Review

Multiple reaction monitoring for quantitative biomarker analysis in proteomics and metabolomics ☆

Neil R. Kitteringham [a,*,1], Rosalind E. Jenkins [a,1], Catherine S. Lane [b], Victoria L. Elliott [a], B. Kevin Park [a]

[a] MRC Centre for Drug Safety Science, Department of Pharmacology, University of Liverpool,
The Sherrington Building, Ashton Street, Liverpool, UK
[b] Applied Biosystems, Lingley House, Birchwood Boulevard, Warrington, UK

ARTICLE INFO

ABSTRACT

The conventional pipeline for biomarker development involves a discovery phase, typically conducted by mass spectrometry (MS), followed by validation and clinical application, usually on an alternative platform, such as immunoassay. Whilst this approach is suitable for the development of single biomarkers, with the current drive towards larger panels of multiplexed biomarkers, the process becomes inefficient and costly. Consequently, the emphasis is now shifting towards performing full biomarker discovery, qualification and quantification on the same technology platform. The ease of multiplexing and ability to determine protein modifications makes MS an attractive alternative to antibody-based technologies. In addition, developments in quantitative MS, through the application of stable isotope labelling and scanning techniques, such as multiple reaction monitoring (MRM), have greatly enhanced both the specificity and sensitivity of MS-based assays to the point that they can rival immunoassay for some analytes. This review focuses on the application of MRM for quantitative MS analysis, particularly with respect to proteins and peptides.

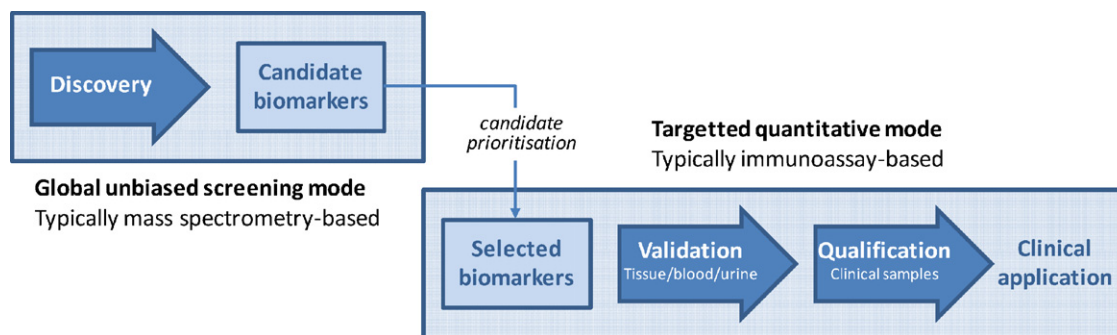© 2008 Elsevier B.V. All rights reserved.

Contents

Fig. 1. The conventional biomarker development pipeline, indicating the two principal stages of the development process – *discovery* and *validation/qualification* – prior to clinical application. Traditionally, the initial discovery phase is conducted on a mass spectrometry-based platform, whilst validation and ultimate clinical application generally involves the development of an antibody-based protocol, such as ELISA.

1. Introduction

The identification of biomarkers for diagnosis, prognosis, therapy monitoring and toxicity is a major goal of biomedicine in the 21st century [1–8]. The emergence of the 'omics technologies has brought this goal closer in the last 20 years but there is no doubt that, despite rapid technological advances, there remain huge challenges. Patient heterogeneity, variation during sample acquisition and storage, sample stability, dynamic range of the analytes, modification status of analytes, macromolecular interactions and other factors all profoundly influence the observed qualitative and quantitative characteristics of a potential biomarker. Currently, the most commonly applied pipeline for biomarker development involves a global discovery phase on small numbers of samples followed by validation of the potential biomarker with large numbers of patient samples, before it is eventually adopted as a clinical tool (Fig. 1).

Discovery usually involves the application of genomic [9–11], proteomic [1,4,7] and/or metabolomic [12–14] approaches, with validation by an alternative analytical methodology, such as immunoassay. Clinical application of the biomarker almost invariably utilises an antibody-based diagnostic approach such as ELISA or dipstick. Whilst this process has the potential to deliver clinically important markers, particularly within the cancer field, it may not represent the most efficient process since it requires the independent and sequential development of at least two analytical methodologies. Furthermore, the "low hanging fruit" biomarkers, those that are abundant, easy to detect and easy to quantify, have already been characterised and there is an increasing emphasis on multiple biomarkers being more specific and diagnostic for a given clinical condition [3,15–20]. This means that there will be a requirement for multiplexed patient screens with all the associated problems of cost and antibody production.

For these reasons, attention is turning increasingly to the possibility of conducting both biomarker discovery and validation on the same platform, thereby obviating the need for parallel assay development. The main obstacle to this approach is that the discovery phase is most commonly undertaken by either mass spectrometry (MS) or nuclear magnetic resonance (NMR), neither of which is ideally suited to high throughput quantitative analysis. In MS, this problem is being addressed by the application of *stable isotope* techniques which allow relative quantification between differentially labelled samples or, through the inclusion of internal labelled standards, absolute quantification [21–27]. These approaches provide the opportunity not only to measure protein levels but also to quantify specific protein post-translational modifications (PTM) [28–36]. We and other groups have also exploited these approaches in order to quantify non-physiological modification of proteins, for example by drugs or their metabolites, which may provide toxicological alerts during pre-clinical pharmaceutical safety testing [37–40]. These techniques are proving to be very useful for the discovery of potential biomarkers, but in order for the target to be detected with high specificity and sensitivity in clinical samples that exhibit a high degree of matrix noise, more sophisticated MS approaches are now being considered.

Table 1 summarises the approach and the technology required during the different phases of biomarker development using MS as the principal platform technology. The discovery phase is already very well catered for with numerous high speed and high resolution MS models being available. For validation of potential biomarkers in clinical samples, the MS-based technique of multiple reaction monitoring (MRM) is now being explored. MRM is not a new technique: it has been employed by small molecule mass spectrometrists for over 30 years and is widely used in drug metabolism studies for quantification of metabolites [41–46]. However, the application

Table 1
The biomarker development pathway, indicating the nature of the analysis in the three phases of discovery, validation and clinical application.

	Discovery phase	Validation/qualification phase	Clinical application
Type of analysis	Global	Specific	Specific
Goal	Maximum number of quality IDs	Accurate quantification of candidate biomarkers	
Sample number	Low	Medium	High
MS platforms	MALDI	Triple quadrupole	Triple quadrupole
	Q-ToF	Trip quadrupole/ion trap	Ion trap
	SELDI		Trip quadrupole/ion trap
	Ion trap		
	Orbitrap/FT-ICR		
Desired properties	High resolution	High sensitivity	High throughput
	Short duty cycle		

Mass spectrometry platforms applicable to each phase of the process are indicated.

of MRM to protein and peptide analysis has only been adopted recently because of the emergence of new MS instrumentation. The low mass range of many instruments used for metabolite identification precluded their use for MRM analysis for all but the smallest peptides, and it is only through the introduction of triple quadrupole instruments (QqQ) with extended mass ranges that MRM has become available to the protein mass spectrometrist. This review will describe the rudiments of the MRM approach, and will highlight the utility and power of MRM as a quantitative tool for biomarker analysis in the fields of metabolomics and, particularly, proteomics.

1.1. What is multiple reaction monitoring?

Molecules that have undergone a thorough characterisation during the discovery phase may be detected with enhanced specificity and sensitivity using *ion monitoring* techniques such as selective ion monitoring: during this process, the MS analysis time is focussed only on analytes of specific masses, while all others are excluded. Even greater specificity may be achieved by fragmenting the analyte, and monitoring both parent and one or more product ions simultaneously. This *reaction* must be defined (or predicted) to allow selection of the appropriate parent/product ion pairs, or *transitions*, for the analyte of interest. This process, known as *single (SRM)* or *multiple reaction monitoring* where several target ions are screened, is not the only approach suitable for quantification of known analytes, but it is one of the most sensitive [47] (Fig. 2).

2. MS platforms amenable to MRM analysis

The essential requirement for MRM is the ability to detect a specific precursor ion, to isolate that ion for collision-induced fragmentation and, finally, to detect a specific product ion following fragmentation. Thus, only instruments compatible with a sequential workflow are capable of undertaking a true MRM experiment. Any QqQ instrument is capable of MRM analysis, but selection of the optimal platform will be dependent on the nature and quantity of analytes under investigation. Probably the largest discriminator in choosing a QqQ system is whether or not a confirmatory, full-scan MS/MS spectrum of the compound of interest is required. Quadrupole mass analysers are at their most sensitive when they are transmitting a single *m/z* value. If they are used for full-scan analysis, their sensitivity is greatly compromised because most of the ions generated from the source are wasted; the sensitivity difference between MRM and full-scan MS/MS can be orders of magnitude. In addition, the time taken for a quadrupole to scan over a range of *m/z* suitable for a full-scan MS/MS spectrum is generally not compatible with a chromatographic timescale.

For traditional low molecular weight (LMW) quantitative analysis, MRM performed on triple quadrupole mass spectrometers is the industry standard [48]. The diverse range of fragmentation mechanisms for LMW compounds, coupled with the ready availability of standards means that there is usually no need for confirmatory MS/MS of the precursor ion. However, for proteomic and metabolomic workflows the situation is different. The increasing sample complexity associated with these analyses introduces an ever greater chance of 'false positive' transitions being registered during the MRM run. Thus, it is important, particularly in the development stage of the analysis, to be able to confirm the identity of the precursor ion giving rise to a specific transition. This step can also assist in selecting the most intense fragments for MRM design, where no standard compounds are available. In such cases, a triple quadrupole-linear ion trap hybrid instrument (QqQ-LIT) such as the

Q TRAP® [49,50] has advantages. Ion traps are capable of very high efficiencies because the time to fill the trap and generate a complete mass spectrum can be very short. A Q TRAP® instrument is able to switch rapidly between triple quadrupole and linear ion trap modes in order to acquire linear ion trap full-scan MS/MS confirmatory data of sensitivity equal to the MRM itself, and on a timescale suited to on-line chromatography. This last type of approach, in which the detection of a specific MRM transition automatically triggers a full product ion scan, has been referred to as MRM-initiated detection and sequencing (MIDAS) (Fig. 3) [51].

This approach has proved particularly successful for protein modification profiling (see Section 4.4). Nevertheless, high-end triple quadrupole instruments that lack the ion trap functionality can be used in conjunction with other instruments in a two-step procedure, whereby a sample is analysed first by MRM (typically utilising multiple MRMs per peptide) and, in a second analysis, on a discovery-based instrument to obtain confirmatory MS/MS data.

Where a large number of MRMs are required, a second discriminator in the selection of a triple quadrupole system will be the ability of the QqQ to perform the necessary number of MRMs on a chromatographic timescale, without loss of sensitivity. The generation of multiply charged peptide ions increases the requirement for multiple transitions in order to quantify the precursor ion accurately. In addition, there is a need to monitor at least two to three peptides per protein in order to be confident that peptide abundance accurately reflects protein abundance. Consequently, hundreds of transitions may be required in a MRM assay in order to cover the necessary peptides and ionisation states for all proteins of interest. It is therefore an advantage to maximise the number of transitions that can be monitored simultaneously within an acceptable cycle time and without loss of sensitivity. The ability to schedule individual MRMs within the chromatographic gradient enables the MS to monitor each transition within its own predefined temporal window. This allows a much greater number of transitions to be measured over the entire analysis without compromising either the sensitivity of detection or the number of data points across the chromatographic peak.

3. Use of multiple reaction monitoring for the analysis of small molecules

MRM has been used as a quantitative technique for analysis of LMW chemicals for over 30 years. The first use of the term MRM appeared in a 1978 publication describing chlorine isotope effects [42], however the technique itself had already been applied a year earlier by Baty and Robinson to monitor the plasma levels of the drug phenytoin and its metabolites [41]. Since then, through ever improving triple quadrupole technology, MRM has become the method of choice for low molecular weight chemical analysis. It is a particularly powerful technique for the analysis of complex drug metabolic pathways where both the parent drug and multiple metabolites can be monitored simultaneously with high sensitivity and precision. Numerous examples of the application of MRM for the analysis of low molecular weight chemicals with biological relevance exist in the literature and these span a broad range of analytes including endogenous compounds, therapeutic agents and their metabolites, environmental toxicants and compounds of abuse or malicious intent. Recent examples of the use of MRM for analysing endogenous compounds in humans include the measurement of vitamins [52], steroids [53–55] and neurotransmitters [56,57]. MRM analyses of drugs and their metabolites are too numerous to list comprehensively, but include the measurement of therapeutic agents, such as warfarin [58], triazolam [59], nevirapine [60] and antibiotics [61], the measurement of drugs of
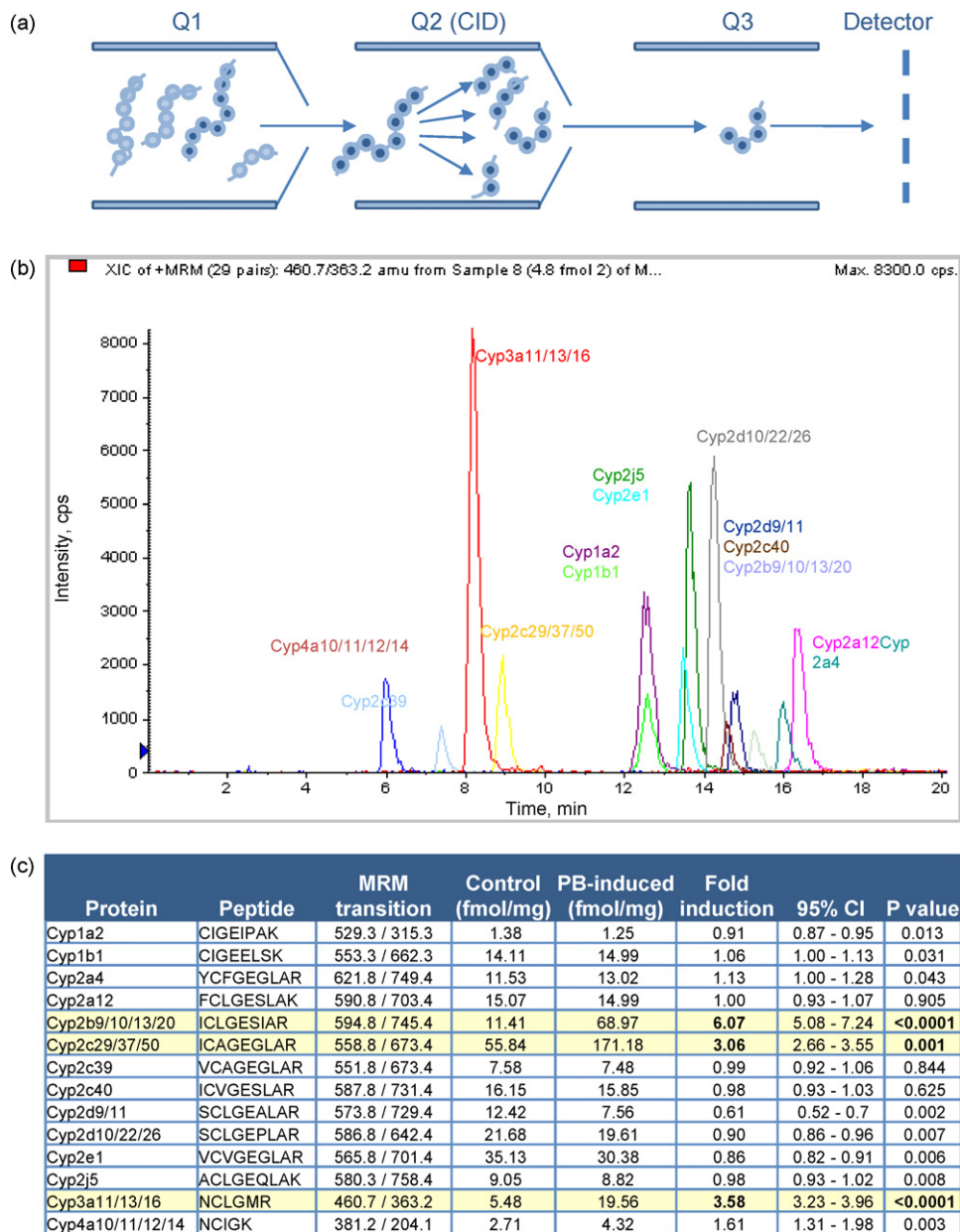
Fig. 2. (a) Schematic of the multiple reaction monitoring (MRM) scanning technique on a triple quadrupole mass spectrometer. The targeted parent ion is selected in the first quadrupole (Q1) and enters the second quadrupole (Q2) where it undergoes collision-induced dissociation. One or more fragment ions are then selected according to the predefined transitions and the ensuing signal provides the spectral counts for quantification. Where more than one transition is selected for a given precursor ion, the accumulative counts are used for quantification. (b) MRM MS trace for 14 different analytes measured simultaneously in the same run. The trace represents an overlay of the MRM spectra for synthetic peptides representative of 14 cytochrome P450 isoforms. In each case the peptides were labelled with light ICAT reagent to allow them to be used as spiked internal standards for the absolute quantification of cytochromes P450 in mouse liver microsomes. A rapid 10 min gradient from 2 to 35% acetonitrile was used to separate the 4.8 fmol of each P450 peptide. Notice that the MRM response varies for the different peptide sequences, reflecting differences in ionisation of the individual analytes. (c) Comparison of the cytochrome P450 profiles of liver microsomes prepared from control mice and from mice pre-treated with phenobarbitone (PB) for 3 days prior to removal of the liver. For each peptide the sequence and MRM transition ion masses used is given along with the absolute amounts found in control and PB-induced microsomes. Cytochromes P450 that were significantly induced by PB treatment are indicated by the *P*-values and yellow shading. Values are means of four individual animals in each case. All data obtained from reference [21]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

abuse, such as heroin [62,63], cocaine [64,65] and cannabinoids [64,66], and the assay of performance enhancers, such as androgens [67] and other stimulants [68]. In addition, MRM has been utilised for the analysis of low molecular weight chemicals present in plants [69,70], fish [71] and contaminated water courses [72], demonstrating the versatility and breadth of application of the technique for the routine quantification of low molecular weight products.

3.1. MRM and metabolomics

The term *metabolomics* (often used interchangeably with *metabonomics*[2]) was initially coined by Oliver et al. [74] by analogy

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[2] There is some disagreement among workers in the field as to whether the terms metabolomics and metabonomics are interchangeable or, in fact, have subtly differ-
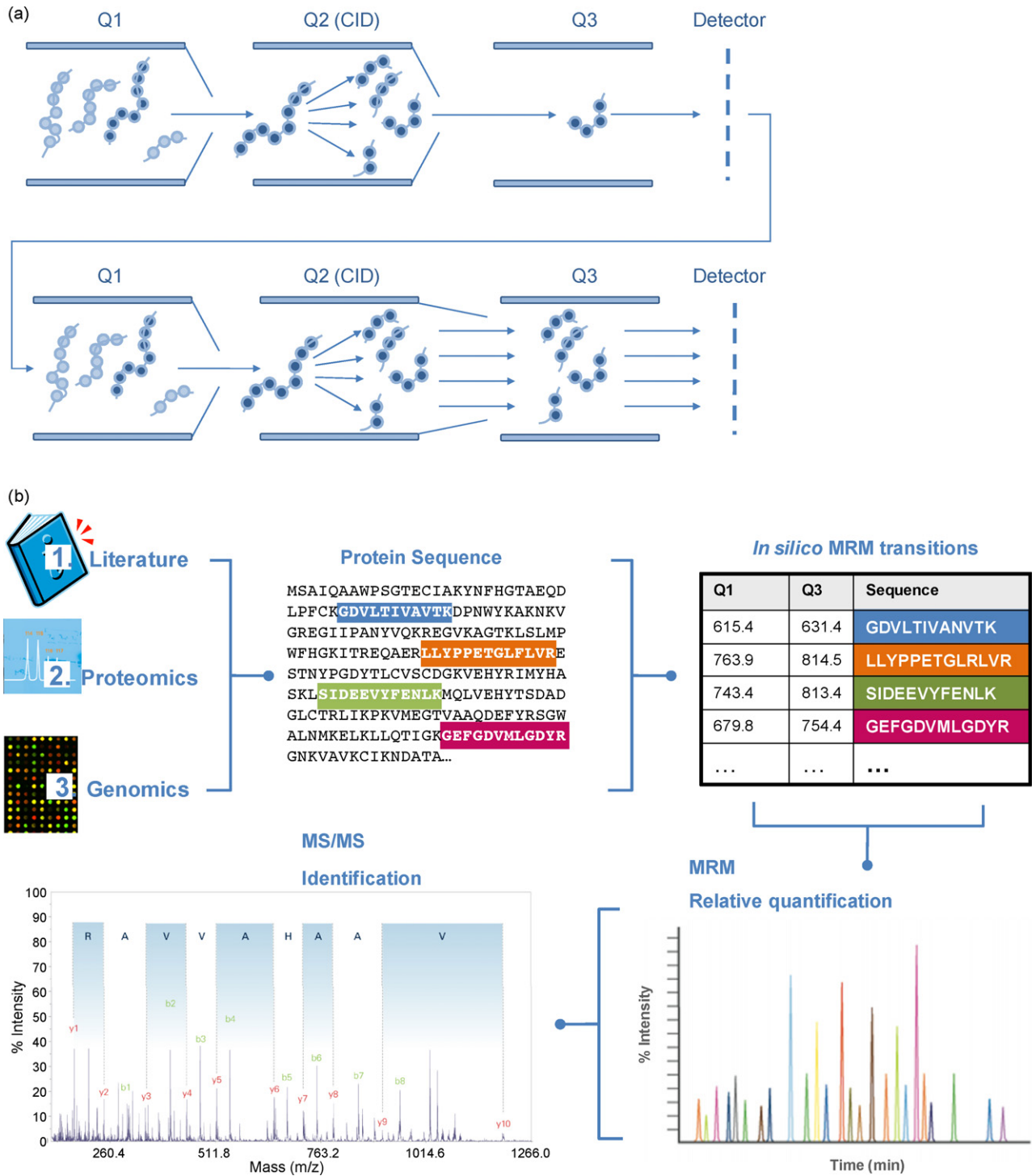
Fig. 3. (a) Schematic of the MIDAS (MRM-initiated acquisition and sequencing) scanning protocol for peptide quantification and identification on a triple quadrupole-ion trap mass spectrometer. The first stage is identical to the standard MRM experiment described in Fig. 2, however, in the MIDAS protocol, the detection of a predefined transition then triggers the MS to perform a full-scan precursor ion MS/MS in order to confirm the identity of the parent ion. (b) Overview of a MIDAS experiment indicating the use of various information sources to design peptide transitions and the combined quantification and identification stages of the procedure.

to the global analysis of genes (genomics) or proteins (proteomics). Hence, metabolomics refers to the global analysis of metabolites

ent meanings. The term metabonomics was first introduced by Nicholson et al. [73] and has become associated with NMR-based and primarily human metabolic profiling, whereas the term metabolomic is generally associated with MS-based analysis and is broader ranging with respect to species.

produced by the actions of biochemical processes. Despite the extensive use of MRM for the analysis of small molecules, including both endogenous and xenobiotic metabolites, the adoption of this technique within metabolomic studies is limited. This reflects the complexity of the metabolome, estimated to comprise over 7000 molecular species in humans [75], such that a comprehensive global approach to metabolomics would be outside the scope of targeted MS approaches. Adoption of a less rigid definition

of metabolomics, however, to include targeted screening of *postulated* metabolites or where a range of compounds displaying a common structural motif might represent a selective metabolomic profile, allows various types of selected ion monitoring techniques to be utilised in a discovery model to screen simultaneously for a large number of metabolites. For example, both endogenous and exogenous molecules undergo biotransformation in the liver which may be predicted to involve various common conjugation reactions, such as glucuronidation, sulfation and glutathione conjugate formation. MRM approaches have been used to screen human urine for glucuronide metabolites as potential surrogate biomarkers for human genetic variation. Lutz et al. [76] administered a low dose of dextromethorphan to volunteers with known cyp2D6 polymorphism status. Through quantitative analysis of urinary dextromethorphan–glucuronide conjugates followed by principal component analysis of the data, it was possible to differentiate "slow" and "rapid" metabolizers among the volunteers and, in addition, the study revealed previously unidentified metabolic pathways. An *in vitro* method for predicted metabolite screening in rat liver microsomes, suitable for early stage preclinical drug metabolic stability testing, was developed by Shou et al. [77] using MRM-triggered information-dependent acquisition (IDA) on a QqQ-ion trap instrument. Using model drug compounds bufuralol, propranolol, imipramine, midazolam, verapamil and diclofenac, the authors were able to show that the MRM-triggered IDA approach was as sensitive as a standard MRM experiment but afforded the advantage of simultaneous metabolite identification within the same chromatographic run. Metabolic stability is an important factor in the chemical design of potential new drug candidates and a rapid screening procedure will allow "go/no go" decisions to be made early in the discovery programme [78]. Similarly, Gao et al. [44] defined a methodology for rapid metabolite profiling in a hepatocyte model using MRM-triggered IDA on a QqQ-ion trap mass spectrometer. Hepatocytes have the advantage over microsomes of containing a full complement of drug metabolizing enzymes and, therefore, should provide a metabolic profile more relevant to the *in vivo* situation. Using 48 transitions to screen for common phase I and phase II metabolites and then enhanced-product ion scans to confirm metabolite identification, the authors were able to compare the metabolic profiles of a probe compound across hepatocytes derived from rodent, dog, monkey and human liver. Li et al. [79] employed a method incorporating both conventional MRM acquisition of parent compound and the MRM-triggered IDA of potential metabolites within the same scan cycle during the same LC–MS/MS run in order to quantify *in vivo* levels of both parent drug and metabolites in primate blood samples.

The products of most phase II (conjugative) metabolic biotransformations are likely to fragment to generate an identical product, which can be used to perform constant neutral loss or precursor ion scanning experiments resulting in the identification and quantification of the parent ion. An essential feature of MRM is that both the parent ion and one or more transitional product masses are known. This precludes its use in screening for completely unknown metabolites, however by utilising data obtained from constant neutral loss experiments "theoretical" MRM transitions may be constructed allowing the simultaneous analysis of several hundred compounds in a biological matrix. This approach has been adopted to screen for glutathione conjugates or their related mercapturic acid derivatives of both naturally occurring products and drugs in rat blood and urine [80,81]. Mercapturic acids are secondary products of glutathione conjugation and are regarded as potential urinary biomarkers of exposure to toxic metabolites, since chemically reactive species such as electrophiles and free radicals are generally eliminated by this pathway [82]. Most drugs associated with adverse drug reactions can form chemically reactive metabolites [83,84] and consequently are likely to be eliminated, at least partially, as glutathione-derived metabolites. Using a "training set" of 16 synthetic mercapturates (*N*-acetylcysteine thioethers), Scholz et al. [80] compared with the sensitivity of constant neutral loss and theoretical MRM techniques for analysis of rat urine. Both methods conducted on a 4000 Q TRAP instrument were highly sensitive (limits of detection 60–1630 pmol/ml depending on the compounds) and, when coupled to an IDA scan, were capable of providing confirmatory product identification. Similarly, Mueller et al. [85] have succeeded in developing a screening procedure for over 300 commonly prescribed drugs that are likely to be found in either accidental clinical or forensic intoxication cases. Once again, the use of a QqQ-ion trap instrument allowed both quantitative analysis and identification simultaneously within the same analytical run, providing a significant efficiency gain when compared with a previous study by the same group [86] on a standard QqQ instrument in which 238 drugs were quantified simultaneously, but identification of the compounds relied solely on the transition ions monitored.

These examples of multi-analyte screening procedures indicate that, although MRM would not be applicable as a primary screening tool for metabolomic studies, developments in triple quadrupole technology and of hybrid instruments such as quadrupole-ion traps, make possible the simultaneous analysis of several hundred metabolites of endogenous or exogenous origin and provides the technology for targeted metabolomic studies following the initial discovery driven phase.

4. MRM for proteomic analysis

4.1. Selection of MRMs

There are a number of criteria to consider when selecting the optimal transitions for MRM analysis [21,87]. The target peptides should not exhibit any enzyme missed cleavage sites, nor should they be susceptible to post-translational modification, unless it is the purpose of the assay to quantify PTMs. They should be of a size to be accommodated by the mass range of the QqQ instrument, in the range of 7–30 amino acids, and they should uniquely identify the protein of interest. Each MRM transition requires an optimised set of MS parameters for maximum sensitivity. Most studies use at least two peptides per protein and up to two different charge states for each of the parent ions in combination with two different fragment ions for each peptide: this means that each protein quantification will be based on at least eight MRM transitions.

However, designing and validating hundreds of individual peptide transitions for the quantitative analysis of complex samples is a major bottleneck in the MS biomarker pipeline. *In silico* methods are available, and continue to be refined, which facilitate the process. These employ fairly sophisticated algorithms to deliver putative MRM transitions based on a combination of theoretical rules and empirical observation for optimal peptide MS/MS. Anderson and Hunter used computational methods alone to derive MRMs for 30 plasma proteins and found these to be reasonably reliable for the analysis of spiked peptide standards in a human plasma digest [47]. However, only 11 of the 30 were detectable as native proteins within the same plasma digest, indicating the limitations of *in silico* methods alone for analysis of complex samples. Thus, there is as yet no computer-based method that can entirely replace 'real-life' data when it comes to generating reliable MRM transitions, and for that reason Jaffe et al. [88] have derived an 'MRM pipeline' referred to as Accurate Mass Inclusion Screening (AIMS). The purpose of AIMS is to facilitate the configuration of sensitive and accurate MRM assays guaranteed to detect native proteins in biological fluids. The AIMS

protocol involves the compilation of a comprehensive list of candidate biomarkers based on data derived from typical discovery mode LC–MS/MS peptide analyses (or other sources of candidate proteins, e.g. literature, gene array, etc.) followed by targeted confirmatory MS/MS. This intermediate verification stage is essential to eliminate false positives and to ensure that the potential biomarkers are indeed visible within the biological fluid most likely to be analysed in the clinical setting. The verification stage involves the construction of an inclusion list that instructs the mass spectrometer to concentrate only on peptides that originate in one of the targeted proteins. In this way, relatively high sensitivity may be achieved on instruments primarily designed to deliver high mass accuracy, since analysis time is not wasted on irrelevant high abundance ions. The high resolution achievable in such instruments is, however, critical to the accurate detection of the postulated peptide analytes, and provides the confidence and MS information required to proceed to the MRM phase of analysis. Software packages capable of using such data iteratively to select the most appropriate MRMs, such as MRMPilot (Applied Biosystems), QuanOptimise (Waters), SRM Workflow (Thermo-Fisher) and MassHunter Optimiser (Agilent), are just becoming commercially available and should rapidly increase the uptake of this approach as a routine quantitative tool in proteomics.

4.2. Plasma biomarkers

There are now several examples of extensive MRM studies conducted on samples from both human and other species. The "Holy Grail" of biomarker discovery is to identify diagnostic compounds that can be detected and quantified in human plasma or urine. Whilst this is readily achievable for low molecular weight markers, it provides a major challenge when looking for proteins or peptides. It has been estimated that human plasma contains proteins that span 10 orders of magnitude of concentration [89], and includes resident plasma proteins (high and medium abundance) and signalling proteins such as hormones and cytokines (low abundance). High abundance species comprise a small population of approximately 20 proteins, including albumin, haptoglobin, hemopexin and the immunoglobulins, which between them account for 99% of the total protein content [89]. These are resident members of the blood and, as such, are unlikely to provide informative biomarkers *per se* (although albumin has been suggested to represent a potential repository of information since it binds multiple proteins and peptides derived from other tissues [90]). Tissue leakage products are the most likely source of potential biomarkers, but they are present at vanishingly low levels: the exquisite sensitivity of MRM-MS, which can on occasion rival antibody-based analysis, provides the most promising currently available technology to detect these low level protein markers in blood.

Some early demonstrations of the proof of principle for MRM-based quantification of proteins in biological matrices include the measurement in serum of C-reactive protein [91] and prostate specific antigen (PSA) [92]. The former is a plasma protein that may be detected at basal levels (0.25 μg/ml, 9.6 nM) in healthy plasma but is very readily quantified in plasma from patients with non-erosive (∼1.2 μg/ml, 46.2 nM) or erosive (∼20.1 μg/ml, 0.77 μM) rheumatoid arthritis [91]. In the second study, PSA spiked into unfractionated female serum was detected at a lower limit of 4.5 μg/ml (1.6 μM) using isotopically-labelled internal standards for four tryptic peptides [92]. However, as emphasized by the authors, the elevated levels of PSA routinely detected in prostate cancer patients by immunoassay are only 10 ng/ml (3.6 nM).

The intermediate abundance proteins may provide a far richer vein of informative markers since their levels may fluctuate dramatically in response to a given stimulus and their expression

levels are well within the limit of detection of the modern MS. In a seminal study by Anderson and Hunter, MRM experiments were designed for 53 of the most common high and medium abundance plasma proteins, using a variety of *in silico* and information-directed methods to derive the transitions, and 47 of these proteins were successfully detected with intra-assay variability (CV < 25%) [47]. Interestingly, depletion of the six most abundant plasma proteins using a multiple affinity removal system (MARS, Agilent) failed to increase the number of peptide transitions detected, but did improve the CVs associated with multiple analyses. The proteins selected for this study spanned a range of approximately four orders of magnitude with albumin representing the most abundant protein (∼50 mg/ml) and L-selectin the least abundant (∼1 μg/ml), indicating the applicability of the method for analysing poorly represented proteins in a highly complex biological matrix. However, as pointed out by the authors, the variability associated with repeated quantification of lower abundance proteins was much greater than for higher abundance analytes. Poor reproducibility (e.g. CV consistently >10%) would compromise the applicability of MRM-based methods for robust routine measurement which would be required for a clinical diagnostic screen. In order to enhance the robustness of the method, stable isotope-labelled peptides for 30 of the selected proteins were synthesised as a single concatenated gene product, trypsin digested and spiked into plasma samples to provide internal standards, thereby allowing quantification at the absolute level [47]. It remains to be seen whether the promise of the approach indicated by these relatively high abundance proteins can be fulfilled when the MRM approach is applied in a similar way to low or trace abundance analytes.

There are very few publications describing the use of MRM for detection of low abundance human plasma biomarkers, and they are mostly demonstration studies. Keshishian et al. spiked six proteins into immunodepleted human plasma and showed that they could be detected at levels of 1–10 ng/ml and over two orders of magnitude [93] using MRM and isotope-labelled standards. Similarly, Stahl-Zeng et al. spiked recombinant transmembrane protein 27 (TMEM27) into whole human serum and, following isolation of the glycosylated proteins, showed that they could detect the protein at a level of 0.1 ng/ml [94]. Several groups have come close to identifying true plasma biomarkers of disease using a stepwise approach: first, tissue proteins over- or under-expressed in cancer tissue were identified; the altered expression levels in tissue were confirmed by ELISA and/or MRM-MS with and without isotopically-labelled standards; and the potential biomarkers were then detected in serum by affinity enrichment and MRM-MS [95,96]. These studies proposed fibulin-2 as a marker for breast cancer in a mouse model of the disease [95] and confirmed the utility of carcinoembryonic antigen as a marker of lung cancer [96].

4.3. Tissue biomarkers

Although the chief focus of attention for quantitative analysis of candidate biomarkers has been the accessible human biological fluids, such as blood and urine, these are not the exclusive source of valuable markers. Indeed, the poor rate of biomarker discovery using plasma as the target proteome has prompted many to pursue other sources of material, such as tissue extracts, subcellular fractions, interstitial fluids, bile and cell secretomes. Biomarkers are also required to investigate animal models of disease or for pre-clinical drug safety testing and, in this case, a wide variety of biological fluids or tissue samples may provide the origin of the protein markers. An early example of the use of absolute MRM quantification to define expression changes in a family of liver proteins was a study carried out on cytochrome P450 enzymes (CYPs)

in mice (see Fig. 2b and c). CYPs represent a large multigene super-family of enzymes responsible for the phase I metabolism of most therapeutic drugs. Although over 150 different isoforms exist in both rodents and humans, those in families 1–4 account for the metabolism of the vast majority of xenobiotics. A feature of most CYPs is the fact that their expression can be highly induced by a wide range of xenobiotics. They are also responsible for the forma-tion of most drug-derived chemically reactive metabolites. Whilst CYPs do not conform to the classical definition of a biomarker, induction of CYPs by new drug candidates is routinely monitored during pre-clinical safety assessment within the pharmaceutical industry, because of the risk of initiating adverse drug reactions [97]. Diagnostic MRM transitions were designed for a set of 16 major isoforms utilising a single tryptic peptide for each [21], and standard peptides were chemically synthesised and labelled with light ICAT reagent. Microsomes were isolated from mice exposed to different inducing agents and were labelled with heavy ICAT reagent. This enabled all 16 CYPs to be identified and quantified in a single LC–MRM-MS run, providing one of the first definitive assessments of the absolute levels of CYPs in mouse liver. More-over, it was found to be more sensitive for some isoforms than the traditional immunoassays used for semi-quantitative induction studies as well as providing a level of isoform discrimination not achievable with commercially available antibodies [21]. Although the number of publications utilising MRM for the quantification of tissue proteins is small (see for example [98–100]), this number is likely to increase rapidly as the power of the approach becomes more widely acknowledged. Clearly, tissues represent more het-erogeneous and dynamic sources of biomarkers than plasma or urine, since infiltration by secondary cell types, e.g. macrophages or lymphocytes may alter the proteomic profile substantially, and this must be taken into consideration when interpreting the relevance of novel biomarkers. However, when used as a preliminary source of candidate biomarkers which are subsequently validated in an alternative compartment, this may not be a complication. If, alter-natively, the intention is to develop a diagnostic tissue biomarker, then care needs to be taken to ensure that any qualitative or quan-titative changes truly reflect the causative pathology, rather than a secondary change in tissue composition.

4.4. MRM as a tool for protein modification profiling

Protein modifications, whether they are endogenous or exoge-nous, represent a major untapped source of information for understanding the fundamental biology of complex systems and for gaining a deeper insight into the pathogenesis of disease. In addition, they represent a huge repository of potential biomark-ers with the possibility of exquisite diagnostic specificity. As such, much effort has been expended by many groups to enhance the quantitative power of protein modification screening. Although phosphorylation has been the primary focus of attention, several groups are now attempting to devise methodologies for a wide range of other modifications, and frequently this involves the use of an MRM approach. Examples of the application of MRM-based mass spectrometry encompass assays for phosphorylation, acety-lation, glycosylation (please see below), nitrosylation/oxidation [101], ubiquitinylation [102] and folate modification [103], as well as applications involving the measurement of xenobiotic modifica-tions, such as drugs.

4.4.1. Phosphorylation
Phosphorylation has a predominant role in intracellular sig-nalling, which makes phosphoproteins attractive candidates for biomarkers of aberrant cellular behaviour. Discovery proteomics is the approach to exploit where novel phosphorylation on multiple

sites are the subject of investigation. The development of high reso-lution mass spectrometers providing rapid scan speeds has greatly facilitated the high throughput screening for PTMs, as exemplified by Olsen et al. who have successfully identified over 6000 phos-phorylation sites on 2244 different proteins [104]. However, where a single or small number of proteins are the focus of investigation, or if it is desirable to quantify specific modifications on previously characterised proteins, as is likely to be the case for the develop-ment and application of selective biomarkers, then a more targeted approach such as MRM may be preferable.

Unwin et al. focused on the phosphorylation profile of the yeast cell cycle protein Cyclin B, comparing a MIDAS-based approach with the standard precursor ion scanning method conducted on the same instrument [51]. The results showed a >10-fold enhancement in sensitivity, principally due to noise reduction, but in addition far fewer false positives were recorded due to the increased selectivity inherent to the MRM approach. Similarly, an MRM experiment was designed to monitor the phosphorylation of a transcription factor, myocyte enhancer factor 2A (MEF2A) that is involved in the devel-opment of cardiac and skeletal muscle cells [105]. This protein is under the regulation of a phosphorylation "switch" such that modi-fication at different sites will either enhance transcriptional activity or depress through targeting of MEF2A for degradation. Using *in silico* prediction alone, the authors were able to design 58 tran-sitions for potential serine/threonine or tyrosine phosphorylations and conducted an MRM-triggered MS/MS scan protocol (the MIDAS approach described above) on a hybrid QqQ-ion trap instrument. They were able to identify unambiguously six phosphorylated peptides, two more than were identified by a neutral loss exper-iment undertaken on the same sample. An MRM approach was also exploited to assay the phosphorylation sites on focal adhe-sion kinase (FAK), a 125 kDa enzyme involved in the regulation of integrin signalling and, consequently, in vascular wall development and repair [106]. FAK is subject to phosphorylation on multiple sites through the action of Src and through autophosphorylation, and this study aimed to identify and quantify these modifications as well as to differentiate between Src-mediated and FAK-mediated events. Eleven peptides containing a total of 17 potential phos-phorylation sites were selected and, on the basis of information obtained from MIDAS and precursor ion scanning experiments, 29 different transitions were designed to capture both phosphorylated and non-phosphorylated forms. Six previously uncharacterised phosphorylations were identified and, through differential profil-ing in the presence and absence of Src, it was possible to ascribe the origin of each one [106]. Collectively, these and other studies [107–110] attest to the sensitivity and selectivity of MRM as a pro-cedure for characterizing and quantifying phosphopeptides. Whilst most examples to date have been proof of principle studies, MRM-MS has the potential to provide a fully validated screen for routine monitoring of diagnostic phosphorylation events within a clinical context.

One of the main disadvantages of using MRM is the requirement for fairly simple mixtures so that sensitivity is not compromised, and therefore global analysis of the highly dynamic phosphopro-teome may not be feasible. However, Wolf-Yadlin et al. proposed a scheme [111] whereby cells stimulated with epidermal growth factor (EGF) are processed for iTRAQ labelling, the phosopeptides are isolated by immobilized metal ion affinity chromatography (IMAC) and the targets of interest are identified by discovery LC–MS. The discovery data were used to design the appropriate MRM transitions, taking into account the elution time of the phospho-peptide (scheduled MRM), the charge state of the precursor and the collision energy required for fragmentation. By using two dif-ferent transitions per peptide, the requirement for a full MS/MS scan to confirm identification was reduced. Finally, because the

samples were iTRAQ labelled, the assay could be multiplexed. This strategy enabled the reproducible quantification of 222 phosphopeptides across 7 time points following EGF-stimulation of human mammary epithelial cells in culture, suggesting that global phosphoprotein analysis is possible if an integrated approach is employed [111].

4.4.2. Acetylation

Although it has received far less attention than phosphorylation, acetylation is also a major regulatory modification: indeed its importance has been equated to that of phosphorylation [112] and it is possibly involved in as varied a range of cellular functions. Griffiths and co-workers applied a MIDAS-based approach similar to the one developed by the same group for phosphorylation analysis [51,113] to determine the sensitivity of detection of acetylated peptides derived from bovine serum albumin. Once again they found that the MRM method offered >10-fold enhanced sensitivity compared with precursor ion scanning for the immonium ion of acetyl lysine minus NH_3 (mass 126.1 Da). Detection was achieved within the low fmol (on-column) range and the utility of the application was confirmed by analysing naturally acetylated peptides derived from cytokeratin 8 isolated from a cell lysate by 2D-gel electrophoresis. Using a total of 84 MRM transitions for potential acetylation sites, the authors were able to identify five novel acetylated peptides [113], confirming that the method has the selectivity and sensitivity to identify naturally modified proteins present within a biological matrix.

4.4.3. Glycosylation

The mammalian glycome consists of up to several thousand glycan structures and may be larger even than the proteome [114]. Aberrations in the glycosylation machinery, both anabolic and catabolic, can have a profound effect on processes as diverse as cell adhesion, cell signalling and endocytosis [114] with congenital disorders of glycosylation (CDGs) affecting the central and peripheral nervous system, the endocrine system and coagulation. Preliminary evidence also suggests that alterations to the serum glycome may provide markers of cancer progression [115,116]. It is therefore clear that a full understanding of human diseases will require an in-depth and quantitative map of the glycome. Serum proteins are highly glycosylated, contributing greatly to the complexity of the 2D-PAGE map of blood proteins, and may provide a simple read-out of the human glycome in health and disease. In one study, MRM transitions were designed for the glycosylation sites on transferrin and α1-antitrypsin and appropriate isotopically-labelled standards were synthesised, revealing a correlation between the *N*-glycosylation site occupancy and the severity of disease in CDG patients [117]. Similarly, defects in the glycosylation of properdin were found by MRM-MS analysis to be associated with Peters Plus syndrome, confirming the condition as a newly defined CDG [118].

4.4.4. Non-physiological protein modifications

Phase I and phase II metabolism in humans can lead to the formation of metabolites that are capable of modifying cellular macromolecules such as proteins and DNA. These chemically reactive metabolites (CRMs) are negative indicators in the drug development pipeline as they can lead to the malfunction of critical cellular proteins, and result in organ toxicity or immune-mediated hypersensitivity reactions [119,120]. The pharmaceutical industry have adopted small molecule trapping agents such as glutathione to study adduct formation following bioactivation of new drugs [121], but a much more realistic indication of covalent modification may be achieved if modification of proteins can be measured. We utilised MRM-MS to analyse the modification of human glutathione-*S*-transferase pi with the CRM of paracetamol, and were able to show that adducts could be detected when the protein was exposed *in vitro* to a molar ratio of drug to protein of 1:10,000, comparable to *in vivo* levels of exposure [38]. Other studies using this approach for the detection of chemical adducts are as yet rare, but they include assessment of exposure to agents of biological warfare [122] and industrial chemicals [123], and mapping of the covalent modification of proteins with the lipid peroxidation product 4-hydroxy-2-nonenol [124].

5. Limitations of MRM for targeted protein quantification

Whilst MRM mass spectrometry shows considerable promise for protein quantification, the technique is still in its infancy for proteomic applications and many technological hurdles need to be overcome if the technique is to establish itself as the method of choice for high molecular weight analytes, as it has for low molecular weight species. The level of complexity of a tryptic digest of unfractionated plasma is several orders of magnitude greater than the equivalent sample typically analysed for metabolite quantification. The examples provided above clearly indicate the power of MRM methodology for sensitive quantification of high and intermediate abundance proteins within complex mixtures. What remains unclear is the potential for the MRM technique to determine trace peptides within a complex "sea" of high abundance interfering peptides. As set out by Keshishian et al. [93], the principal technological limitation for MRM of proteins in complex biofluids is the relatively low resolution of triple quadrupole mass spectrometers. With resolutions typically in the range of 1000–3000 there is a considerable potential for interfering ions being selected in either the Q1 or Q3 analysers since ions differing by as little as 2 Da would have overlapping isotopic distributions. A further confounding factor is in-source fragmentation of abundant peptides whereby fragment ions of such peptides, rather than the precursors themselves, represent a source of interference. In the longer term the utility of MRM within the field of routine clinical measurement of diagnostic disease biomarkers relies upon technological developments to enhance the mass accuracy of triple quadrupole, or equivalent, mass spectrometers and to overcome the issue of in-source fragmentation. It will also be necessary to develop existing separation technologies and strategically combine them in order to enrich the target analytes prior to quantification. Such techniques include multi-dimensional liquid chromatography (e.g. MudPIT) [125–127], affinity depletion/enrichment such as Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) [25,128,129], IMAC or titanium dioxide for isolation of phosphopeptides [130–134], combined fractional diagonal chromatography (COFRADIC) [135,136], and so on. Nonetheless, the prospects are good for the adoption of MRM-MS as an essential element in the quantitative proteomics toolbox.

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