [94], however, due to the idiosyncratic nature of antibody generation the development of reliable antibodies for screening can be problematic at best. Because MRM can detect peptides at low ng/mL levels [13,95,96] and is applicable to all peptides it is thought that this method may provide the most promise for biomarker validation and screening.

Other label free methods for biomarker identification are largely untested in the clinical proteomics arena, including spectral feature analysis where the peptide sequence is not identified and quantification is carried out by comparison of spectral features from separate LC/MS runs [23,97–99]. However, this method generates high error rates [100,101], therefore it is generally accepted that further studies are required to verify any changes in abundance and to determine the identity of these spectral features [21].

2.2. Future challenges and directions

Recent advances in LC–MS/MS-based techniques for clinical proteomic biomarker discovery and validation have offered much hope for superior patient care, particularly for cancer diagnosis and treatment where the potential gains for individualised therapy are huge. However, the complexity, variation and dynamic range of proteins present within bio-fluids (such as plasma) are major obstacles to these methods to accurately quantify changing protein levels. In

(a) Multiple Reaction Monitoring (MRM)



(b) MRM quantification by stable isotope dilution

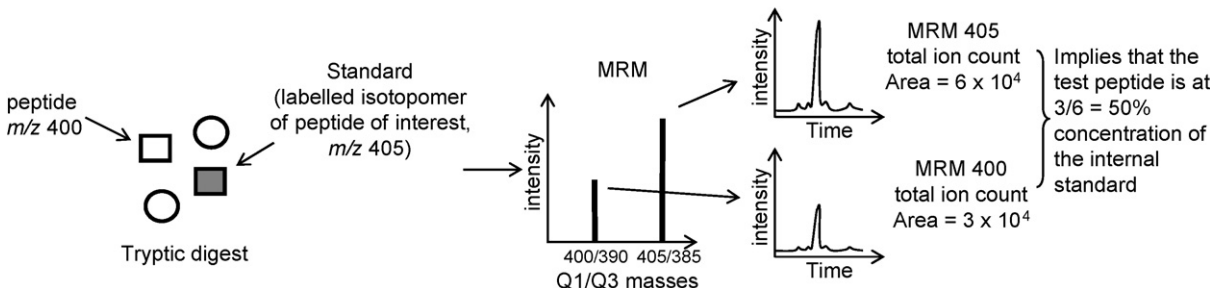


Fig. 2. Schematic overview of multiple reaction monitoring (MRM) for biomarker quantitation (adapted from [103]). (a) Specific peptide detection by MRM. In this example peptides from a tryptic digest enter the first quadrupole (Q1) and a diagnostic peptide (m/z 400) eluting at a specific time during liquid chromatography (LC) is isolated and enters the collision cell (Q2). Collision induced dissociated (CID) fragments this peptide and a specific product ion (m/z 390) if generated, is selected to enter the third quadrupole (Q3) where it then reaches the detector. This filtering dramatically reduces the background resulting in a significantly increased signal to noise and greater sensitivity. (b) Absolute quantification by MRM. Inclusion of an isotopically labelled standard peptide allows for MRM transitions to be monitored for the test and standard peptide. The mass difference imparted by the isotopomer enables the test and standard peptide to co-elute during LC and be monitored for different MRM transitions in parallel. As the concentration of the standard is known, the ratios of the total signal generated by each peptide can be calculated and thus used for absolute quantitation purposes.

addition to problems presented by the analyte itself, many other factors such as specimen collection, handling and processing (fasting samples, freeze-thaw effects, life style variations, for example), pre-fractionation methodology, instrumentation set-up, database mining, statistical analysis and data storage will all lead to increased costs and decreased throughput and thus affect the ultimate success of LC-MS/MS-based biomarker discovery [102].

Traditional drug development within the pharmaceutical industry follows a process from discovery through to pre-clinical development and clinical testing, typically involving large scale screening of multiple analytes. In contrast, basic research is dominated by studies of individual molecules. Therefore, in order for clinical proteomics by MS-based methods to be successful it is essential that the gap between these two disciplines is bridged [1]. One of the major challenges is the translation of pre-clinical animal studies into human subjects. MS offers the exciting prospect of bypassing this problem by moving directly into human bio-fluid samples for discovery-based medicine. In addition, there is potential to reduce the number of patients required for clinical testing by carrying out well designed pre-clinical studies in well characterised animal and/or cell line models [3].

It is essential that novel biomarker profiles are carefully validated and it is possible that routine application may be carried out by immunoassays, which can also present huge challenges. These include issues surrounding antibody reliability and sensitivity and the ability for multiplexing interactions which all impact upon their cost effectiveness. In the case of clinical proteomics it is likely that multiple novel candidates will be identified and thus multiple reaction monitoring/stable isotope dilution (MRM/SID-MS) by triple quadrupole MS may be more feasible, allowing for greater throughput, accuracy, sensitivity and throughput than antibody development [13,30]. Perhaps the most important factor for the realisation of personalised medicine provided by novel biomarkers identified by MS is the need for careful and rigorous validation of these markers though rationally designed, large scale clinical studies [1]. These can only be successfully realised by close working relationships between discovery labs and clinical centres. This highlights the major challenge faced by translational medicine, in particular clinical proteomics, but with careful planning it is hoped that the potential provided by continual developments in LC-MS/MS methods for relative and absolute protein quantification will lead to advances in the way diseases such as cancer are diagnosed and managed.

References

- [1] L. Beretta, *Nat. Methods* 4 (2007) 785.
- [2] R. Aebersold, M. Mann, *Nature* 422 (2003) 198.
- [3] C.L. Sawyers, *Nature* 452 (2008) 548.
- [4] N.L. Anderson, N.G. Anderson, *Mol. Cell. Proteomics* 1 (2002) 845.
- [5] G. Marko-Varga, A. Ogiwara, T. Nishimura, T. Kawamura, K. Fujii, T. Kawakami, Y. Kyono, H.K. Tu, H. Anyoji, M. Kanazawa, S. Akimoto, T. Hirano, M. Tsuboi, K. Nishio, S. Hada, H. Jiang, M. Fukuoka, K. Nakata, Y. Nishiwaki, H. Kunito, I.S. Peers, C.G. Harbron, M.C. South, T. Higenbottam, F. Nyberg, S. Kudoh, H. Kato, *J. Proteome Res.* 6 (2007) 2925.
- [6] M. Mayr, J. Zhang, A.S. Greene, D. Gutterman, J. Perloff, P. Ping, *Mol. Cell. Proteomics* 5 (2006) 1853.
- [7] T. Pisitkun, R. Johnstone, M.A. Knepper, *Mol. Cell. Proteomics* 5 (2006) 1760.
- [8] Y. Ogata, M.C. Charlesworth, L. Higgins, B.M. Keegan, S. Vernino, D.C. Muddiman, *Proteomics* 7 (2007) 3726.
- [9] J. Li, J. Zhao, X. Yu, J. Lange, H. Kuerer, S. Krishnamurthy, E. Schilling, S.A. Khan, S. Sukumar, D.W. Chan, *Clin. Cancer Res.* 11 (2005) 8312.
- [10] J.L. Noble, R.S. Dua, G.R. Coulton, C.M. Isacke, G.P. Gui, *Eur. J. Cancer* 43 (2007) 2315.
- [11] T.M. Pawlik, H. Fritsche, K.R. Coombes, L. Xiao, S. Krishnamurthy, K.K. Hunt, L. Pusztai, J.N. Chen, C.H. Clarke, B. Arun, M.C. Hung, H.M. Kuerer, *Breast Cancer Res. Treat.* 89 (2005) 149.
- [12] E.R. Sauter, S. Shan, J.E. Hewett, P. Speckman, G.C. Du Bois, *Int. J. Cancer* 114 (2005) 791.
- [13] N. Rifai, M.A. Gillette, S.A. Carr, *Nat. Biotechnol.* 24 (2006) 971.
- [14] R. Etzioni, N. Urban, S. Ramsey, M. McIntosh, S. Schwartz, B. Reid, J. Radich, G. Anderson, L. Hartwell, *Nat. Rev. Cancer* 3 (2003) 243.
- [15] M. Bantscheff, M. Schirle, G. Sweetman, J. Rick, B. Kuster, *Anal. Bioanal. Chem.* 389 (2007) 1017.
- [16] W.M. Old, K. Meyer-Arendt, L. Aveline-Wolf, K.G. Pierce, A. Mendoza, J.R. Sevinisky, K.A. Resing, N.G. Ahn, *Mol. Cell. Proteomics* 4 (2005) 1487.
- [17] J. Lill, *Mass Spectrom. Rev.* 22 (2003) 182.
- [18] A. Gilchrist, C.E. Au, J. Hiding, A.W. Bell, J. Fernandez-Rodriguez, S. Lesimple, H. Nagaya, L. Roy, S.J. Gosline, M. Hallett, J. Paiement, R.E. Kearney, T. Nilsson, J.J. Bergeron, *Cell* 127 (2006) 1265.
- [19] H. Liu, R.G. Sadygov, J.R. Yates 3rd, *Anal. Chem.* 76 (2004) 4193.
- [20] M.P. Washburn, D. Wolters, J.R. Yates, 3rd, *Nat. Biotechnol.* 19 (2001) 242.
- [21] A.I. Nesvizhskii, O. Vitek, R. Aebersold, *Nat. Methods* 4 (2007) 787.
- [22] M.K. Dayarathna, W.S. Hancock, M. Hincapie, *J. Sep. Sci.* 31 (2008) 1156.
- [23] D. Radulovic, S. Jelveh, S. Ryu, T.G. Hamilton, E. Foss, Y. Mao, A. Emili, *Mol. Cell. Proteomics* 3 (2004) 984.
- [24] E.F. Petricoin, A.M. Ardekani, B.A. Hitt, P.J. Levine, V.A. Fusaro, S.M. Steinberg, G.B. Mills, C. Simone, D.A. Fishman, E.C. Kohn, L.A. Liotta, *Lancet* 359 (2002) 572.
- [25] J.R. Barr, V.L. Maggio, D.G. Patterson Jr., G.R. Cooper, L.O. Henderson, W.E. Turner, S.J. Smith, W.H. Hannon, L.L. Needham, E.J. Sampson, *Clin. Chem.* 42 (1996) 1676.
- [26] E. Kuhn, J. Wu, J. Karl, H. Liao, W. Zolg, B. Guild, *Proteomics* 4 (2004) 1175.
- [27] M.C. Pietrogrande, N. Marchetti, F. Dondi, P.G. Righetti, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 833 (2006) 51.
- [28] P. Weingarten, P. Lutter, A. Wattenberg, M. Blueggel, S. Bailey, J. Klose, H.E. Meyer, C. Huels, *Methods Mol. Med.* 109 (2005) 155.
- [29] E.A. Sheta, S.H. Appel, I.L. Goldknopf, *Expert Rev. Proteomics* 3 (2006) 45.
- [30] T.D. Veenstra, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 847 (2007) 3.
- [31] E.B. Altintas, A. Denizli, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 832 (2006) 216.
- [32] D.M. Lubman, M.T. Kachman, H. Wang, S. Gong, F. Yan, R.L. Hamler, K.A. O'Neil, K. Zhu, N.S. Buchanan, T.J. Barder, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 782 (2002) 183.
- [33] F. Yan, B. Subramanian, A. Nakeff, T.J. Barder, S.J. Parus, D.M. Lubman, *Anal. Chem.* 75 (2003) 2299.
- [34] H. Skalnikova, P. Rehulka, J. Chmelik, J. Martinkova, M. Zilvarova, S.J. Gadhre, H. Kovarova, *Anal. Bioanal. Chem.* 389 (2007) 1639.
- [35] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, *Nat. Biotechnol.* 17 (1999) 994.
- [36] P.L. Ross, Y.N. Huang, J.N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlett-Jones, F. He, A. Jacobson, D.J. Pappin, *Mol. Cell. Proteomics* 3 (2004) 1154.
- [37] R.D. Unwin, A.D. Whetton, *Blood Rev.* 21 (2007) 315.
- [38] K.C. Hansen, G. Schmitt-Ulms, R.J. Chalkley, J. Hirsch, M.A. Baldwin, A.L. Burlingame, *Mol. Cell. Proteomics* 2 (2003) 299.
- [39] R. Ahrends, S. Pieper, A. Kuhn, H. Weisshoff, M. Hamester, T. Lindemann, C. Scheler, K. Lehmann, K. Taubner, M.W. Linscheid, *Mol. Cell. Proteomics* 6 (2007) 1907.
- [40] C.P. Vaughn, D.K. Crockett, M.S. Lim, K.S. Elenitoba-Johnson, *J. Mol. Diagn.* 8 (2006) 513.
- [41] J. Zhang, D.R. Goodlett, E.R. Peskind, J.F. Quinn, Y. Zhou, Q. Wang, C. Pan, E. Yi, J. Eng, R.H. Aebersold, T.J. Montine, *Neurobiol. Aging* 26 (2005) 207.
- [42] J. Zhang, D.R. Goodlett, J.F. Quinn, E. Peskind, J.A. Kaye, Y. Zhou, C. Pan, E. Yi, J. Eng, Q. Wang, R.H. Aebersold, T.J. Montine, *J. Alzheimer's Dis.* 7 (2005) 125.
- [43] A. Schmidt, J. Kellermann, F. Lottspeich, *Proteomics* 5 (2005) 4.
- [44] A. Thompson, J. Schafer, K. Kuhn, S. Kienle, J. Schwarz, G. Schmitt, T. Neumann, R. Johnstone, A.K. Mohammed, C. Hamon, *Anal. Chem.* 75 (2003) 1895.
- [45] F.Y. Che, L.D. Fricker, *Anal. Chem.* 74 (2002) 3190.
- [46] M.O. Glocker, C. Borchers, W. Fiedler, D. Suckau, M. Przybylski, *Bioconjug. Chem.* 5 (1994) 583.
- [47] J. Ji, A. Chakraborty, M. Geng, X. Zhang, A. Amini, M. Bina, F. Regnier, *J. Chromatogr. B Biomed. Sci. Appl.* 745 (2000) 197.
- [48] X. Zhang, Q.K. Jin, S.A. Carr, R.S. Annan, *Rapid Commun. Mass Spectrom.* 16 (2002) 2325.
- [49] Y.H. Lee, H. Han, S.B. Chang, S.W. Lee, *Rapid Commun. Mass Spectrom.* 18 (2004) 3019.
- [50] D.E. Mason, D.C. Liebler, *J. Proteome Res.* 2 (2003) 265.
- [51] J.L. Hsu, S.Y. Huang, S.H. Chen, *Electrophoresis* 27 (2006) 3652.
- [52] J.L. Hsu, S.Y. Huang, N.H. Chow, S.H. Chen, *Anal. Chem.* 75 (2003) 6843.
- [53] C. Ji, N. Guo, L. Li, *J. Proteome Res.* 4 (2005) 2099.
- [54] A. Pierce, R.D. Unwin, C.A. Evans, S. Griffiths, L. Carney, L. Zhang, E. Jaworska, C.F. Lee, D. Blinco, M.J. Okoniewski, C.J. Miller, D.A. Bitton, E. Spooncer, A.D. Whetton, *Mol. Cell. Proteomics* 7 (2008) 853.
- [55] K.J. Reynolds, X. Yao, C. Fenselau, *J. Proteome Res.* 1 (2002) 27.
- [56] X. Yao, A. Freas, J. Ramirez, P.A. Demirev, C. Fenselau, *Anal. Chem.* 73 (2001) 2836.
- [57] K.C. Rao, R.T. Carruth, M. Miyagi, *J. Proteome Res.* 4 (2005) 507.
- [58] G. Hergenroeder, J.B. Redell, A.N. Moore, W.P. Dubinsky, R.T. Funk, J. Crommett, G.L. Clifton, R. Levine, A. Valadka, P.K. Dash, *J. Neurotrauma* 25 (2008) 79.
- [59] R. Ralhan, L.V. Desouza, A. Matta, S. Chandra Tripathi, S. Ghanny, S. Datta Gupta, S. Bahadur, K.W. Siu, *Mol. Cell. Proteomics* (2008).
- [60] L.V. DeSouza, J. Grigull, S. Ghanny, V. Dube, A.D. Romaschin, T.J. Colgan, K.W. Siu, *Mol. Cell. Proteomics* 6 (2007) 1170.

- [61] L. DeSouza, G. Diehl, E.C. Yang, J. Guo, M.J. Rodrigues, A.D. Romaschin, T.J. Colgan, K.W. Siu, *Proteomics* 5 (2005) 270.
- [62] L. DeSouza, G. Diehl, M.J. Rodrigues, J. Guo, A.D. Romaschin, T.J. Colgan, K.W. Siu, *J. Proteome Res.* 4 (2005) 377.
- [63] T.C. Poon, *Expert Rev. Proteomics* 4 (2007) 51.
- [64] K.A. Baggerly, J.S. Morris, K.R. Coombes, *Bioinformatics* 20 (2004) 777.
- [65] K.A. Baggerly, J.S. Morris, S.R. Edmonson, K.R. Coombes, *J. Natl. Cancer Inst.* 97 (2005) 307.
- [66] O.J. Semmes, Z. Feng, B.L. Adam, L.L. Banez, W.L. Bigbee, D. Campos, L.H. Cazares, D.W. Chan, W.E. Grizzle, E. Izbicka, J. Kagan, G. Malik, D. McLerran, J.W. Moul, A. Partin, P. Prasanna, J. Rosenzweig, L.J. Sokoll, S. Srivastava, S. Srivastava, I. Thompson, M.J. Welsh, N. White, M. Winget, Y. Yasui, Z. Zhang, L. Zhu, *Clin. Chem.* 51 (2005) 102.
- [67] D. Agranoff, D. Fernandez-Reyes, M.C. Papadopoulos, S.A. Rojas, M. Herbster, A. Loosemore, E. Tarelli, J. Sheldon, A. Schwenk, R. Pollok, C.F. Rayner, S. Krishna, *Lancet* 368 (2006) 1012.
- [68] R.T. Pang, T.C. Poon, K.C. Chan, N.L. Lee, R.W. Chiu, Y.K. Tong, R.M. Wong, S.S. Chim, S.M. Ngai, J.J. Sung, Y.M. Lo, *Clin. Chem.* 52 (2006) 421.
- [69] T.C. Poon, K.C. Chan, P.C. Ng, R.W. Chiu, I.L. Ang, Y.K. Tong, E.K. Ng, F.W. Cheng, A.M. Li, E.K. Hon, T.F. Fok, Y.M. Lo, *Clin. Chem.* 50 (2004) 1452.
- [70] M.G. Gravett, M.J. Novy, R.G. Rosenfeld, A.P. Reddy, T. Jacob, M. Turner, A. McCormack, J.A. Lapidus, J. Hitti, D.A. Eschenbach, C.T. Roberts Jr., S.R. Nagalla, *JAMA* 292 (2004) 462.
- [71] U. Ruetschi, A. Rosen, G. Karlsson, H. Zetterberg, L. Rymo, H. Hagberg, B. Jacobsson, *J. Proteome Res.* 4 (2005) 2236.
- [72] A. Busch, S. Michel, C. Hoppe, D. Driesch, U. Claussen, F. von Eggeling, *J. Histochem. Cytochem.* 53 (2005) 341.
- [73] O. Carrette, I. Demalte, A. Scherl, O. Yalkinoglu, G. Corthals, P. Burkhard, D.F. Hochstrasser, J.C. Sanchez, *Proteomics* 3 (2003) 1486.
- [74] T.C. Poon, T.T. Yip, A.T. Chan, C. Yip, V. Yip, T.S. Mok, C.C. Lee, T.W. Leung, S.K. Ho, P.J. Johnson, *Clin. Chem.* 49 (2003) 752.
- [75] V. Paradis, F. Degos, D. Dargere, N. Pham, J. Belghiti, C. Degott, J.L. Janeau, A. Bezeaud, D. Delforge, M. Cubizolles, I. Laurendeau, P. Bedossa, *Hepatology* 41 (2005) 40.
- [76] E.E. Schwegler, L. Cazares, L.F. Steel, B.L. Adam, D.A. Johnson, O.J. Semmes, T.M. Block, J.A. Marrero, R.R. Drake, *Hepatology* 41 (2005) 634.
- [77] N.T. Zinkin, F. Grall, K. Bhaskar, H.H. Otu, D. Spentzos, B. Kalmowitz, M. Wells, M. Guerrero, J.M. Asara, T.A. Libermann, N.H. Afdhal, *Clin. Cancer Res.* 14 (2008) 470.
- [78] F. Taguchi, B. Solomon, V. Gregorc, H. Roder, R. Gray, K. Kasahara, M. Nishio, J. Brahmer, A. Spreafico, V. Ludovini, P.P. Massion, R. Dziadziszko, J. Schiller, J. Grigorieva, M. Tsybin, S.W. Hunsucker, R. Caprioli, M.W. Duncan, F.R. Hirsch, P.A. Bunn Jr., D.P. Carbone, *J. Natl. Cancer Inst.* 99 (2007) 838.
- [79] M. Han, Q. Liu, J. Yu, S. Zheng, *J. Clin. Lab. Anal.* 22 (2008) 131.
- [80] B.L. Adam, Y. Qu, J.W. Davis, M.D. Ward, M.A. Clements, L.H. Cazares, O.J. Semmes, P.F. Schellhammer, Y. Yasui, Z. Feng, G.L. Wright Jr., *Cancer Res.* 62 (2002) 3609.
- [81] J.Y. Engwegen, H.H. Helgason, A. Cats, N. Harris, J.M. Bonfrer, J.H. Schellens, J.H. Beijnen, *World J. Gastroenterol.* 12 (2006) 1536.
- [82] Z. Zhang, R.C. Bast Jr., Y. Yu, J. Li, L.J. Sokoll, A.J. Rai, J.M. Rosenzweig, B. Cameron, Y.Y. Wang, X.Y. Meng, A. Berchuck, C. Van Haaften-Day, N.F. Hacker, H.W. de Bruijn, A.G. van der Zee, I.J. Jacobs, E.T. Fung, D.W. Chan, *Cancer Res.* 64 (2004) 5882.
- [83] J. Mueller, F. von Eggeling, D. Driesch, J. Schubert, C. Melle, K. Junker, *Eur. Urol.* 47 (2005) 885.
- [84] A. Vlahou, P.F. Schellhammer, S. Mendrinos, K. Patel, F.I. Kondylis, L. Gong, S. Nasim, G.L. Wright Jr., *Am. J. Pathol.* 158 (2001) 1491.
- [85] A. Goncalves, B. Esterni, F. Bertucci, R. Sauvan, C. Chabannon, M. Cubizolles, V.J. Bardou, G. Houvenaegel, J. Jacquemier, S. Granjeaud, X.Y. Meng, E.T. Fung, D. Birnbaum, D. Maraninchi, P. Viens, J.P. Borg, *Oncogene* 25 (2006) 981.
- [86] A.J. Cheng, L.C. Chen, K.Y. Chien, Y.J. Chen, J.T. Chang, H.M. Wang, C.T. Liao, I.H. Chen, *Clin. Chem.* 51 (2005) 2236.
- [87] S.A. Gerber, J. Rush, O. Stemman, M.W. Kirschner, S.P. Gygi, *Proc. Natl. Acad. Sci. U.S.A.* 100 (2003) 6940.
- [88] D.R. Barnidge, G.D. Hall, J.L. Stocker, D.C. Muddiman, *J. Proteome Res.* 3 (2004) 658.
- [89] N.L. Anderson, N.G. Anderson, L.R. Haines, D.B. Hardie, R.W. Olafson, T.W. Pearson, *J. Proteome Res.* 3 (2004) 235.
- [90] Y. Lu, P. Bottari, R. Aebersold, F. Turecek, M.H. Gelb, *Methods Mol. Biol.* 359 (2007) 159.
- [91] D.S. Kirkpatrick, S.A. Gerber, S.P. Gygi, *Methods* 35 (2005) 265.
- [92] L. Anderson, C.L. Hunter, *Mol. Cell. Proteomics* 5 (2006) 573.
- [93] D.R. Barnidge, M.K. Goodmanson, G.G. Klee, D.C. Muddiman, *J. Proteome Res.* 3 (2004) 644.
- [94] B. Schweitzer, S. Roberts, B. Grimwade, W. Shao, M. Wang, Q. Fu, Q. Shu, I. Laroche, Z. Zhou, V.T. Tchernev, J. Christiansen, M. Velleca, S.F. Kingsmore, *Nat. Biotechnol.* 20 (2002) 359.
- [95] G.S. Omenn, P. Ping, *Expert Rev. Proteomics* 2 (2005) 9.
- [96] D.J. States, G.S. Omenn, T.W. Blackwell, D. Fermin, J. Eng, D.W. Speicher, S.M. Hanash, *Nat. Biotechnol.* 24 (2006) 333.
- [97] J.D. Jaffe, D.R. Mani, K.C. Leptos, G.M. Church, M.A. Gillette, S.A. Carr, *Mol. Cell. Proteomics* 5 (2006) 1927.
- [98] X.J. Li, E.C. Yi, C.J. Kemp, H. Zhang, R. Aebersold, *Mol. Cell. Proteomics* 4 (2005) 1328.
- [99] H. Zhang, E.C. Yi, X.J. Li, P. Mallick, K.S. Kelly-Spratt, C.D. Masselon, D.G. Camp, R.D. 2nd, C.J. Smith, R. Kemp, Aebersold, *Mol. Cell. Proteomics* 4 (2005) 144.
- [100] J. Listgarten, A. Emili, *Mol. Cell. Proteomics* 4 (2005) 419.
- [101] W.J. Qian, J.M. Jacobs, T. Liu, D.G. Camp, R.D. 2nd, Smith, *Mol. Cell. Proteomics* 5 (2006) 1727.
- [102] E. Marrer, F. Dieterle, *Chem. Biol. Drug Des.* 69 (2007) 381.
- [103] R.D. Unwin, C.A. Evans, A.D. Whetton, *Trends Biochem. Sci.* 31 (2006) 473.
- [104] A. Wolf-Yadlin, S. Hautaniemi, D.A. Lauffenburger, F.M. White, *Proc Natl Acad Sci U S A* 14 (2007) 5860.