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Review

Combinatorial peptide ligand libraries: The conquest of the 'hidden proteome' advances at great strides

The combinatorial peptide ligand library (CPLL) is compared here with the immuno-depletion method for evaluating their respective abilities in digging deeper and deeper into the low-abundance proteome. A recent report suggested in fact that immuno-subtraction for biomarkers discovery in sera does not perform so well, since it results in a meagre 25% increase in identified proteins compared with unfractionated plasma, leaving little capacity to sample lower abundance proteins. On the contrary, CPLLs permit from 300 to 600% increments in detection abilities, as amply demonstrated in several reports. Moreover, when dealing with large sample volumes, an amplification factor of up to four orders of magnitude for trace proteins could be demonstrated, with 80% capture efficiencies even in large (up to 1 L) sample volumes. At present, the lower detection ability of CPLLs has been evaluated at 1 ng/mL (traces of casein additives in white wines).

Keywords:
Hexapeptide ligands / Immuno-depletion / Low-abundance proteome / Peptide libraries
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1 Introduction

We have already extensively reviewed the combinatorial peptide ligand library (CPLL) methodology in quite a large number of articles [1–10]. In one instance, detailed protocols for solid-phase peptide library users have been described [11]. In another couple of investigations, the capturing ability of these libraries as a function of the oligopeptide length has also been explored [12] and even when using just single amino acids attached to the beads [13]. Therefore, in the present review, we will cover only the most recent advances not yet previously described. In fact, we will compare the CPLL technique with some of the most popular enhancing methods, such as immuno-depletion as well as other methodologies for capturing specific population of sub-proteomes. It must be emphasized that the picture at present is not as rosy as depicted at the beginning of this decade, when biomarker discovery seemed to be at hand. According to Mitchell [14], in fact, proteomics is retrenching in that many of the expectations have not been met. This is particularly true in the case of biomarkers. According to the last count, >1250 presumptive biomarkers have been reported so far in the proteomic field, but such vast literature has not produced a single approved biomarker. It has generated expenditures of hundreds of millions of dollars in research that lead nowhere! Most of this has been caused by lack of validation of any of those presumptive biomarkers, but certainly a great part of this failure is also due to the fact that treasure hunting for precious biomarkers in sera (plasma) has been performed in a blindfolded way, i.e. having no map of such a treasure. At present, the only new test approved by FDA in November 2009 is the OVA1 for ovarian cancer [15]. It took 7 years of hard work to get there! This study, utilizing SELDI technology, involved multi-institutional analyses encompassing more than 600 individuals. The test in current use (cancer antigen 125, CA125) did not have the ability to discriminate between malignant and benign ovarian tumors and did not permit detection of early-stage ovarian cancer. The novel OVA1 test approved exploits a panel of seven SELDI markers: inter-α-trypsin inhibitor heavy chain 4 (ITIH4); transthyretin; apolipoprotein A1; hepcidin; β2-microglobulin; transferrin and connective tissue activating peptide III (CTAP3), in combination with the old CA125 test. There is clearly plenty of room for improvement!

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Abbreviations: CPLL, combinatorial peptide ligand library**; IgG,** immunoglobulin G**; IgY,** immunoglobulin Y**; HAP,** high-abundance proteins**; LAP,** low-abundance proteins

2 The pit and the pendulum

2.1 Subtraction approaches

Not precisely a horror story like the famous one by Edgar Allan Poe of 1842, this subtitle is meant to describe the

Colour Online: See the article online to view Figs. 1–3 in colour.

oscillation of scientists between the two extremes: the subtraction and enrichment approaches (the latter ones to be described ahead). The two approaches are nicely illustrated in Fig. 1, taken from an extensive review of Fang and Zhang [16]. Both methodologies can then be followed up by further downstream processes (e.g. SDS-PAGE, 2-D electrophoresis, SEC, HPLC and the like) in turn followed by MS analysis (although one can go directly to MS if the first steps have been already successful as such in digging into the deep proteome). Immuno-affinity separation of proteins using immunoglobulin G (IgG) or immunoglobulin yolk (IgY) as first reported by Pieper et al. [17] had become quite popular and had generated great hopes in biomarker discovery [18, 19]. It had been increasingly accepted as the most effective sample preparation process in plasma proteomics studies. The purpose of this process is to specifically remove top high-abundance proteins (HAP) from plasma or serum samples to achieve broader proteome coverage. Originally, monoclonal antibodies with high affinity to a unique epitope on the macromolecule were adopted to completely deplete the targeted proteins. However, such antibodies would not recognize truncated proteins or differences in post-translational modifications of the same gene product or even misfolded epitopes to which they are designed, creating thus unexpected issues of specificity. Using polyclonal antibodies will in principle result in a better depletion since they recognize many regions on the target surface. Moreover, since IgY polyclonal hens' egg yolk antibodies exhibit less cross-reactivity and are quite evolutionary distant from mammals IgG-based antibodies, they are today preferred for HAP depletion. Their advantage extends also to the fact that the Fc region does not bind to other proteins as it is the case for mammalian antibodies. Several companies have developed and marketed commercial kits for separation or subtraction of HAPs. The technology and products were also developed for digging deeper into the proteome and enriching low-abundance proteins (LAP) for biomarker discovery.

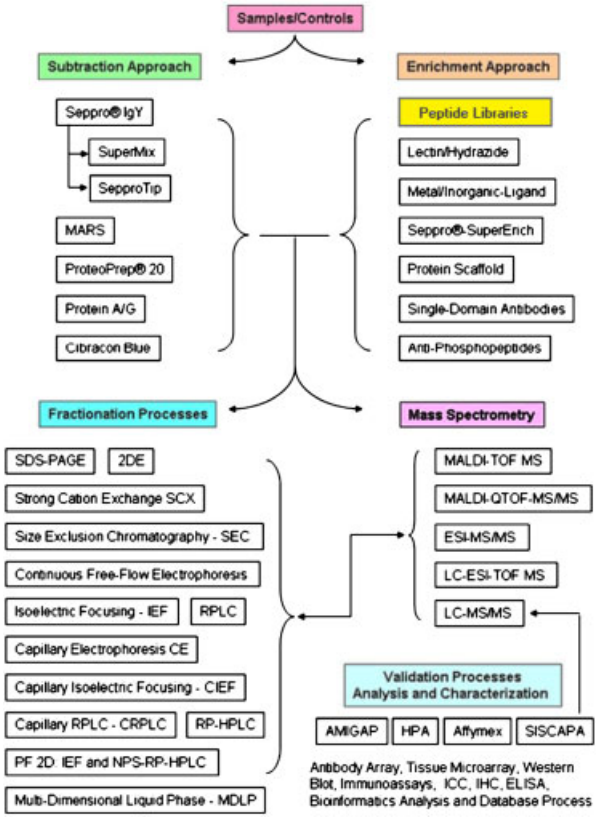

Figure 1. Technologies and methods in proteomic sample preparation are categorized into two major approaches: subtraction and enrichment. Their relative positions, inter-relationship and possible connections to downstream fractionation processes and mass spectrometry analysis are illustrated. How to select proper technologies and combine them to form a best fit workflow depends upon the needs and purposes of each proteomic study. An independent process for validating and analyzing proteomic studies is also depicted. Certain technologies are listed as examples of applications. In addition to AMIGAP, HPA, SISCAPA and those conventional assay methods, Affymex, a database tool for optimizing subtraction approach, is listed (from Fang and Zhang [16], by permission).

Although subtraction methods appear to be very popular today, they are not immune to severe drawbacks, as illustrated in Fig. 2. It is readily apparent that they are only operative in the upper part of the proteomic scale, i.e. they are efficient in removing the HAPs, but they do not cover the entire range of protein concentrations, down to MAP (medium-abundance proteins) or to LAP, whereas the enrichment approaches, in quite a few cases, seem to be able to cover almost the entire dynamic range in sera spanning some 12 orders of magnitude (Fig. 2, lower part). In other words, once the top 10 or 20 most abundant proteins in sera are removed, the MAP and LAP components remain at minimum, just as dilute as prior to treatment (in most cases the resulting sample is significantly more dilute) and thus they are not readily visible. As another major drawback, such immuno-depletion columns can only handle limited sample volumes (typically 20–100 μL): in such limited volumes, the LAPs might be present in such minute quantities that they could still be undetectable in MS analysis. Only methods that can work under large overloading conditions can concentrate and enrich the LAP class to a considerable extent. A third, most disturbing feature, is the fact that, when operating under immuno-subtraction, there is always the risk of co-depletion of other species either bound to the target protein being immuno-subtracted, or because of spurious interactions with the antibody columns. This last phenomenon could assume devastating proportions, e.g. Shen et al. [20], when depleting sera with just two antibody columns, against human serum albumin (HSA) and IgGs, reported a substantial loss of additional proteins, which amounted to another 815 species in the case of HSA depletion and to another 2091 species (not including IgG) in the case of IgG depletion. To complete this picture, it should be mentioned that immuno-depletion is by definition highly specific and usable only for one organism at a time, which

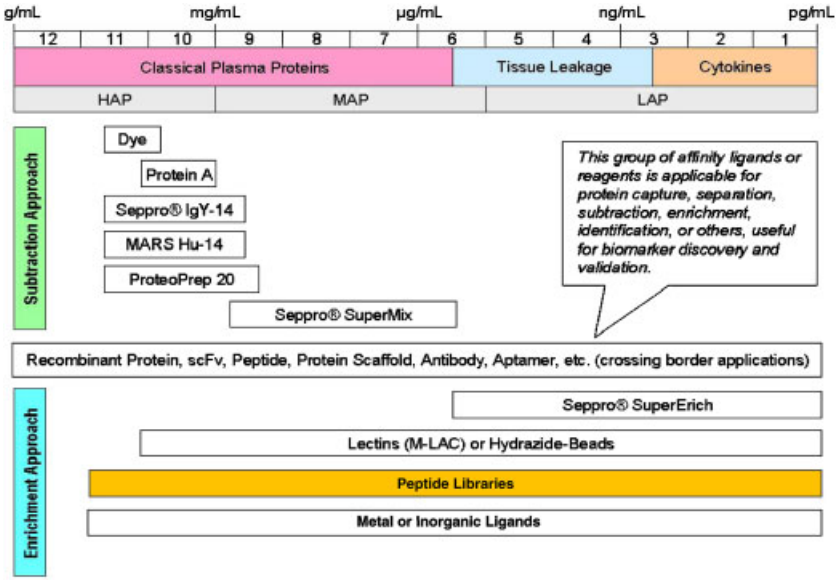

Figure 2. Affinity separation and enrichment technologies are put into the context of human plasma proteome, which has a dynamic concentration range of 10^{11}–10^{12} magnitude (from mg/L to pg/mL, depicted at the top). Each technology or method has its own position and scope of action. It is clear that subtraction technologies have more specific functional areas and those for enrichment and detection appear to be covering the full range of the proteome. The decision to choose the proper technology, single or in combination, depends upon the needs of different experiments and the proficiency of different researchers. The application results may vary accordingly (modified from Fang and Zhang [16], by permission).

strongly limits the universality of its application. Under such conditions, projects aiming at biomarker discovery seem rather unrealistic when exploiting such tools. This concern had also been voiced by other groups as well [21–23].

Although such immuno-subtraction (or depletion) techniques have been largely exploited up to the present, it has not at all been clear how effective they could possibly be in permitting biomarker discovery. The only deep evaluation of subtraction approaches has come recently from Tu et al. [24] and, alas, their report tolls a feral message for such methodologies. They evaluated the effects of top 7/top 14 immuno-depletion on the shotgun proteomic analysis of human plasma. Their goal was to evaluate the impact of immuno-depletion on detection of proteins across all ranges of abundances. On the positive side, they stated that the depletion columns afforded highly repeatable and efficient plasma protein fractionation, with relatively few non-targeted proteins capture by the depletion columns (this suggesting that the antibody columns had been substantial improved since the report of Shen et al. [20]). Additionally, analyses of unfractionated and immuno-depleted plasma by peptide IEF, followed by liquid chromatography tandem mass spectrometry (LC-MS/MS), demonstrated enrichment of non-targeted plasma proteins, as assessed by MS/MS spectral counting. However, either top 7 or top 14 immuno-depletion resulted in a meagre 25% increase in identified proteins compared with unfractionated plasma. Although 23 low-abundance (<10 ng/mL) plasma proteins were detected, they accounted for only 5–6% of total protein identifications in immuno-depleted plasma. In both unfractionated and immuno-depleted plasma, the 50 most abundant plasma proteins accounted for 90% of cumulative spectral counts and precursor ion intensities, leaving little capacity to sample lower abundance proteins. Their conclusions: 'untargeted proteomic analyses using current LC-MS/MS platforms – even with immuno-depletion – cannot be expected to efficiently discover low-abundance, disease-specific biomarkers in plasma'.

2.2 Enrichment approaches

Among different enrichment approaches other than CPLLs, one can briefly recall here aptamer microarrays adopted to identify low-abundance targets in complex serum samples [25], peptide affinity media able to act also under chaotropic conditions [26] and of course the classical approaches for protein families, such as lectin affinity chromatography for glycoproteins, especially when coupled to magnetic bead arrays [27], phophoproteome (and phosphopeptide) capture via, e.g. titanium dioxide beads, often coupled to pre-enrichment on anion-exchangers [28]. Other approaches regard Global Chemoselective Fractionation (GCF) via, e.g. cleavable thiol-reactive compounds capturing cysteinyl peptides [29]. A company called Caprotec (Capture Protein Technology) also offers a variety of small synthetic molecules able to interrogate and isolate families of native proteins via cleavable covalent linkers (in general bi-functional or tri-functional molecules).

Yet, in this scenario, perhaps the most striking technology still remains the CPLL method, that was described just 5 years ago [30] and seems to be able to bring about a big revolution in discovery of low-abundance proteomes. We briefly review here the properties of CPLLs: the library consists of a mixture of porous beads on which hexapeptides are singularly covalently attached. Depending on the number of amino acids used, a library contains a population of millions of different ligands (e.g. 11, 24 or 64 millions starting, respectively, from 15, 17 or 20 different amino acids). Basically, when a complex protein extract is exposed to such a ligand library in large overloading conditions, each

bead with affinity to an abundant protein will rapidly become saturated, and the vast majority of the same protein will remain unbound. In contrast, trace proteins will not saturate the corresponding partner beads but are captured in progressively increasing amounts as the beads are loaded with additional protein extract. Thus, a solid-phase ligand library enriches for trace proteins, while concomitantly reducing the relative concentration of abundant species (something that, of course, immuno-depletion methods cannot possibly perform). While absolute protein quantitation after treatment is no longer possible because of the reduction in the dynamic protein concentration range, the proportionality is maintained, authorizing a relative quantitation as described [31, 32]. This is an important feature for the detection of up-regulated and down-regulated situations when searching for biomarkers. Although we had achieved remarkable results with this methodology, it has recently been criticized in at least two reports (without counting the arguments raised by Solon and Cato) [33]. In one, Bandow [34] while comparing CPLLs (commercialized under the trade name ProteoMiner) with immuno-depletion (Seppro IgY14 System) concluded that in both cases the detectable protein spots in 2-D maps of the different plasma fractions recovered with both methods contained exclusively HAPs normally present in plasma at concentrations between 1 μg and 40 mg/mL, a most disappointing result, to say the least. In another, Keidel et al. [35] asserted that ProteoMiner beads do not act at all according to millions of specific affinities of each hexapeptide with a partner protein, but simply 'according to a general hydrophobic mechanism, where diversity in surface ligands plays only a negligible role'. Both papers [34, 35] indeed seemed to celebrate the funeral of CPLLs, not even in a solemn ceremony. Yet, by taking a close look at such reports, one can see flaws in their arguments and/or experimental protocols. In the first case [34], it was surprising to find that the elution of the captured proteins from ProteoMiner was performed via a mixture of 4 M urea and 1% CHAPS, a too mild protocol ineffective to desorb all proteins. Such an eluant will not even elute 30% of the captured species and certainly not those having high affinity for the hexapeptide ligands, i.e. those trace proteins that had to compete hard with the overwhelming presence of the high-abundance species that might have had lower affinities for the same baits! As a result of the insufficient elution protocol, only the high- to medium-abundance species were desorbed, thus leaving onto the beads the precious booty of LAPs! We have in fact reported that, in order to recover $>99\%$ of the bound species, the beads have to be boiled in 4% SDS containing 30 mM DTT [36]. Other harsh elution protocols have also been suggested [3] but they are most probably less effective than boiling SDS solutions. Moreover, the protein capture was performed at high ionic strength values, thus discouraging binding via ionic interactions, which are the most prominent ones on the peptide baits [10], whose hydrophilicity and charge state is largely superior to their hydrophobicity. This last argument is also valid in the case of Keidel et al. [35]: to state that ProteoMiner acts via a simple hydrophobic mechanism suggests a superficial knowledge of the mechanism of action of such hexapeptides and of the basic rules governing immunoaffinity and the properties of epitopes on proteinaceous surfaces. These authors may have neglected the fundamental functional difference between a mixed bed (e.g. a ligand library) and a homogeneous bed. This is easily understandable by a simple reading of the protein capturing ability of just single amino acids [12] or of peptides of different lengths (from di- up to hexapeptides) [13]. These two papers teach that the picture is totally different and if their voice were to be compared with the brilliant tonality of a Rossini's opera (the celebre cavatina of Figaro 'Largo al factotum' of the Barbiere di Siviglia) it would appear of a rather splintered quality, like a note sounded on a length of cracked bamboo. Table 1 should surely dissipate such clouds and resurrect the CPLL creature in the context of proteomics studies. In fact, in all systems we investigated, we usually detected from three to six times as many proteins as in the control. Which means not the meagre 25% more reported in the case of immuno-depletion [24], but rather 300–600% more. Before closing this section we would like to report the data of Colzani et al. [43] as nicely illustrated in Fig. 3: when analyzing the secretome of two human breast cancer cell lines in culture (called MDA-MB-231, panel A, and MCF-7, panel B), the number of validated human hits in ProteoMiner treated was 400–600% higher than in control, untreated samples (note for instance that, in panel A, last column to the right, 19 proteins were confidently validated as human in the data from the raw conditioned growth medium, while the number increased to 114 in the ProteoMiner-treated sample, this indicating a six-fold increase in protein discovery. The bars 'hits in mammalian database' refer to the identification of all proteins in the cell culture medium, which contains also calf serum; the bars

Table 1. Unique gene products detected in different proteomes (the 'literature best' column photographs the situation available in the literature at the time of publication of each article quoted in the third column)

Sample	Literature best[a)]	Number of species captured[b)]
Human urine	96	495 [37]
Human serum	800	3869 [38]
Egg white	78	148 [39]
Egg yolk	115	255 [40]
Cow's whey	75	149 [41]
Red blood cell cytoplasm	252	1578 [31]
Cerebrospinal fluid	550	1213 [32]
Spinach leaf cytoplasm	100	322 [42]

a) The references to this column are to be found in the relevant paper quoted in next column, since in each of these papers abundant references are made to all previous literature available at the time of publication.

b) In square brackets, the relevant literature reference pertaining to each proteome analyzed with ProteoMiner is given.

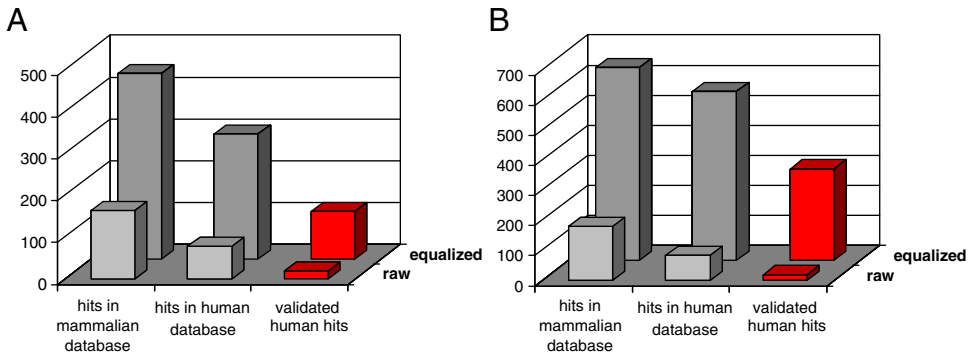

Figure 3. Proteins identified in MDA-MB-231 (A) and MCF-7 (B) conditioned media before (raw) and after ProteoMiner treatment. The raw and ProteoMiner-treated culture media were analyzed by GE-LC/MS (GE: gel electrophoresis). The number of protein hits in the mammalian and human databases, together with the number of proteins validated as genuinely human (containing at least two D8-labeled peptides, where D8 stands for octa-deuterated valine added to the culture medium) are shown in the graph. Together, the 114 proteins identified in the MDA-MB-231 CM and the 303 proteins identified in the MCF-7 CM form a global non-redundant data set of 328 validated proteins. The term 'equalized' refers to samples treated with ProteoMiner, since before their launch by Bio-Rad it was named 'Equalizer beds' (from Colzani et al. [43], by permission).

'hits in human database' now refer only to the cell secretome; finally, the bars 'validated human hits' refer to just those proteins in the secretome that could be correctly assigned to unique gene products). The applicability of ProteoMiner to biomarker discovery has also been elegantly demonstrated in [44].

3 From ProteoMiner to ProteoWiner

There are rumors that in the year 1111 AD the German bishop Johannes Defuk (Johann Fugger in German) made a pilgrimage to Rome, taking the well known (in those days) Via Francigena. He had a friar scouting the territory to mark in each village the best tavern in town, in which, in the evening, they could rest drinking wine to the health of the Pope. The tavern would be marked by the friar with the secret symbol 'est' (the verb 'to be' in Latin, meaning here it is). At one point of the pilgrimage the tavern had been marked by a triple 'est', which induced the bishop to precipitously enter the cellar and start drinking wine nonstop till his belly burst and he landed up in Heaven (or Hell?) and not in Rome. If you visit the town of Montefiascone (close to Rome) you will find a white wine with the label 'EST, EST, EST', in memory of this event. Italian wines have been celebrated since antiquity, starting perhaps with the highly famous Falernum, a most costly wine appreciated by Emperors and patricians in ancient Rome (Julius Caesar spent a fortune on this wine in the year 47 AC to celebrate his victories described in De Bello Gallico). Even throughout the Renaissance Popes and Cardinals greatly appreciated these wines, as nicely told in the detective and gastronomic novels by Barrière [45]. Modern wines, however, might be quite different from those drunk by our ancestors. One of the main reasons is that the residual grape proteins, which survived the fermentation process, slowly aggregate leading to amorphous sediments or flocculates, causing turbidity. A haze or deposit in bottled wine indicates that the product is unstable, has a low commercial value and is therefore unacceptable for sale. For these reasons, it has become customary, especially in white wine, to remove the residual proteins remaining in the finished product, so as to prevent haze formation and sediment in the bottled wines available for sales. Among the fining agents, one of the most popular is casein derived from bovine milk. However, caseins are also known as major food allergens and, therefore, according to the Directive 2007/68/EC of the European Community (EC), 'any substance used in production of a foodstuff and still present in the finished product' must be declared on the label, especially if it originates from allergenic material. Due to the fact that caseins are nearly insoluble at the pH of white wines and that they form insoluble complexes with phenolic compounds, they are considered to be almost completely coagulated and thus eliminated by precipitation after treatment, so no wine maker has reported the presence of caseins in their fined product. Yet, classical chemistry laws suggest that traces of caseins should remain even after their massive co-precipitation with residual grape proteins. Unfortunately, the official ELISA test of the EC has a too low sensitivity limit of 200 μg casein per liter [46]; in other words, not enough to detect traces of it. Just out of curiosity, we have applied the CPLL technique to such analyses, in order to see what could be the lowest possible detection limit. Tiny amounts of beads (barely 100 μL) were added to as much as 1 L of white wine and any captured proteinaceous compound eluted in boiling 4% SDS and 30 mM DTT. As shown in Fig. 4, we could detect as little as 1 μg casein per liter, a 200-fold increment of sensitivity as compared with the ELISA test [47]. But there is even more to be stated in this regard. Just as our paper [47] appeared in *J. Proteomics*, another one was published in *J. Chromatogr. A* [48]: in this last paper, the authors stated 'when fined wine samples were considered, the lowest added concentration for which the peptide marker could be detected was 50 μg/mL (the peptide marker referring to

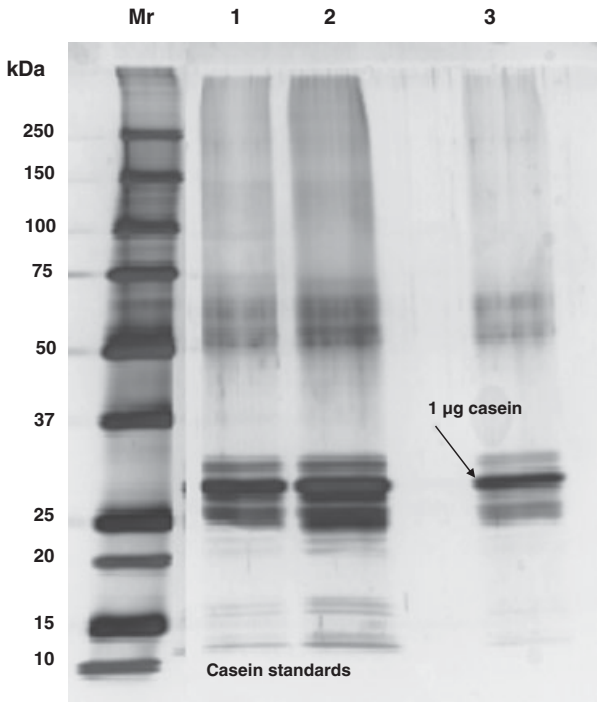

Figure 4. SDS-PAGE of casein standards and a casein eluate from ProteoMiner in a wine-like mixture consisting of 12.5% ethanol in water at pH 3.3 (with acetic acid) added with 1 μg/L of casein. M_r: molecular mass standards; tracks 1, 2: 1 and 2 μg casein standards; track 3: casein recovered from the wine-like mixture. Detection by silver staining (modified from Cereda et al. [46], by permission).

casein digests, as identified by MS) [48]. Now, if we are not mistaken, this means that our CPLL treatment for harvesting and detecting minute traces of caseins in white wines (as well in red wines) [49] has a sensitivity 50 000 times better than the MS method of Monaci et al. [48] (it goes without saying that we too identified the captured caseins via MS). In a third paper, the proteome content of beers was also explored to the maximum extent, permitting visibility of plenty of residual barley proteins (>20) and as many as 40 trace proteins of *Saccharomyces cerevisiae* that nobody ever dreamed to detect (current literature on this topic listed barely two such residual proteins!). So, if you will read what might go down in history as the 'drunkard's trilogy' [47, 49, 50] you will appreciate more and more that the CPLL technology is here to stay and that it might turn out to be the best one for biomarker discovery.

4 Concluding remarks

One might wonder what is behind the curious title of the previous section (ProteoMiner versus ProteoWiner). Well, since our report on traces of caseins in all white wines thus treated [47], plenty of colleagues have written to us stating that now they knew why, after drinking white wines, they experienced stomach ache and even head aches (such minute traces of casein will never give an anaphylactic shock, to be sure, but certainly could produce some general disturbances in the organism of allergic persons). So, just as the ProteoMiner has been patented, we are now offering a 'ProteoWiner', i.e. tiny capsules of CPLL beads that, added to your bottle of white wine, will harvest residual casein traces and let you drink it with full peace of mind!

In conclusion, our data on the alcoholic beverages trilogy [47, 49, 50] have allowed us to quantify some important aspects of the CPLL technology, such as: (i) 80% efficiency of capture of traces of proteinaceous material; (ii) ability to treat large sample volumes (1 L and more) in a simple experimental set-up and (iii) signal amplification factors reaching up to 10 000 folds (four orders of magnitude). Let us drink to that!

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The authors have declared no conflict of interest.

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