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Review

Biomarker discovery from body fluids using mass spectrometry☆

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Abstract

Systems analysis of body fluids by mass spectrometry (MS) is an upcoming field of biomarker research. This approach is extremely attractive because it does not require biomarker candidates and the sample preparation is simple. However, during the development of the technique strong critical comments were made on the poor reproducibility, the special characteristics of blood as a source of peptides and on the frequent non-adequate statistical analysis of the data. Here we discuss the efforts made in the last few years to develop suitable protocols, which could lead to biomarker discovery from body fluids by mass spectrometry. Our review focuses on the systems analysis of non-digested blood serum or plasma samples by MALDI-TOF and SELDI-TOF.

Keywords: Biomarker; Mass spectrometry; Serum analysis; High throughput

Contents

1. Introduction

Early diagnosis and treatment result in the best prognosis of many diseases. However, despite the enormous effort invested during the last 20 years in the search of novel diagnostic techniques, the advances in the field have been modest. The development of new technologies in the last decade in the field of genomic and proteomic analysis has brought novel optimism to the search for improved biomarkers [1–6].

One of the novel strategies employed for the discovery of new biomarkers is the analysis of the peptides and proteins contained in plasma or blood serum by mass spectrometry (MS). Blood has two properties that make it attractive for the search of

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biomarkers. On one hand, it is more easily accessible than other body tissues. On the other hand, it perfuses all the other tissues of the body, so it carries not only "plasma-specific" proteins but also proteins derived from other tissues. Thus, the proteins circulating in blood may reflect the biological status of the body. In order to analyse the circulating proteins and peptides, the cellular components of blood are removed, either in the presence of anticoagulants, which yields plasma, or after blood coagulation, which yields serum [7,8].

The analysis of proteins and peptides contained in blood-derived samples by mass spectrometry was initially limited to the identification of spots in 2-DE gels [8]. In 2002 for the first time, the use of peak patterns of mass spectra from serum samples as a fingerprint that allows the distinction of cases and controls in ovarian cancer [9], prostate cancer [10,11] and breast cancer [12] was published. Serum samples were analysed with the SELDI-TOF technology. Briefly, samples are first incubated on chips whose surface is coated with a protein-fractionating resin (for instance C18 or strong cationic exchange). Then, the unbound compounds are washed away, the chips are overlaid with an energy-absorbing matrix and finally spectra are acquired by using laser ionization and TOF separation mass spectrometry [13]. The resulting peak intensities are supposed to correlate with the concentrations of diverse peptides in the blood plasma. The statistical analysis of the whole set of cases and controls uncovers those peaks whose intensities significantly differ between both sample populations. The same approach is also employed with conventional MALDI-TOF instruments. Here, the peptides and proteins are extracted from the sample before deposition on the target plate [14–21].

One of the major advantages of these procedures is that no pre-selection of biomarker candidates is required because many molecules (all in the range of detection) are screened in a single experiment. Moreover, in principle, no biological knowledge about the patho-physiology of the disease is required. From the experimental point of view, these methodologies are simple and can be easily automatized. Initially, the impossibility of the direct identification of the molecules causing the peaks was not considered a drawback, since the distinction between cases and controls is provided by the pattern itself, regardless the nature of the molecules.

In this review we will focus our attention in the analysis of serum and plasma samples by SELDI-TOF and MALDI-TOF without prior enzymatic digestion, independently of the molecular mass range analyzed. In the literature, the word "peptidomics" is employed to refer to the low-molecular-weight proteome (less than 15×10^3) [3,21].

2. The critics

Despite the initial optimism, several concerns arose regarding the use of the spectral patterns as biomarkers. Already in 2002 it was pointed out that the positive-predictive value of the obtained peptide patterns was insufficient to be used as an earlier marker of diseases with very low frequency in the population, such as ovarian cancer [22–24]. The possibility that these putative markers are not specific for the tumours but rather reflect epiphenomena accompanying them was also suggested [25]. In the following years, other aspects in the use of SELDI-TOF and mass spectrometry for the search of biomarkers in plasma/serum were heavily criticised. These critics can be grouped in three classes: questions regarding the experimental design and the statistical processing of the data; questions regarding the technical limitations of mass spectrometry and concerns with respect to the biological meaning of the obtained data.

2.1. Experimental design

The pitfalls found in the experimental design and in data mining were diverse. Most of the studies were based on the comparison of only two groups of samples: blood from individuals with a specific disease and blood from healthy individuals (case versus control studies). When the putative biomarkers found in some of these works were identified, it turned out that they represented proteins whose expression increased as a non-specific reaction of the organism. Frequently they belonged to the family of acute-phase response proteins, and were a side effect of multiple diseases rather than a marker for the disease itself [4,17]. Additionally, these studies often used inadequate algorithms to develop classifiers, which led in many cases to the overfitting of the classification model, i.e. to the finding of peak patterns that fit almost perfectly the data set used for building up the classifier but failed when applied to different data sets. This artefact appeared when the number of samples were too small relative to the high dimensionality of the data, and could be prevented by employing an adequate number of samples, overfitting-resistant algorithms and appropriate validation of the resultant model [4,26–30]. Another common mistake was the lack of quality control of the spectra: improper calibration of the spectrometers, failure to check the reproducibility of the spectra after changes in the machines or in the protocol, the inclusion of spectral regions with high noise into data mining, the non-alignment of the spectra before comparison, and others. These shortcomings lead to results that could not be reproduced, neither in different laboratories nor in the same laboratory [27,31–36]. Finally, the use of "features" (any m/z value) or peaks (local maxima in the spectra) for the classification of the samples is still a topic of discussion [6,29–31,37–40].

2.2. Mass spectrometry techniques

The ability of mass spectrometry to quantitatively assess alterations of the protein concentration was also questioned. The variables that affect the intensity of the signals are not completely understood. It is known that the compounds present in a sample may alter the ionization efficiency of a certain protein. Indeed, the differences in the composition of two samples may cause the signals derived from a certain molecule whose concentration is identical in both samples appear with different intensity [4,6,41]. Another important limitation of the use of MALDI-TOF and SELDI-TOF in this way is that the direct identification of the potential biomarkers is not possible. Thus non-informative signals (acute-phase response, diverse

artefacts, etc.) cannot be filtered out. Moreover, these non-informative signals may be identified as biomarkers by the classification algorithms employed to compare the spectra. To circumvent this problem, tandem mass spectrometry techniques and/or cumbersome purifications are required [16,17,21,42–45]. The SELDI-TOF technology was criticized in particular. The differences between the spectra obtained by using different batches of chips and the drift and the noise of the spectrometers contributed to the poor reproducibility of the results [13,26,46].

2.3. Physiologic and clinical relevance of the data obtained

Finally, the biological significance of the data generated by mass spectrometric analysis of blood serum or plasma was examined. It is well known that the concentration of the proteins present in the blood covers at least 10 orders of magnitude, ranging from albumin (35–50 mg/ml in serum) to IL6 (0–5 pg/ml in serum) [8]. Ninety-seven percent of the proteins found in plasma belong to one of the seven major groups of high-abundant plasma proteins: albumin, immunoglobulins, fibrinogen, α-1 antitrypsin, α-2 macroglobulin, transferrin and lipoproteins [3]. Many researchers consider these proteins not informative enough to be used as biomarkers of diseases of most tissues [3,4,6]. The remaining 3% is a complex mixture of middle and low abundance proteins, including proteins from the family of the complements, hormones (like insulin or erythropoietin), other proteins originated from normal tissue secretion (such as cytokines) or from tissue leakage upon cell death or damage (for instance, myoglobin released in myocardial infarction). The dynamic range of protein amount that can be detected in a single mass spectrum (2–3 orders of magnitude depending on the instrument) is insufficient to cover the range present in a blood sample. Because of that, the concentration in blood of any protein released from a tissue as consequence of a certain disease may be too low to be detected by mass spectrometry, particularly when only a few cells are affected. The signals of the most abundant, non-informative proteins obscure the signals of those present at concentrations that are several orders of magnitude lower [4,6,17,47]. Furthermore, when an unspecific binding material, such as partition resins, is used to extract the proteins from the sample, the high-abundance proteins compete and may interfere with the binding of the low abundant ones. Then, the intensity of the spectral signal of a low abundant protein may simply differ between two samples because of the presence of other blood components [4]. Another important influence is the intrinsic variability of the protein concentrations in circulating plasma depending on a number of factors, like genetic background, sex, age, nutritional status, lifestyle of the individual (including smoking and diet), medical treatment, bed rest and others [8,48]. Also the way the blood sample is collected influences the resulting spectra. Factors such as the position of the individual during the sampling or the use of atourniquet can affect the concentration of proteins in the collected blood. Moreover, there are notorious differences in the proteomes of serum and plasma and both are extremely sensitive to changes in the protocol to prepare and store them [3,4,8].

In summary, it is important to know that differences in the spectral patterns between blood samples are not always due to the specific disease investigated, but may be caused by artefacts from the analytical procedure or by the natural variability of the blood. The artefacts result in apparent but not real differences in protein concentration and have diverse causes, like differences in sample processing or preparation as well as intrinsic errors from the experimental techniques. On the other hand, with the simple methodology reviewed here, only differences in the concentration of the most abundant proteins (for instance haptoglobin, amyloid A protein, apolipoprotein A, transthyretin) can be detected. These variations are supposed to be related with acute-phase response and other unspecific reactions [4,6,8].

The other concern of this approach for the biomarker discovery is the lack of identification of the putative biomarkers. Even though the differences in the spectral patterns may constitute a biomarker on its own, the identification of the proteins that generate discriminating signals has several advantages. First, it provides a way of designing independent assays (for instance, ELISA) to validate the mass spectrometry results. Besides, these alternative assays may have lower costs and easier implementation in clinical practice. Also, once the identity of the peaks is known, it is possible to filter out the signals with no or very low predictive value, caused by artefacts (noise, cell lysis during serum and plasma preparation, etc.) or by unspecific responses. Lastly, the identity of the proteins that permit the correct classification of the samples can provide biological information about the studied disease. It has been suggested that the proteomic profiling of blood samples has more utility as a screening tool to preselect a few candidates in the search for biomarkers for a specific condition (for instance, a certain cancer). These candidates must be tested and validated with other techniques in order to find the most appropriate [4,6,27].

3. The efforts

Not to discourage researchers, all these concerns resulted in a series of efforts to make the proteomic analysis of serum or plasma samples a valid tool for the development of new biomarkers. These efforts address all the steps in the process of development, from the design and planning of the studies to the validation of the results, including sampling, sample preparation for MS, processing of the individual spectra and data mining. The strategies employed can be classified into five groups: (1) improvement of the spectral reproducibility, (2) increase of the detection range to monitor the low-abundance proteins, (3) application of technologies that allows protein quantitation, (4) identification and indexing in databases the human plasma proteins and (5) improvement of the reproducibility of analysis of the spectra (processing, data mining and interpretation of the results).

Here we will focus on the first four groups. The critics and the solutions to overcome them are also summarised in Table 1. The issues regarding the processing of the spectra, the data mining and the interpretation of the results are reviewed elsewhere [2,6,27,30].

Table 1
Scheme of the drawbacks described in this review for the use of MALDI- and SELDI-TOF spectra from blood peptides as biomarkers

Critics	Solutions
Limitations of MALDI- and SELDI-TOF (Section 2.2)	
MS is not a quantitative technique	Differential isotopic labelling and use of internal standards (Section 3.3)
Protein identification not possible	Construction of databases of plasma and serum proteins (Section 3.4)
Influence of the experimental conditions for the acquisition of the spectra	Standarization of the protocols (Section 3.1) Regular calibration and control of the instruments (Section 3.1) Use of standards (Section 3.1) Automatization (Section 3.1)
Blood characteristics (Section 2.3)	
The range of protein concentration in blood is higher than the range of detection by MS	Depletion of the most abundant proteins (Section 3.2)
	Fractionation of the samples (Section 3.2)
Natural variability of the protein concentration in blood	Track of all the variables that might influence the protein content in blood, such as age, gender or diet of the donor (Section 3.1)
Influence of the protocol for sample preparation in the protein content of plasma and serum	Standarization of the procedure for sample collection (Section 3.1)

3.1. Efforts to improve the reproducibility of the spectra

The variables that affect the reproducibility of the spectra fall in two major groups: the protocol employed to prepare the sample and the spectrometer conditions during acquisition. On the sample preparation, one of the most important issues is the choice between plasma and serum. As mentioned above, plasma is obtained after removing the cellular elements of the blood by centrifugation whereas serum is prepared by allowing the blood to clot to separate cellular particles. Because of that, serum contains peptides derived from the coagulation process, such as fibrinogen fragments. Also a certain extent of proteins from the sample could be lost due to retention in the protein network formed during coagulation. For these reasons, the composition of the plasma seems to be closer to that one of the circulating blood and is thus preferable over serum. However, the optimization of the MS techniques for the use of serum is also attractive because it is the most abundant form in which blood samples are archived. Plasma has as well some disadvantages. It can be contaminated with peptides produced by the activation of the platelets as well as with intracellular molecules from the cellular elements present in the blood due to a non efficient removal or to cell lysis during sample handling [3,21,49].

The differences between plasma and serum are not limited to the proteins of the coagulation cascade and intracellular contaminants. During blood clotting, enzymes that are not involved in the coagulation may also be active. Especially, proteases may digest some of the proteins in the sample, which alters the peak pattern of the mass spectrum. Some researchers consider that these enzymatic activities should be prevented because they are spurious and can affect the reproducibility of the analysis [21,47,49,50], whereas other investigators postulate that these reactions are an indirect evidence for the presence of low-concentration enzymes in the circulating blood plasma. Since a single enzyme molecule can act on a number of substrate molecules, it is easier to detect the effect of the enzymatic activity, e.g. the peptides resulting from a certain proteolysis, than the enzyme itself, i.e., the protease. Thus, a small amount of a certain protease released from a specific tissue upon certain conditions (for instance, a disease) can be monitored as changes in the MS pattern of serum due to the proteolysis of high and middle-abundance proteins. The same reasoning can be applied for any enzyme that alters the mass of the proteins, such as kinases, phosphatases, acetyltransferases, and others. From this point of view, the inhibition of such an enzymatic activity during sample handling reduces the amount of information that can be obtained [6,16].

Proteolytic activity in plasma has also been reported, even though the time to prepare plasma (and thus the time window for the proteolysis) is shorter than the time necessary to prepare serum [21,47,49,51].

Independently of the choice between serum and plasma, a large number of variables related to sample collection, handling and processing can alter the resulting spectra. In order to prevent the finding of artefactual biomarkers, it is important to eliminate as many of these variables as possible. Every single factor that may influence the outcome of the analysis must be tracked, from the medical history of the blood donor to the acquisition of the spectra. Several studies have addressed these questions. When preparing plasma, the nature of the anticoagulant added to the blood and the protocol employed to remove the cellular components influences the protein content of the sample [18,21,47,49,52]. Similarly, when preparing serum, the material of the tube for blood collection (plastic or glass) and the conditions of the clotting affect the peak pattern [15,20]. Other important factors are the temperature of handling [18,20,21,49] the storage conditions [18,20,49], the number of freezing and thawing cycles [15,18,49] and, if used, the chemical nature of protease inhibitors [49]. Even the way the samples are desalted and pre-processed for the spectra acquisition can contribute to a significant degree of variation among the spectra from a single spectrum (Fig. 1).

The other factors described above in Section 2, which are related with the particularities of the individual from which the sample was taken, i.e. sex, age, lifestyle or drug treatment, are

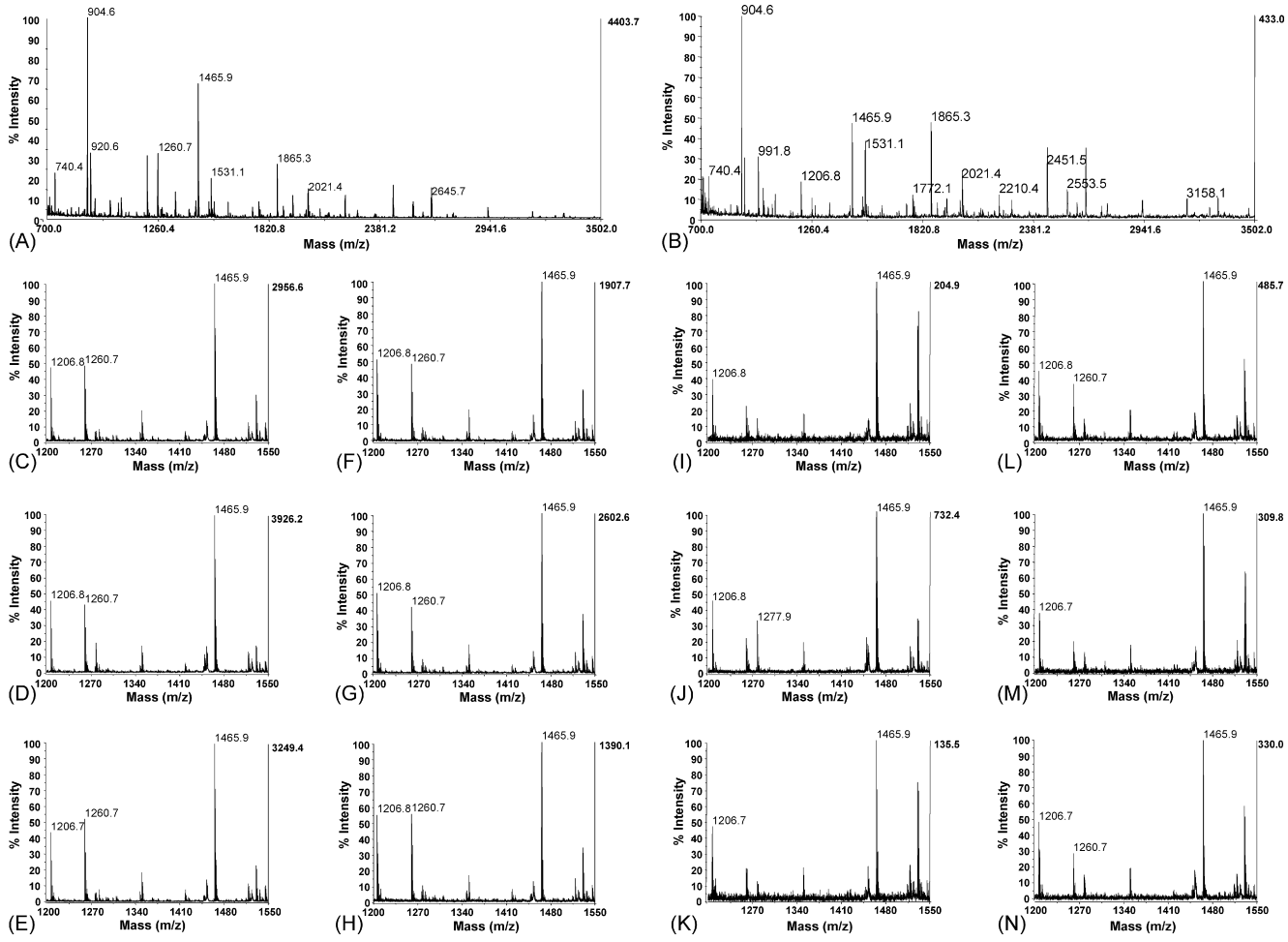

Fig. 1. Influence of the protocol employed to desalt the samples. Aliquots of the same pool of serum from 25 anonymous donors were thawed and desalted using C18 resin in a robot (Tecan). All the spectra were acquired automatically with the same settings on a Voyager STR (Applied Biosystems). A and B: spectra of two aliquots processed in parallel and the same way with exception of the elution from the C18 resin. In spectrum A, the sample was eluted with a solution containing 50% acetonitrile and 0.1% TFA, spotted onto the target plate and finally mixed with the matrix (indirect elution). In spectrum B, the sample was eluted directly with the matrix solution. C–N: magnifications of the region 1200–1550 for spectra from different aliquots. Samples were desalted in parallel two different days (first day: C, D, E, I, J and K; second day: F, G, H, L, M and N) employing freshly prepared solutions. C–H: the peptides were eluted in the indirect way from the resin. I–N: the peptides were eluted from the resin with the matrix solution. Note the influence of the elution method in the relative intensities of the peaks 1206.8 vs. 1260.7 and 1465.9 vs. 1531.1. Also, note that the noise is higher in the spectra obtained after direct elution of the samples.

expected to affect the obtained spectra [8,49]. However, these variables have not been extensively explored and may be not easy to control. In a recent study it was shown that alterations in the diet influence the protein content of blood and subsequently the MALDI-TOF spectrum of the serum [48].

Serum and plasma samples must be desalted in order to get MS spectra of good quality. Usually, resins that bind peptides and proteins are employed to separate these compounds from inorganic salts and other substances. In the case of the SELDI-TOF, these resins are coating the chip that will be used as a target plate. In the case of MALDI-TOF, the extraction is performed before depositing the sample on the target plate. In both situations, it is possible to select materials whose binding characteristics depend on different physicochemical properties, such as solubility, electric charge, etc. However, each protein displays different affinities for the different resins and not all of the proteins present in a sample bind to a certain resin with the same efficiency. Thus, it is obvious that the resin employed to extract the proteins from the sample will affect the resulting spectra [14,19,47]. Moreover, differences in the spectra depending on the batch of the resin employed have been reported [15,26]. Other variables of the extraction process, such as the concentration and volume of the analyte, the number of washes of the resin, the composition of the washing and elution solutions, the shape of the resin bed (length and size), and others also influence the spectra [14,19,53].

Once the proteins have been extracted from the sample, it is necessary to mix them with the matrix before acquiring spectra with SELDI-TOF or MALDI-TOF spectrometers. Mass spectrometry is based on the detection of the ions (positive or negative) derived from the sample. In MALDI-TOF and SELDI-TOF, the analyte molecules are ionized by energy transfer from the matrix molecules. The matrixes are diverse chemicals that absorb UV light and transfer the energy to the analyte. Their chemical properties, the proportion in which they are mixed with the analyte and the way in which they co-crystallize

with the sample influence the spectra and their reproducibility [14,15,19,53].

Finally, spectra are acquired. As in the previous steps, also a large number of variables in the acquisition process influence the outcome. The matrix is excited by bombardment with UV light. The number of laser shots and the light intensity affect the quality of the spectra [14,15]. The regular calibration of the instruments and a strict control of their performance to correct possible drifts is extremely important for the reproducibility of the results [26,27,31,48,54–56].

In order to reduce the experimental variability, several groups have successfully automatized one or more of these steps (peptide extraction, the addition of the matrix and spectra acquisition) [14–16,55,56].

From a more global point of view, studies that address the inter-laboratory reproducibility demonstrate that, under tight control of every step from sample collection to recording of the spectra, it is possible to achieve an inter-laboratory variation in the spectra similar to the intra-laboratory one [55,56]. The importance of obtaining good reference materials for the fine adjustment of protocols and spectrometers in order to get the desired reproducibility between different laboratories and along time has been highlighted [14–16,26,27,30,49,55–57].

In summary, it is possible to achieve a good reproducibility of the spectra (including inter-laboratory reproducibility) if the sample handling, storage and the instrument performance are strictly controlled.

3.2. Efforts to increase the dynamic concentration range

As mentioned above, the dynamic range of the amount of protein that can be detected in a single MALDI-TOF or SELDI-TOF spectrum is about 2 orders of magnitude, whereas the range of protein concentration in the blood stretches approximately 10 orders of magnitude [6]. To overcome this disadvantage, two non-exclusive strategies have been developed. One consists of the selective depletion of the most abundant proteins in the plasma and the second one, on the fractionation of the sample.

There are diverse procedures to remove the most abundant proteins from serum and blood samples, including ultrafiltration or the usage of diverse antibodies attached to solid supports.

For the depletion of albumin, the simplest procedures are based on the different solubilities of the proteins. For instance, by adding acetonitrile to the serum, a pellet enriched in globular proteins, including albumin, is formed, whereas the supernatant is depleted in these macromolecules [48]. Alternatively, if the serum is mixed with sodium chloride and ethanol, a protein pellet is formed but most of the albumin remains in the supernatant [58,59]. The inconvenience of these methods is the poor selectivity because proteins that bind albumin and proteins with a solubility similar to that of albumin may not be separated [58]. Another possibility is the use of dyes, like Cibacron blue, that bind and deplete most of the albumin in the sample. The dyes are usually covalently attached to solid supports which constitute the bed of spin-columns. These dye-based methods are fast, cheap and the columns are disposable, which prevents the cross-contamination between samples. However, the selectivity is poor and other less abundant proteins are removed as well [57,60,61]. Echan et al. have proposed that this lack of specificity may become an advantage, since it may permit the use of these resins for fractionation [60].

For the removal of IgG, bacterial protein A and G linked to microbeads can be used, but these proteins do not bind efficiently to all the IgG groups and, as result, the depletion is not complete. Like in the case of the dyes for the albumin, this feature may allow the use of these resins to fractionate the samples, rather than to deplete the IgG [57].

The use of antibodies for the removal of albumin, immunoglobulins and other high-abundance proteins is at present the most efficient and specific method. The major drawback is the high cost. Affinity-purified polyclonal IgG antibodies raised against albumin and other high-abundance proteins have been linked to solid supports for this purpose. They are commercially available from different manufacturers, usually as cocktails that can bind not only albumin and IgGs but also several other proteins, for instance transferrin, haptoglobin, α-1-antitrypsin, IgAs. These resins are reported to be robust, reusable, highly specific and to yield reproducible results [60–63]. They can even deplete the peptides resulting from the proteolysis of the targeted proteins [60]. However, it seems that variations in the protocol, e.g. the number of washes of the resin or the composition of the buffers, have a strong influence on the proportion of non-targeted proteins that are retained on the beads [60,61,64]. As an alternative, avian polyclonal IgY antibodies have been raised against several high-abundance proteins of the human plasma: albumin, IgGs, transferrin, α-1-antitrypsin, IgAs, IgMs, α-2-macroglobulin, haptoglobin, and high-density lipoproteins (HDL, mainly ApoA-I and ApoA-II), orosomucoid (α-1-acid glycoprotein) and fibrinogen. Like the IgG antibodies, the IgY antibodies are crosslinked to a solid support and can be employed in cocktails. The resulting resins are also reusable and give reproducible results. The use of IgY has some advantages over IgG: IgY is less cross-reactive than IgG with heterologous human proteins, and large amounts of IgY are secreted by the immunized hens into the egg yolk, which is more easily accessible than the rabbit blood. However, the IgY resins also retain some non-target proteins and the exact protocol used for the depletion influences this unspecific effect [65].

Additionally, Fu et al. recommend removing the lipids from plasma and serum samples because they may interfere with the protein depletion and with the subsequent analysis of the samples. Among the different methods to remove the lipid component of the blood, centrifugation of the serum samples is the one that produces less protein losses. However, proteins complexed with triglycerides and other lipoproteins are removed together with the lipid layer [58]. Indeed, ultracentrifugation of plasma has been used to separate and analyze those proteins that interact with HDL [66].

Up to date, all the methodologies developed to deplete the high-abundance proteins from plasma and serum samples remove as well, at least in part, some middle and low abundant proteins, including proteins leaked from tissue and cytokines. There are two non-exclusive explanations for this.

On one hand, it is known that albumin and other high-abundance proteins bind some less abundant peptides in the blood and act as their carriers. In the depletion procedure, these peptides may not detach from the high-abundance proteins and thus are also retained with them. On the other hand, it is possible that the reagents used for the depletion show a certain degree of cross-reactivity with non-targeted proteins [57,60–62,64,65,67,68]. Some studies suggest that the analysis of the protein fraction that is retained with the depletion targets can provide information over the interactions between plasma proteins and even can be useful in the search for biomarkers [61,62,64,65,67,69–72]. Moreover, putative biomarkers like the cancer-associated, SCM-recognition, immunodefense-suppressing and serine protease protection (CRISPP) peptide and biomarkers currently used in the clinical setting have been found bound to the high-abundance proteins after the depletion procedure [64,70,72].

Another limitation of the depletion reagents is that they may not remove all the degraded or oxidized forms of the targeted proteins. The same may be true for isoforms and molecules carrying post-translational modifications [60].

Major efforts are currently made to develop even more selective and efficient depletion methods. On one hand, it is necessary to minimize the loss of potential biomarkers during the depletion, since their detection in a fraction enriched in albumin or other proteins may not be possible. On the other hand, the incomplete removal of albumin and the other abundant proteins from plasma, even if only a small proportion is left, may keep the range of protein concentrations in the sample still too large to detect the less abundant proteins [57].

Despite the limitations of the current depletion techniques, several groups have demonstrated that the removal of the high-abundance proteins significantly increases the detection of the low-abundance proteins in plasma [57,60–63,65,67].

After the depletion of albumin, IgG and other high-abundance proteins, the middle-abundance proteins like ferritin or the components of the complement system can still impede the detection of the less abundant proteins. The fractionation of the sample is another way of favouring their detection. In some cases, the fractionation is done just after the depletion, whereas in others the fractionation is the unique separation step for the samples. The fractionation increases the number of spectra per sample, adding one more dimension to the data obtained: with no fractionation, the data are bi-dimensional (signal intensity and m/z), whereas with fractionation, the fraction number constitutes the third dimension. This increases the time required to acquire, process and analyze the data.

Fractionation is mostly used in combination with protein digestion and tandem mass spectrometry. These technologies are employed for the discovery of proteins rather than to compare samples and are described or reviewed elsewhere [57,67,69,73–75]. The separation methods based on gel electrophoresis usually require the digestion of the fractions in order to release the analyte from the gel. In contrast, liquid chromatography and various affinity purification methods do not entail digestion of the sample and hence allow the analysis of intact peptides and proteins.

The simplest fractionation procedure takes advantage of the desalting step required for any MALDI-TOF analysis (Fig. 1). Usually, desalting is performed by flushing or incubating the sample in a small bed of chromatographic particles (ionic exchange resins, partition chromatography beads, etc.). Then, the resin is immediately washed with an aqueous solution and finally, the bound sample is eluted in a few microliters of an adequate elution buffer, which detaches virtually all the proteiniaceous material bound to the resin. However, it is possible to perform a step-wise elution with two or more different solutions. The bed is flushed sequentially with the elution buffers, starting with the milder one and finishing with the one able of removing the most strongly bound molecules. Each eluate constitutes a fraction which is analysed afterwards by MS [17,47].

More sophisticated and with a much higher resolution, HPLC has been employed by several groups to study the low-mass constituents of the proteome. The samples are separated in several fractions that are then individually analyzed by MALDI-TOF. In some cases, the sample mixed with the adequate buffer was loaded directly onto the chromatographic column [49,76], whereas in others the high-molecular weight proteins were removed before the separation [21]. Given the high diversity of stationary phases commercially available, the use of HPLC allows the performance of orthogonal separations. In other words, it is possible to fractionate the sample by multiple different fractionation principles, e.g. first by the isoelectric point of the peptides and then partition chromatography or ionic exchange [76].

One of the major inconveniences of HPLC fractionations is the low throughput of the technique. Samples have to be separated sequentially, one after the other and the time for each separation is relatively long (0.5–1 h per sample) [21,76]. This not only increases the time for the analysis but also augments the risk of poor reproducibility of the results due to the changes of the column over time. Additionally, the same protein can be present in different fractions, which challenges the comparison of its abundance between samples.

The use of antibodies or dyes to deplete the sample in high-abundance proteins has been already commented, but other affinity separations can also be employed to select specifically a certain fraction of the sample proteome. For instance, a multilectin column permits the capture of the glycosylated proteins in the sample. No substantial differences have been found between the glycoproteome of plasma and serum when analysed by LC–MS, perhaps because the glycosylation increases the solubility and the stability of the plasma proteins. It has been suggested that these properties of the glycoproteins may also strengthen the reproducibility of their detection even if there is some variability in the collection and storage of the sample [77]. Alternatively, glycoproteins can be first oxidized and then covalently trapped by reaction of the resulting aldehydes with beads coated with hydrazide. The analysis of the bound fraction has allowed the identification of proteins present in plasma at very low concentrations [78]. As far as we know, no data have been published reporting the use of none of these columns before MALDI-TOF or SELDI-TOF analysis of the undigested samples.

The use of peptide libraries linked to a solid support is another way to enrich the sample in low-abundance proteins. It is assumed that each peptide on the library has high affinity for one or a few proteins present in the serum sample, thus the library can simultaneously bind similar amounts of many different serum proteins. These proteins are eluted and subsequently analysed. No data are reported on the reusability of the beads or about the loss of low-abundance proteins bound to the excess of the most abundant proteins [79].

Finally, antibodies raised against inter-α-trypsin inhibitor heavy chain 4 (ITIH4) have been used to capture the ITIH4-derived peptides from serum and plasma samples. The analysis of these peptides revealed the association of the proteolytic patterns of ITIH4 with specific disease conditions [80].

3.3. Application of methodologies that allow protein and/or peptide quantitation

The factors that govern the intensity of the signals in mass spectrometry are not well understood. As result, the signal of a certain analyte present in identical concentration in two samples may have different intensity due to interferences with other analytes and other factors [4,6,41]. The application of methodologies that allow an acceptable quantitation of the proteins in the sample are under study. The most common methods used in peptide and protein mass spectrometry are based on differential isotopic labelling, which permits to compare the relative abundance of proteins between two samples. In general, each sample is chemically modified with a reagent highly enriched in a certain isotope variant. For instance, one of the samples is modified with the reagent highly enriched in light isotopes (^{1}H, ^{12}C, etc.) whereas the other one is modified with a chemically identical compound but enriched in heavy isotopes (^{2}H, ^{13}C). Samples are then mixed, processed and the spectra acquired. A certain peptide from the sample labelled with the light isotopes will give a signal at *m/z* some units lower than the same peptide from the sample labelled with the heavy isotopes. In order to be labelled, peptides must have certain reactive groups, like the sulfhydryl group of cystein or amino groups at the N-termini or in lysine residues [81,82].

Spiking the samples with a reference material is another possibility. If the putative biomarkers have been identified, it is possible to synthesize a set of peptides chemically identical to them but labelled with rare isotopes (for instance, a few positions enriched in ^{13}C, ^{15}N or ^{2}H). These peptides can be used as an internal standard, mixing known amounts with the sample as in the classical isotopic dilution approach. Due to the chemical identity with the analyte, the signals of both the standard and the analyte should suffer the same interferences. In contrast with the differential isotope labelling, this method, known as AQUA (absolute quantitation) allows the absolute quantitation of the analyte [83]. AQUA can only be applied in the later steps of biomarker development, such as validation studies, after the identification of the candidate biomarkers.

3.4. Identification of the human plasma proteins and generation of adequate databases

The methodology described in this review does not allow the direct identification of the compounds that originate the peaks in the spectra and thus non informative signals (acute-phase response, diverse artefacts, etc.) cannot be filtered out. Therefore, the identification of the putative biomarkers found is a must for the development of assays that permit the validation by independent techniques.

The construction of good plasma protein databases may also facilitate the design of the experiment: the knowledge of all the proteins contained in the plasma allows the estimation of the proteins which are more likely to be detected with a certain protocol.

Additionally, it may allow the preselection of candidate biomarkers and optimize the analytical procedure for their detection.

Databases where the analytical conditions that led to the detection of each protein are specified could allow, in the future, the assignment of the spectral signals of samples just by knowing the protocol for the sample preparation. Then, it would be easy to filter out the signals from non-informative proteins before the algorithms for classifier development are applied. A similar approach is currently investigated for the analysis of tryptic digests by LC–MS [73].

But not only the description of the plasma proteome is useful. The knowledge of the characteristic proteome of a certain tissue during a specific disease can be used to identify candidate biomarkers and afterwards to focus the analysis of the blood samples to detect them [84].

At the moment, several databases are under construction. For a comment and comparison, see ref. [57].

4. Conclusion

In the last few years, it has been demonstrated that MALDI-TOF and SELDI-TOF technologies have diverse limitations for the search of biomarkers in the intact pool of proteins contained in the human plasma. Taking these limitations into strict consideration is the first step to improve the experimental designs and techniques, and to generate valid tools for the discovery of new biomarkers. The possibility to screen a multitude of proteins or peptides in a single spectrum is attractive enough to justify the efforts to optimise all the different aspects of these methodologies, including their reproducibility, the expansion of the detection range and the quantitation. In the next few years we will hopefully see the desired results of these efforts.

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References

[1] L.L. McCabe, B.L. Therrell Jr., E.R.B. McCabe, Mol. Genet. Metab. 77 (2002) 267.

[2] F. Vitzthum, F. Behrens, N.L. Anderson, J.H. Shaw, J. Proteome Res. 4 (2005) 1086.
[3] I. Schulte, H. Tammen, H. Selle, P. Schulz-Knappe, Expert Rev. Mol. Diagn. 5 (2005) 145.
[4] E.P. Diamandis, Mol. Cell. Proteomics 3 (2004) 367.
[5] R. Etzioni, N. Urban, S. Ramsey, M. McIntosh, S. Schwartz, B. Reid, J. Radich, G. Anderson, L. Hartwell, Nat. Rev. Cancer 3 (2003) 243.
[6] M.A. Gillette, D.R. Mani, S.A. Carr, J. Protome Res. 4 (2005) 1143.
[7] J.E. Katz, P. Mallick, D.B. Agus, B. J. U. Int. 96 (2005) 477.
[8] N.L. Anderson, N.G. Anderson, Mol. Cell. Proteomics 1 (2002) 845.
[9] E.F. Petricoin 3rd, 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.
[10] 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.
[11] Y. Qu, B.L. Adam, Y. Yasui, M.D. Ward, L.H. Cazares, P.F. Schellhammer, Z. Feng, O.J. Semmes, G.L. Wright Jr., Clin. Chem. 48 (2002) 1835.
[12] J. Li, Z. Zhang, J. Rosenzweig, Y.Y. Wang, D.W. Chan, Clin. Chem. 48 (2002) 1296.
[13] F. Simpkins, J.A. Czechowicz, L. Liotta, E.C. Kohn, Pharmacogenomics 6 (2005) 647.
[14] J. Villanueva, J. Philip, D. Entenberg, C.A. Chaparro, M.K. Tanwar, E.C. Holland, P. Tempst, Anal. Chem. 76 (2004) 1560.
[15] J. Villanueva, J. Philip, C.A. Chaparro, Y. Li, R. Toledo-Crow, L. DeNoyer, M. Fleisher, R.J. Robbins, P. Tempst, J. Proteome Res. 4 (2005) 1060.
[16] J. Villanueva, D.R. Shaffer, J. Philip, C.A. Chaparro, H. Erdjument-Bromage, A.B. Olshen, M. Fleisher, H. Lilja, E. Brogi, J. Boyd, M. Sanchez-Carbayo, E.C. Holland, C. Cordon-Cardo, H.I. Scher, P. Tempst, J. Clin. Invest. 116 (2006) 271.
[17] J.M. Koomen, L.N. Shih, K.R. Coombes, D. Li, L.-C. Xiao, I.J. Fidler, J.L. Abbruzzese, R. Kobayashi, Clin. Cancer Res. 11 (2005) 1110.
[18] S. Baumann, U. Ceglarek, G.M. Fiedler, J. Lembcke, A. Leichtle, J. Thiery, Clin. Chem. 51 (2005) 973.
[19] A.K. Callesen, S. Mohammed, J. Bunkenborg, T.A. Kruse, S. Cold, O. Morgensen, R.P. Christensen, W. Vach, P.E. Jørgensen, O.N. Jensen, Rapid Commun. Mass Spectrom. 19 (2005) 1578.
[20] M. West-Nielsen, E.V. Hogdall, E. Marchiori, C.K. Hogdall, C. Schou, N.H. Heegaard, Anal. Chem. 77 (2005) 5114.
[21] H. Tammen, I. Schulte, R. Hess, C. Menzel, M. Kellmann, T. Mohring, P. Schulz-Knappe, Proeomics 5 (2005) 3414.
[22] B. Rockhill, Lancet 360 (2002) 169.
[23] D.C. Pearl, Lancet 360 (2002) 169.
[24] M. Elwood, Lancet 360 (2002) 170.
[25] E.P. Diamandis, Lancet 360 (2002) 170.
[26] M.A. Rogers, P. Clarke, J. Noble, N.P. Munro, A. Paul, P.J. Selby, R.E. Banks, Cancer Res. 63 (2003) 6971.
[27] Z. Feng, R. Prentice, S. Srivastava, Pharmacogenomics 5 (2004) 709.
[28] Y. Yasui, M. Pepe, M.L. Thompson, B.-L. Adam, G.L. Wright Jr., Y. Qu, J.D. Potter, M. Winget, M. Thornquist, Z. Feng, Biostatistics 4 (2003) 449.
[29] M. Wagner, D.N. Naik, A. Pothen, S. Kasukurti, R.R. Devineni, B.-L. Adam, O.J. Semmes, G.L. Wright Jr., BMC Bioinformatics 5 (2004) 26.
[30] M. Hilario, A. Kalousis, C. Pellegrini, M. Müller, Mass Spectrom. Rev. 25 (2006) 409.
[31] K.A. Baggerly, J.S. Morris, K.R. Coombes, Bioinformatics 20 (2004) 777.
[32] J.M. Sorace, M. Zhan, BMC Bioinformatics 4 (2003) 24.
[33] E.P. Diamandis, Clin. Chem. 49 (2003) 1272.
[34] K.R. Coombes, H.A. Fritsche Jr., C. Clarke, J.-N. Chen, K.A. Baggerly, J.S. Morris, L.-C. Xiao, M.-C. Hung, H.M. Kuerer, Clin. Chem. 49 (2003) 1615.
[35] K.R. Coombes, S. Tsavachidis, J.S. Morris, K.A. Baggerly, M.-C. Hung, H.M. Kuerer, Proteomics 5 (2005) 4107.
[36] K.A. Baggerly, J.S. Morris, S.E. Edmonson, K.R. Coombes, J. Natl. Cancer Inst. 97 (2005) 307.
[37] Y. Yasui, D. McLerran, B.-L. Adam, M. Winget, M. Thornquist, Z. Feng, J. Biomed. Biotechnol. 2003 (2003) 242.
[38] J.S. Morris, K.R. Coombes, J. Koomen, K.A. Baggerly, R. Kobayashi, Bioinformatics 21 (2005) 1764.
[39] T.W. Randolph, B.L. Mitchell, D.F. McLerran, P.D. Lampe, Z. Feng, Mol. Cell. Proteomics 4 (2005) 1990.
[40] X. Wang, W. Zhu, K. Pradhan, C. Ji, Y. Ma, O.J. Semmes, J. Glimm, J. Mitchell, Proteomics 6 (2006) 2095.
[41] R. Aebersold, M. Mann, Nature 422 (2003) 198.
[42] J.J. Hlavaty, A.W. Partin, M.J. Shue, L.A. Mangold, J. Derby, T. Javier, S. Kelley, A. Stieg, J.V. Briggman, G.M. Hass, Y.J. Wu, Urology 61 (2003) 1261.
[43] 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 Gaaften-Day, N.F. Hacker, H.W.A. de Bruijn, A.G.J. van der Zee, I.J. Jacobs, E.T. Fung, D.W. Chan, Cancer Res. 64 (2004) 5882.
[44] B. Ye, D.W. Cramer, S.J. Skates, S.P. Gygi, V. Pratomo, L. Fu, N.K. Horick, L.J. Licklider, J.O. Schorge, R.S. Berkowitz, S.C. Mok, Clin. Cancer Res. 9 (2003) 2904.
[45] W.C.S. Cho, T.T.C. Yip, C. Yip, V. Yip, V. Thulasiraman, R.K.C. Ngan, T.-T. Yip, W.-H. Lau, J.S.K. Au, S.C.K. Law, W.-W. Cheng, V.W.S. Ma, C.K.P. Lim, Clin. Cancer Res. 10 (2004) 43.
[46] Z. Xiao, D. Prieto, T.P. Conrads, T.D. Veenstra, H.J. Issaq, Mol. Cell. Endocrinol. 230 (2005) 95.
[47] J.M. Koomen, D. Li, L.-C. Xiao, T.C. Liu, K.R. Coombes, J. Abbruzzese, R. Kobayashi, J. Proteome Res. 4 (2005) 972.
[48] B.L. Mitchell, Y. Yasui, J.W. Lampe, P.R. Gafken, P.D. Lampe, Proteomics 5 (2005) 2238.
[49] A.J. Rai, C.A. Gelfand, B.C. Haywood, D.J. Warunek, J. Yi, M.D. Schuchard, R.J. Mehigh, S.L. Cockrill, G.B.I. Scott, H. Tammen, P. Schulz-Knappe, D.W. Speicher, F. Vitzthum, B.B. Haab, G. Siest, D.W. Chan, Proteomics 5 (2005) 3262.
[50] D.E. Misek, R. Kuick, H. Wang, V. Galchev, B. Deng, R. Zhao, J. Tra, M.R. Pisano, R. Amunugama, D. Allen, A.K. Walker Jr., P. Strahler, G. Andrews, S. Omenn, S.M. Hanash, Proteomics 5 (2005) 3343.
[51] G.S. Omenn, Export Rev. Proteomics 3 (2006) 165.
[52] B.B. Haab, B.H. Geierstanger, G. Michailidis, F. Vitzthum, S. Forrester, R. Okon, P. Saviranta, A. Brinker, M. Sorette, L. Perlee, S. Suresh, G. Drwal, J.N. Adkins, G.S. Omenn, Proteomics 5 (2005) 3278.
[53] H.C. Cordingley, S.L. Roberts, P. Tooke, J.R. Armitage, P.W. Lane, W. Wu, S.E. Wildsmith, Biotechniques 34 (2003) 364.
[54] D. de Seny, M. Fillet, M.A. Meuwis, P. Geurts, L. Lutteri, C. Ribbens, V. Bours, L. Wehenkel, J. Piette, M. Malaise, M.P. Merville, Arthritis Reum. 52 (2005) 3801.
[55] 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. Ibicka, J. Kagan, G. Malik, D. McLerran, J.W. Moul, A. Partin, P. Prasanna, J. Rosenzweig, L.J. Sokoll, S. Srivastava, I. Thompson, M.J. Welsh, N. White, M. Winget, Y. Yasui, Z. Zhang, L. Zhu, Clin. Chem. 51 (2005) 102.
[56] A.J. Rai, P.M. Stemmer, Z. Zhang, B.-L. Adam, W.T. Morgan, R.E. Caffrey, V.N. Podust, M. Patel, L.-Y. Lim, N.V. Shipulina, D.W. Chan, O.J. Semmes, H.-C.E. Leung, Proteomics 5 (2005) 3467.
[57] G.S. Omenn, D.J. States, M. Adamski, T.W. Blackwell, R. Menon, H. Hermjakob, R. Apweiler, B.B. Haab, R.J. Simpson, J.S. Eddes, E.A. Kapp, R.L. Moritz, D.W. Chan, A.J. Rai, A. Admon, R. Aebersold, J. Eng, W.S. Hancock, S.A. Hefta, H. Meyer, Y.-K. Paik, J.-S. Yoo, P. Ping, J. Pounds, J. Adkins, X. Qian, R. Wang, V. Wasinger, C.Y. Wu, X. Zhao, R. Zeng, A. Archakov, A. Tsugita, L. Beer, A. Pandey, M. Pisano, P. Andrews, H. Tammen, D.W. Speicher, S.M. Hanash, Proteomics 5 (2005) 3226.
[58] Q. Fu, C.P. Garnham, S.T. Elliot, D.E. Bovenkamp, J.E. Van Eyk, Proteomics 5 (2005) 2656.
[59] D.A. Colantonio, C. Dunkinson, D.E. Bovenkamp, J.E. van Eyk, Proteomics 5 (2005) 3831.
[60] L.A. Echan, H.-Y. Tang, N. Ali-Khan, K. Lee, D.W. Speicher, Proteomics 5 (2005) 3292.
[61] N. Zolotarjova, J. Martosella, G. Nicol, J. Bailey, B.E. Boyes, W.C. Barrett, Proteomics 5 (2005) 3304.
[62] K. Bjorhall, T. Miliotis, P. Davidsson, Proteomics 5 (2005) 307.
[63] R. Pieper, Q. Su, C.L. Gatlin, S.-T. Huang, N.L. Anderson, S. Steiner, Proteomics 3 (2003) 422.
[64] M. Zhou, D.A. Lucas, K.C. Chan, H.J. Issaq, E.F. Petricoin 3rd, L.A. Liotta, T.D. Veenstra, T.P. Conrads, Electrophoresis 25 (2004) 1289.

[65] L. Huang, G. Harvie, J.S. Feitelson, K. Gramatikoff, D.A. Herold, D.L. Allen, R. Amunngama, R.A. Hagler, M.R. Pisano, W.-W. Zhang, X. Fang, Proteomics 5 (2005) 3314.
[66] F. Rezaee, B. Casetta, J.H.M. Levels, D. Speijer, J.C.M. Meijers, Proteomics 6 (2006) 721.
[67] Y. Shen, J. Kim, E.F. Strittmatter, J.M. Jacobs, D.G. Camp 2nd, R. Fang, N. Tolié, R.J. Moore, R.D. Smith, Proteomics 5 (2005) 4034.
[68] J. Granger, J. Siddiqui, S. Copeland, D. Remick, Proteomics 5 (2005) 4713.
[69] P. He, H.-Z. He, J. Dai, Y. Wang, Q.-H. Sheng, L.-P. Zhou, Z.-S. Zhang, Y.-L. Sun, F. Liu, K. Wang, J.-S. Zhang, H.-X. Wang, Z.-M. Song, H.-R. Zhang, R. Zeng, X. Zhao, Proteomics 5 (2005) 3442.
[70] M.S. Lowenthal, A.I. Mehta, K. Frogale, R.W. Bandle, R.P. Araujo, B.L. Hood, T.D. Veenstra, T.P. Conrads, P. Goldsmith, D. Fishman, E.F. Petricoin 3rd, L.A. Liotta, Clin. Chem. 51 (2005) 1933.
[71] M.F. Lopez, A. Mikulskis, S. Kuzdzal, D.A. Bennett, J. Kelly, E. Golenko, J. DiCesare, E. Denoyer, W.F. Patton, R. Ediger, L. Sapp, T. Ziegert, C. Lynch, S. Kramer, G.R. Whiteley, M.R. Wall, D.P. Mannion, G. della Cioppa, J.S. Rakitan, G.M. Wolfe, Clin. Chem. 51 (2005) 1946.
[72] R.L. Moritz, A.B. Clippingdale, E.A. Kapp, J.S. Eddes, H. Ji, S. Gilbert, L.M. Connolly, R.J. Simpson, Proteomics 5 (2005) 3402.
[73] L. Paša-Tolić, C. Masselon, R.C. Barry, Y. Shen, R.D. Smith, Biotechniques 37 (2004) 621.
[74] T. Kislinger, A. Emili, Expert Rev. Proteomics 2 (2005) 27.
[75] J. Martosella, N. Zolotarjova, H. Liu, G. Nicol, B.E. Boyes, J. Proteome Res. 4 (2005) 1522.
[76] T. Linke, A.C. Ross, E.H. Harrison, J. Chromatogr. A (2006) 160.
[77] Z. Yang, W.S. Hancock, T.R. Chew, L. Bonilla, Proteomics 5 (2005) 3353.
[78] T. Liu, W.-J. Qian, M.A. Gritsenko, D.G. Camp 2nd, M.E. Monroe, R.J. Moore, R.D. Smith, J. Proteome Res. 4 (2005) 2070.
[79] L. Guerrier, V. Thulasiraman, A. Castagna, F. Fortis, S. Lin, L. Lomas, P.G. Righetti, E. Boschetti, J. Chromatogr. B 833 (2006) 33.
[80] J. Song, M. Patel, C.N. Rosenzweig, Y. Chan-Li, L.J. Sokoll, E.T. Fung, N.H. Choi-Miura, M. Goggins, D.W. Chan, Z. Zhang, Clin. Chem. (2006) 1045.
[81] W.W. Wu, G. Wang, S.J. Baek, R.-F. Shen, J. Proteome Res. 5 (2006) 651.
[82] A. Leitner, W. Lindner, J. Chromatogr. B 813 (2004) 1.
[83] D.S. Kirkpatrick, S.A. Gerber, S.P. Gygi, Methods 35 (2005) 265.
[84] J.M. Jacobs, J.N. Adkins, W.-J. Qian, T. Liu, Y. Shen, D.G. Camp 2nd, R.D. Smith, J. Proteome Res. 4 (2005) 1073.