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Review

Breakfast at Tiffany's? Only with a low-abundance proteomic signature!

Food science is a complex domain where modern analytical technologies interact with each other. Named as “Foodomics,” this domain assembles a variety of investigational fields such as genomics, transcriptomics, proteomics, peptidomics, and metabolomics. All these technologies are at the service of several goals that are essentially dictated by fundamental understanding, safety, and consistency. In this report, open questions are summarized around proteins and selected technologies recommended for a proper analytical determination not only of composition, but also considering the low-abundance expressed species difficult to detect for their presence, their function, and their adverse effects such as allergic action. In particular, this review covers the proteome of foodstuff and beverages, as detected via combinatorial peptide ligand libraries, such as: bovine and donkey's milk, egg white, and yolk, white and red wine, beer, and a number of nonalcoholic beverages, such as almond's milk and orgeat syrup, Cola, ginger ale, and vinegar.

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Combinatorial ligand libraries / Food chemistry / Low-abundance proteins / Mass spectrometry / Proteomics

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1 Introducing global protein analysis in food

Proteomics investigations with continuously improved technologies started from the elucidation of proteomes to then expand to the identification of differences especially between healthy and pathological situations. The discovery of biomarkers with applications in medical diagnosis and drug development was therefore the natural development. With the advent of novel technologies and the improvement of genomic data banks, proteomics investigations extended toward a variety of domains, one of them being food science. Thus “Foodomics” became the term covering studies in the nutrition domain by means of modern technologies including all those that are currently used in proteomics. In a recent review Herero et al. [1] define Foodomics as a food science domain using modern biology approaches and technologies such as genomics, transcriptomics, proteomics, peptidomics, and/or metabolomics (see scheme of Fig. 1). For all these domains MS and electrophoresis methods are described as powerful tools for investigations of exogenous contaminants (e.g. pesticides), food allergens, and toxins. Foodomics embraces domains that are known as food genomics [2], food allergomics

[3], nutraceutical science [4, 5], and genetically modified food [6, 7].

Deep genomic investigations started years ago and hence a number of data are now available. However, genomics is far from delivering complete information; for example even if, by comparison, molecular components among different plants appear orthologous, their function could be very different [8]. Therefore functional genomics with the objective of assigning a role to each gene is yet a large task to be completed. Transcriptomics, as the next level, made great advancements even if it is not informative enough about protein expression and even worse for posttranslational modifications. This is the reason why proteomics appears irreplaceable in the process of biology/biochemistry knowledge especially with respect to Foodomics where numerous parameters interact with each other. Actually, it probably is the only option to identify and understand posttranslational modifications, interaction of proteins with metabolites (e.g. transport function) and protein–protein interactions. Proteomics also allows determining if food protein components have beneficial or detrimental effects on the organism that receives them.

One goal in current food science is to enhance the level of understanding of food components via the composition and the function of single components. Already in 2004 protein profiles derived from proteomic technologies have been established for assessing food composition, its origin and possible “adulteration” [9]. There are, however, other goals that are also of interest such as how health could be improved by using genetically modified food conferring targeted

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Abbreviation: CPLL, combinatorial peptide ligand library

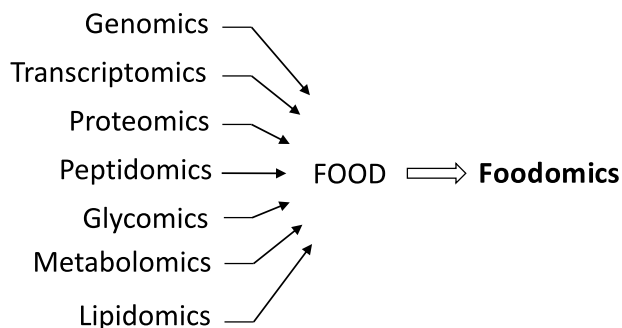


Figure 1. Schematic representation of the dependence of food science on technologies that have been developed separately over the past 20 years. All together when applied to food analysis they form the domain of Foodomics.

pharmacological properties and use of it instead of drugs [10].

Today, the situation is more complex because of the possible presence of genetically modified organisms added to food where they can induce unexpected undesired effects. A transgenic organism is considered safe for human consumption if, by its documented history, is found equivalent to its conventional counterpart. In addition, encoded recombinant information should not generate toxic or allergenic reactions [11]. As reported by Simó et al. [12], investigations about protein expression of transgenic food are of particular interest due to their potential capabilities to induce allergic effects or provide unwanted enzymatic or inhibitory activity and also possible toxic effects. In this respect such transgenic organisms require targeted studies in protein expression alterations that are necessary to decipher when they are present even in a very limited extent. The example taken by Simó et al. was soya beans where no expression differences could be detected. However, it has been reported that variations in storage proteins ratio (that represent more than 80% of the proteome with a large preponderance of 11S- and 7S- globulins) could impact functional and nutritional properties with possible implications in safety [13]. Within this context proteomics investigations are increasingly used to screen and differentiate transgenic plants in order to either evaluate food safety or identify transgenic components in food [14]. Expression changes that are not exclusively the result of transgenicity can be species specific with possible implications in human allergies as described in [15], highlighting the importance of these studies.

There are two reasons justifying the development of transgenic plants for food. The first is related to the economy as a whole. Certain plants are expensive and not productive enough to cover the request; they need too much care and water and are poorly resistant to adventitious agents without massive use of pesticides. To respond to these issues some genetically oriented modifications are necessary for some current plants [16]. The second reason justifying the modification of plant genomes is to enhance expression of specifically targeted molecules that are useful to alleviate

given diseases or to improve the quality of life. Whatever the justification, transgenic versus nontransgenic plants intended to be used as food components must be analyzed to evidence expression differences and possible effects on the receiving host [17, 18]. The proteome equivalence between a regular organism and its genetically modified counterpart becomes thus a real investigational work in proteomics. As an example, soybean was recently scrutinized in a comparative manner [12]. In this case no detectable changes have been observed by using capillary electrophoresis followed by MS. However, the proteome analysis did not consider specific sample enrichment treatments allowing further investigations toward low- and very low-abundance gene product detection.

Globally the term Foodomics assembles modern disciplines and technologies to keep track of all the above food components, origin, and effects. Thus genomics, transcriptomics, proteomics, peptidomics, and metabolomics are part of this field with the aim of discovering the composition, noting differences among samples, exploring nutrition properties and managing the questions around genuineness, quality, expected effect and evidence of agents that are harmful for human health. The complexity is therefore not only played at the level of study disciplines such as genomics versus proteomics or metabolomics, but also in terms of differential expression between species and in terms of selection of investigation technologies. One of them is a global analytical attack involving proteins from few or many organisms depending on the food composition. It is a very ambitious approach since it must federate technologies of protein extraction that are not necessarily the same when dealing with plant extract compared to microorganism and/or animal extracts. Another immense technical challenge is how to deal with the protein dynamic concentration range when the food combines component from animal, plants, and other organisms all having their own extremely large difference in the concentration of high-abundance versus low-abundance proteins.

To reduce the level of complexity, in this review we decided to limit the discussion to protein components of singular food sources and to focus on low-abundance species that represent by themselves a quite complicated issue.

2 Sample treatment prior to protein detection

Depending on the type of foodstuff, the protein content is extremely variable. Moreover, when food is the result of preparations involving blends from vegetable and/or animal origin, the complexity reaches levels that can be quite difficult to manage. In all cases the overall protein analysis requires a pretreatment of the initial material at least to remove non-proteinaceous components and then, if necessary, adopt treatments for unmasking very dilute species. Finally, the specific removal of some proteins or the enrichment of trace components might be desirable.

2.1 Extraction and pretreatment

The largest challenge is represented by the extraction of plant proteins (vegetable-based food) since they are generally accompanied by vegetal pigments, polysaccharides, lipids, and polyphenols. In addition numerous plant proteins are heavily glycosylated, thus adding difficulties for analyses such as, for instance, difficult ionization in MS and abnormal migrations in 2DE. In this respect, plant proteomics teaches how to make a proper cleanup of these samples with the minimal protein losses. Since in differentiated plant tissues soluble protein concentration is generally very low [19], and contains a number of undesirable metabolites interfering with protein separation and analysis [20], before initiating a proteome investigation several points are to be considered. The most common approaches are extraction and precipitation (see Carpentier et al. [21]). For the extraction phase the following general rules apply: (i) with highly viscous material, such as eggs (white and yolk) a dilution with energetic agitation is recommended; (ii) protein extraction should be performed in relatively low ionic strength to prevent the solubilization of nucleic acids and in the presence of small amounts of chelating agents such as EDTA; (iii) for the extraction of proteins engaged within the cell walls, some amounts of nonionic detergent (less than 0.5–1%) and urea (less than 3 M) should be added to the extraction buffer. Finally, since plant extracts contain a quite powerful protease charge, the addition of inhibitors is highly recommended.

The removal of the undesired material mentioned above is performed as a second operation just after the initial crude extraction. Precipitation and/or selective extraction are considered, depending on the context. For instance the removal of lipids requires special attention since their presence can render the subsequent proteomics analysis ineffective. The presence of detergents in the extraction solution should disaggregate lipids while solubilizing hydrophobic proteins. However, if large amounts of lipids are present, as for instance in seeds, chemical delipidation prior to protein extraction might be necessary [22]. Lipid removal is in this case achieved by extraction with hydrocarbon solvents [23] or of mixtures thereof [24], or even containing chlorinated solvents [25]. Partial, but useful, delipidation with ethanol or acetone can also be adopted [26]. Precipitation of proteins using TCA associated with acetone and containing reducing agents allows collecting most proteins as pellets with a supernatant comprising a lot of undesired materials that is eliminated by centrifugation or filtration [27]. Interfering substances can be discarded by using a Tris-HCl solution saturated with phenol followed by protein precipitation with ammonium acetate or ammonium sulphate. This option is especially recommended when the analysis of proteins is based on 2DE. As a variant, the combination of phenol extraction followed by a precipitation with ammonium acetate in methanol has been described by Faurobert et al. [28]. Another less popular method is protein precipitation using a chloroform-methanol mixture with water in 1–4–3 proportions [25]. The following step, which is the resolubilization of precipitated proteins, is not always easy

depending on the nature of proteins. Quite frequently, the presence of zwitterionic detergents and chaotropes is necessary [29].

Animal protein extraction is probably easier than plant. In food, proteins are generally present as tissues and are involved in membranes. The cell membrane is the result of a complex structure involving proteins and lipids. The latter could be structurally different and contain phosphoric esters. Proteins have generally a central hydrophobic segment fully involved in the architecture of the membrane and two extremes that are either exposed to cytoplasm or to the extracellular domain. These proteins are so tightly involved in the membrane structure that is difficult to displace them except when the construct is destroyed by means of buffers containing detergents and possibly chaotropic agents such as urea [30]. Reducing agents, selected cations, sugars, and other chemical agents may also be beneficial to protein solubilization. All these preliminary operations for plant and animal protein extraction do not only remove undesired materials, but also contribute to concentrate proteins that are present in very low amounts and in a quite large number. They are also beneficial when the sample is to be treated with CPLL (combinatorial peptide ligand library). In fact the presence of polyphenols and other polyacidic substances (mucins, nucleic acids, etc.) prevents the proper capture of proteins by peptide libraries. However, the presence of solubilizing agents such as highly concentrated chaotropes and SDS must also be eliminated prior to CPLL treatment by, for instance, protein precipitation or dialysis.

When devising protein extraction methods the compatibility of an initial sample treatment with further processing, such as for instance protein fractionation and proteomic analysis, should be kept in mind. Protein extracts that will be submitted to 2DE analysis should be in fact preferably neutral and in low ionic strength solutions without traces of ionic detergents such as SDS. If the proteome analysis is performed by top-down MS, the protein sample needs also to be cleaned up before being loaded on the MS probe [31]. When using protein arrays (e.g. antibodies) the protein extract should not comprise denaturing agents that prevent the correct formation of antibody-antigen complex. In bottom-up MS where peptides are directly analyzed, there are other rules to follow depending on the method for the separation of peptides. When using for instance capillary electrophoresis the problem of nonspecific binding of molecules on the inner capillary wall results in poor separation efficiency as a consequence of band broadening [32]. Conversely, when using reverse phase chromatography, alkaline peptides may be extremely tightly adsorbed by the solid phase and frequently escape the MS analysis.

2.2 High-abundance protein depletion. Is it applicable?

Evidencing low-abundance proteins from whatever extract is always challenging. There are two main reasons for this

Table 1. Examples of high-abundance (HA) gene products present in various extracts

HA protein	Origin	Prevalence (%)	Reduction method	Selected references
Albumin	Serum, CSF	50–60	Ab, CPLL	[33,34]
IgG	Serum, CSF	10–15	Ab, CPLL	[34,35]
Hemoglobin	Blood cell lysate	85–95	IEX, CPLL	[36,37]
Ovalbumin	Egg white	35–45	CPLL	[38]
Lactalbumin	Milk	30–40	CPLL	[39]
β -Lactoglobulin	Milk	15–20	CPLL	[39]
RuBisCO	Plant leaves	40–60	Ab, CPLL	[40]
Actin	Cell extracts	15–20	CPLL	[41]
Storage proteins	Potato tubers	70–80	IEX	[42]
Endosperm storage proteins	Rice	30–50	Chromatography	[43]

difficulty: (i) the low amount of these proteins falls under the detection limits of the current analytical tools and methods or (ii) few high-abundance proteins are so largely present that the detectability of dilute species is prevented as is the case, for instance, of the detection of sky stars during a sunny day. In Foodomics there are many examples of products that comprise proteins of high abundance: ovalbumin in eggs, RuBisCO in leaves, actin in animal tissues, lactalbumin in milk, to name just a few. Table 1 describes the high-abundance species from several origins and their possible reduction. To facilitate the analytical determinations a relatively common process is to remove all or most of the high-abundance proteins (depletion). Depletion technologies started with the removal of high-abundance proteins from human serum [44,45]. First, current affinity sorbents were used to remove albumin and immunoglobulins G to end up to the generalized use of immunosorbents to remove more specifically several proteins at the same time [46]. Such process is limited to the upper part of protein expression scale and by consequence minor components that represent the largest number of gene products remain as dilute as they were before the treatment and, worst, frequently more dilute. These proteins are thus still undetectable, unless they are submitted to a concentration process. Even so, due to very limited volumes of biological samples submitted to immunosubtraction, the amount of these low-abundance species is frequently below the sensitivity of analytical methods. Moreover with immunosorbents for depletion there is a constant risk of protein codepletion [47] that has nothing to do with the antigen-antibody interaction. This phenomenon can reach devastating proportions [48] with a loss of hundreds of codepleted proteins [49]. The immuno-depletion process is not only protein specific, but also species specific and cannot be used as general tool in Foodomics. In fact, contrary to what is largely applied to human serum, specific antibodies against numerous proteins of different origin are not necessarily available. Thus, the way for revealing low-abundance species is here consequently restricted to the use of tools that are capable to enrich for low-abundance proteins while decreasing the protein dynamic range or are designed for the capture of predesignated species (see next two Sections).

2.3 Enrichment of low-abundance proteins

In the past few years, a general method to significantly shrink the dynamic concentration range by the concomitant decrease of high-abundance and the enhancement of low-abundance species has been described [34,50]. This process is operated by the so-called combinatorial peptide ligand libraries (CPLL) and has been applied to a large number of cases as for example biological fluids [51], cells [37], and tissues [52] from human, animal, and plant origin. These libraries are made of peptides, each of them individually grafted onto distinct beads in many copies. Current CPLL comprises several millions of diverse peptides. When a protein mixture is exposed to such a peptide library specific partnerships between peptides and given proteins are formed like in a multitude of single affinity chromatography units. If the protein loading largely exceeds the binding capacity interesting phenomena take place. High-abundance proteins saturate rapidly the corresponding affinity beads and the protein excess remains in the supernatant while low-abundance species continue to be captured by their partner bead as long as the sample is available. These two concomitant events result in a concentration of low-abundance proteins and a reduction of the amount of high-abundance ones, reducing thus the dynamic range. Another interesting thermodynamic phenomenon takes also place: since a given protein may have an affinity for several peptides (several beads) and one peptide bead may capture several proteins, an intense displacement effect takes place, governed by both the binding constants and the concentration of each protein of the sample. Once the system reaches its own equilibrium, the excess of proteins in the supernatant (those that have saturated the corresponding partner beads) is eliminated by filtration or centrifugation and the captured proteins desorbed by appropriate elution agents. The eluted protein pool comprises all species present in the initial sample but high-abundance proteins are significantly reduced while the low-abundance ones are enriched.

Adsorption and elution are two distinct steps of the entire process both impacting the final result. The adsorption impacts the enrichment of low-abundance proteins: the larger the sample loading, the more dilute species become concentrated and thus detectable. Elution is critical but for other

reasons: (i) it must be strong to desorb all captured proteins, (ii) eluting agents have to be selected to be compatible with subsequent analytical determinations, and (iii) desorption of proteins should be performed sequentially by groups simplifying thus the following analysis. Details of the process and related variants are described in a recent review [53]. Ideally one would start from large amounts of proteins (at least 50 mg) with a volume of CPLL beads of 100 μ L. However, a recent report demonstrated that it is possible to use smaller volumes of dilute biological fluids such as cerebrospinal fluid [54] with a smaller amount of CPLLs. In this case, instead of eluting the captured proteins, it could be preferable to operate the direct trypsinization on the beads, followed by a LC-MS/MS analysis of the obtained peptides [55].

This sample treatment involving CPLLs is highly reproducible as repeatedly reported (see for instance Li et al. [56]), but, as a result of the relative protein concentration change, the absolute quantitative protein determination is questionable. In spite of this change, the proportionality between species of two or more samples evaluated in parallel and under the same conditions is maintained, thus authorizing a relative quantitation, as described [54], with the objective of discovering possible protein markers. It is here important to mention that there are other methods capable to enrich in low-abundance species; however, they do not compress the dynamic concentration range, but rather focus on specific groups of proteins such as phosphoproteins [57, 58] and glycoproteins [59]. Aptamers have also been used on microarrays for the enrichment and identification of low-abundance targets [60].

3 Food proteomic examples and potential applications

Although food is a necessity to the survival of living organisms, it may also represent a number of risks inherent to the composition and origin. Presently, with the industrialization progresses, the food composition has become more and more complex involving a large number of ingredients of vegetal and animal source. To this picture, it is also important to add that primary material for food preparation can come also from genetically modified organisms for reasons that are, as mentioned above, related to improved resistance to given attacks or because the expression of specific components is intended to enhance selected pharmaceutical properties (nutraceuticals).

Within this complex situation global analysis can contribute to understand the protein composition of genuine aliments and their identification. Alternatively, comparative proteomic analysis, such as side-by-side pattern observations, allows improving the knowledge of composition and variability. Finally, the determination of the presence of the allergens could help to evidence the presence of food components that are very difficult to identify when their presence in the food is very low. This last point is extremely important considering

that food allergens are quite common and that they act at trace level.

3.1 Global proteome analysis

Proteome patterns are more and more known in animal, plant, and microbial organisms. The analysis is generally performed either after the separation of protein components or by the direct sequencing of peptides. Most known methods are electrophoresis (mono- and two-dimensional), chromatography, and MS. When they are applied to intact protein samples they are generally classified as top-down proteomics analysis. Bottom-up proteomics analyses at the contrary are performed after having treated the global protein sample with proteolytic enzymes (generally trypsin) and the resulting peptides fractionated and finally analyzed by MS. Whatever the approach the final objective is the identification of all protein components. Nevertheless, to reach the so-called deep proteome (very low concentration proteins) it is necessary to start by an enrichment step. This can be easily done by the use of CPLL capable not only to concentrate low-abundance species, but also to largely reduce interferences due to the presence of very high-abundance proteins. This approach, very popular in fundamental proteomics studies, is not well known in food analysis yet. One main criticism of this technology is the requirement of a relatively large amount of proteins (the larger the better for the enrichment in low-abundance species). Although this limitation is critical for clinical analysis where the biological samples are extremely small, it is not a limiting factor in Foodomics since food can be obtained in relatively large quantity as for instance few grams of proteins or more. To illustrate its capability several examples are given in Section 3.3.

3.2 Targeting specific aspects

Proteins in food can also have other interesting functions than just being a source of energy. Food could carry proteins beneficial to human health. There are for example proteins with antibacterial effect such as lysozyme, a well-known small protein found in egg white [61] and also in milk [62, 63]. From milk the action of lysozyme could also be enhanced by the presence of lactoferrin and lactoperoxidase. These two proteins are extracted from bovine milk and then reinjected in specific preparations to improve the antimicrobial properties [64].

Proteins with anticoagulant properties are present in echinoid worms [65]. Antihypertensive properties are found in tuna proteins [66]. To complete the picture proteins with antioxidant properties and hence antiaging effects, are present in many living organisms and particularly in vegetables [67].

In food preparations, all above pharmaceutical properties can be conferred by simple addition of selected animal or plant tissues or of (semi)purified proteins. Here, the proper analysis of resulting food in terms of consistency and effects

is to be performed by proteomics analysis of the whole food preparation and preferably by means of sample preparation tools that allow a simple access to species that are present in trace amounts.

It is known, however, that not all proteins are good for eating. Food could be a source of adventitious proteins detrimental to individual or collective health such as polypeptidic toxins.

Food can contain powerful allergens of protein origin with dramatic effects. Their stability against proteolysis that normally operates at the level of digestive tract induces direct sensitizations once in the intestinal tract generating sometimes dangerous systemic allergic reactions upon ingestion [68].

The precise prevalence of food allergy is not yet well understood, however it is generally admitted that it rises over time. Allergenic proteins are found in many types of food, but most commons are nuts, fruits, fish, shellfish, seeds, various vegetables, eggs, and milk. Reactions can be extremely rapid and intense even if protein allergens are present in trace amounts. It is thus essential before ingesting food preparations to know exactly the composition, which can only be accessed by a global proteomic analytical approach. Since protein allergens can come from bacterial, animal and plant source a generalized protein analysis is mandatory. In a recent publication Waserman et al. [69] pointed out the danger of some food components and provided data on pathophysiology, diagnosis, and food allergy management. Nevertheless prevention is essential and can be approached by appropriate proteomic analysis.

A specific source of allergy not well documented yet is related to transgenic organisms that could be used as food additives. Even if assessing that the protein expression of genetically modified food might have allergic effects is yet speculation, an in depth analysis including very low-abundance proteins generally undetectable appears more than useful at least as long as the knowledge in this domain is not well developed. CPLs might positively contribute to this enterprise. The technical scheme illustrated in Fig. 2 indicates how the presence of allergens and their identification can be performed. It consists in separating first the protein extract by 2DE (preferably after treatment with CPLs) covering isoelectric points between 3 and 11. The 2D map is stained first with regular protein dye (e.g. colloidal Coomassie Blue), a picture is taken and then it is blotted against the serum of allergic patients. After the entire process, the positive antigen-antibody spots are compared to the gel plate and then excised. Each of them is separately trypsinized and the resulting peptides analyzed by LC-MS/MS for antigen identification. This method allowed already discovering new allergens of low-abundance from various extracts [39, 70, 71].

3.3 Selected proteome analyses of food products

Rice is a very important natural food resource for humans and, as such, it deserves a special attention in terms of food

safety and function. A nice review on the matter has been recently published [72]. While the study of rice proteins is largely underlined for their importance in a variety of domains, the authors made a special focus on the extraction of proteins that was designated as critical to cover at the best the proteomic studies. In this respect, a special sample treatment is described for the largest protein harvesting before proteomic investigations. Elimination of nonproteinaceous material with extraction of difficult proteins is reviewed with indications about how proteins are precipitated and then reconstituted.

Once the proteins are extracted, the selection of low-abundance species is another crucial step in the understanding of protein families that are expressed in low copies. An example is given by RNA-binding proteins that play a central role in the regulation of seed storage proteins [73]. There are several strategies to extract this group of proteins; a well-established one is the use of affinity chromatography using PolyU Sepharose [74]. Among the RNA-binding proteins in rice seeds, 14–3-3 proteins were identified as in the case in *Arabidopsis thaliana* [75]. In the latter plant the number of RNA-binding proteins is predicted to be a group of more than 200 species that is by itself indicative of the amount of work that remains to be done in low-abundance proteins detection and identification in rice.

Among examples of proteomics analysis of important food products are potato tubers [42]. They actually constitute a large part of the diet in northern countries under different forms. As such this plant has stimulated specific genomic modifications in view of better resistance against insects. To this end protease inhibitors have been encoded in plants to prevent insect aggressions. The impact of these manipulations to endogenous proteases has been recently investigated for tubers from potato lines expressing a broad spectrum of protease inhibitors in view of detecting undesired effects. Protein extraction of lyophilized tuber powder was performed in Tris-HCl buffer containing sucrose, $MgCl_2$, and PMSF in the presence of 1% PVP. From the initial extract, proteins were precipitated in methanol/chloroform and separated by centrifugation. They were finally redissolved in 0.5% IPG buffer containing 8 M urea, 2% CHAPS, and 60 mM DTT. Mono-dimensional as well 2DE separations were performed followed by immunodetection and MS. The obtained patterns did not show quantitative significant variation among the lines. However, for few proteins that were independent on transgene expression, some differences appeared. They seemed related to the patatin storage protein complex. On the contrary, protease inhibitor profiles seemed substantially unchanged suggesting no intrinsic allergenic potential with substantial equivalence to nontransgenic standard tubers. Here, again the analysis was restricted to the protein extract with no focus on the low-abundance protein enrichment where the expression could have been largely modified.

Another essential source of nutrients for animals and humans is milk, which today represents a key raw material for food preparations. It comprises proteins of nutritional

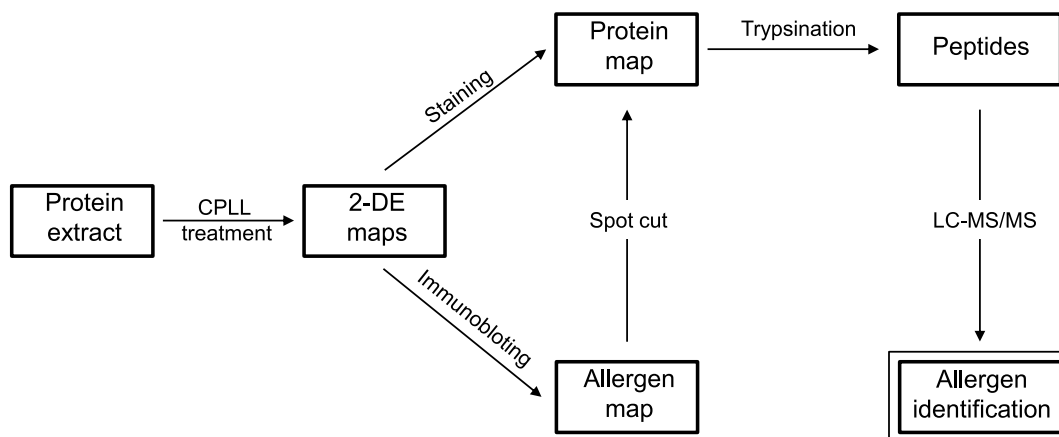


Figure 2. Schematic representation of the process of allergen discovery using first a 2DE separation followed by a reaction with patient's serum containing IgE against presumed allergens from the extract. The identification is operated by LC-MS/MS of individual IgE-positive spots. If the sample is treated with CPLs, additional positive spots can easily be evidenced as a result of "amplification" of the antigen-antibody signal. This is a simple way to detect novel allergens in food.

and health interest possessing, among other effects, bone development/protection, cellular growth and immuno-system stimulation. Bovine milk proteins are brought to regular daily food under different forms and can be modified during processing transformations. As described by Arena et al. [76], thermal treatments modify proteins by the formation of lactose adducts. These modifications need to be monitored because important protein functions might be altered upon physicochemical treatments. The authors have thus analyzed the proteome with the help of CPLs in order to evidence-specific transformations that are of low abundance. After enrichment and protease digestion, the resulting peptides were submitted to a separation on a phenyl-boronic acid sorbent to harvest lactosylated forms.

Large modifications were observed not only on major milk proteins, but also on those that are classified as very low-abundance species that could have important functions on infant nutrition, defence against external agents, and development. The authors concluded that the described modifications, in particular to proteins that are resistant to digestion and act at intestinal level, could enhance the allergic response since lactose molecules grafted to proteins have already been described as hapten-like antigens [77].

As probably all biological materials, milk comprises a large number of low-abundance species that are undetectable with current methodologies and instruments with no preliminary treatment. In a general study, the enrichment process involving CPLs was applied to bovine milk after having eliminated fats and casein and the results of investigation were quite impressing [39]. From 84 gene products found in the untreated milk, 65 additional proteins of presumably low abundance were found. Among them were numerous enzymes involved in sugar metabolism such as UTP-glucose-1-phosphate uridylyltransferase, glucosidases, aldose 1-epimerase, fructose-1,6-bisphosphatase, and tissue alpha-L-fucosidase. It was also very interesting to use the CPL extract for the detection of potential allergens. In this respect isoelec-

tric separations were blotted with the serum of two dozens of patients allergic to bovine milk. A polymorphic alkaline protein was observed with a strong positive signal identified as an immunoglobulin fragment, a minor protein that had been largely amplified by the treatment with CPLs. Few but other potent allergic signals were also found at very acidic protein levels, but they had not been formally identified. The individual allergic protein pattern was always largely heterogeneous underlining the fact that allergy is probably not restricted to a single protein and that a given protein could react differently from patient to patient.

To complete the picture of bovine milk, a recently reported study included a list of more than 500 proteins with the aim of understanding their function [78]. Several expected functional families were found such as transport, immunosystem response and lipid metabolism. Interestingly other groups were found such as proteins involved in anatomical development and cellular proliferation. Clearly the findings from this important dietary product are far from being all discovered and are presumably related to species that are of very low-abundance and probably biologically very active. An in depth investigation was recently performed on donkey's milk, today categorized among the best mother milk substitutes for allergic newborns, due to its much reduced or absent allergenicity, coupled to excellent palatability and nutritional value. By exploiting the CPL technology, and treating large volumes (up to 300 mL) of defatted, decaseinized milk (whey), Cunsolo et al. [79] identified 106 unique gene products, by far the largest description so far of this precious nutrient. Due to poor knowledge of the donkey's genetic asset, only 10% of the proteins could be ascertained via the *Equus asinus* database; the largest proportion (70%) could be designated by homology with the proteins of *Equus caballus*. All these studies confirmed that milk is an important source of active proteins fully involved in the growth and development particularly for newborns. The dynamic changes of low-abundance proteins over a 12-month lactation period has been investigated using CPL

by Liao et al. [80] and interesting data reported. Thirty-eight never-described milk proteins have been reported along with differences in low-abundance proteome patterns all along the lactation period. Outside the fact that such a study contributes to enhance the knowledge in milk protein composition and function, it opens the door to possible development in terms of nutrition needs for advanced artificial formulations.

With the objective of the largest protein identification, the CPLL technology was applied also to other products of food interest as for example egg white [38], egg yolk [81], and spinach leaves [82]. Eggs are largely used as food or food component throughout the world. They bring essential elements to the life, among them proteins with various important functions. They are extensively used in food industry as gelling, emulsifying and foaming agents. In addition to their nutrition importance, egg components are used in the cosmetic and pharma industries. The large majority of investigations performed on egg proteins (white and yolk separately) is limited to detectable species without preliminary sample treatment. However, CPLLs were used to investigate the composition of the deep proteome with a significant degree of success. The egg white proteome is composed of a large number of proteins where 85–90% is represented by ovalbumin, ovotransferrin, ovomucoid, lysozyme, and ovomucin. The remaining proteome includes very many minor undetectable proteins poorly known, but essential to biological processes. From both yolk and egg white the number of identified gene products upon the use of peptide libraries was more than doubled (to 148 for egg white and to 255 for yolk) increasing thus largely the repertoire of known proteins from eggs and hence contributing to a better understanding of nutritional effects. Unfortunately eggs could produce allergic adverse effects with individual reactions and other health risks including asthma. These reactions are attributed to the presence of ovalbumin, but other proteins present in much smaller amounts could have even more sensitization effects. Curiously, Mann and Mann [83] reinvestigated the egg white proteome with the use of the LTQ Orbitrap Velos – a very sensitive MS equipment – and identified just about the same number of proteins as reported in [38]. Their conclusions were that one does not need CPLLs to amplify the signal of the low-abundance proteins, a Velos seemingly doing the job in the untreated material. However, it turns out that most of the proteins reported are not comprised in the list of D'Ambrosio et al. [38] and 44 of them could only be tentatively identified. Thus, one wonders if German eggs are genetically different from Italian eggs, or if, in reality, the presence of the five very high abundance proteins does indeed hamper proper MS analysis.

Spinach leaves are a common green vegetable food-stuff. Recently, the low-abundance proteome analysis was performed by both 2DE and MS after having enhanced proteins of very low concentration [82]. After initial extraction, a protein treatment with CPLLs was performed at three different pHs to capture species that are normally undetectable and thus explore in depth the proteome. The protein recovery from beads was obtained by SDS global elution and the elu-

ates blended together prior to analysis. The number of gene products found exclusively thanks to the use of CPLLs and classified as low-abundance was particularly high, with 208 proteins versus 114 found in the initial extract with an overall gene product count of 322 (Fig. 3). Two main phenomena allowed detecting so many more proteins: the massive reduction of the concentration of RuBisCO and the enrichment of many rare proteins initially present in trace amounts made possible by the use of a relatively large initial volume of spinach extract. The use of CPLLs at three different pHs enlarged the harvesting of species that are particularly acidic and alkaline. Among the enriched cellular components were ribosomal proteins and their complexes. Many of those exclusively found after CPLL treatment were 30S, 40S, 50S and 60S. Other low-abundance proteins found in leaf spinach were, as expected, those proteins related to chloroplast category and those operating in the photosynthetic organelles, for example inorganic pyrophosphatase-1 and phosphoglycerate kinase. From the same category, but involved in the CO₂ fixation and carbohydrate metabolism, were glutamate decarboxylase 2, glucose-1-phosphate adenyltransferase, phosphoglucomutase, fructose bisphosphate aldolase 2, ribulose-phosphate 3-epimerase, and sucrose-phosphate synthase. In spite of numerous novel gene products found many others could not be identified because of lack of genomic data available.

3.4 Selected proteome analyses of beverages

As stated in the introduction, we will limit this survey to beverages that have been explored by the CPLL methodology, in comparison with untreated samples, as reported in the literature. We will only deal with proteomes, although in such beverages also a peptidome screening could be performed with CPLLs. For peptide analyses, one is referred to a series of surveys published almost biannually by Kašička [84–90], which can be used as the Arianna's thread to help the readers out of the labyrinth.

In the last couple of years, we have applied the CPLL technology to the much-unexplored kingdom of beverage proteomes, both alcoholic and nonalcoholic. In the first case, we have analyzed white [91, 92] and red [93] wines, as well as beer [94]. We could indeed demonstrate the unique performance of CPLLs, such as: (i) the ability to handle large sample volumes (one liter and more) in a easy and user-friendly protocol; (ii) sample enhancement factors up to four orders of magnitude; (iii) extremely high detection sensitivities, reaching as little as 1 µg protein/L of sample (in the case of casein detection of white wines), a sensitivity rarely obtained by present-day methodologies. Needless to say, these results were much superior than those obtained with conventional techniques, due to their inability to properly concentrate and "normalize" the relative concentration of the proteomes under analysis [95–102]. Just as an example, in an untreated Recioto dessert white wine [92], we could detect as many as 106 unique gene products, versus, for example, just nine species in Champagne [100] or 28 glycoproteins in a Chardonnay white wine

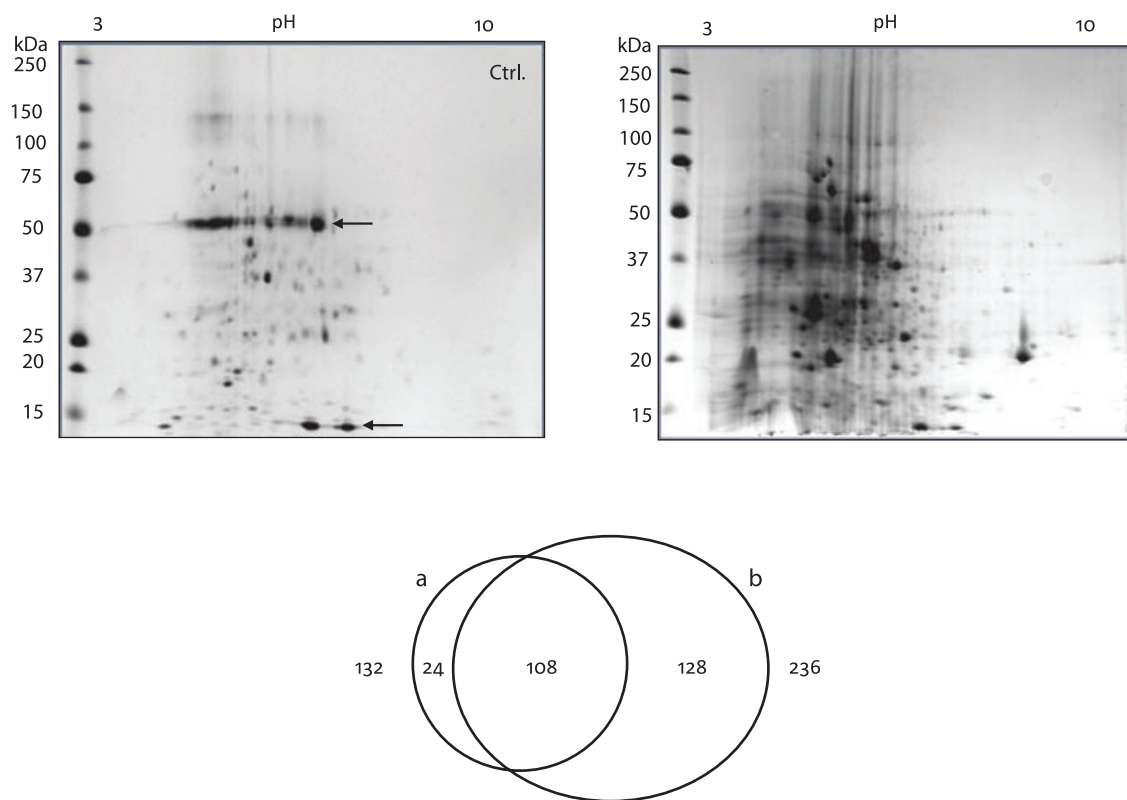


Figure 3. Proteomic analysis of spinach leaves by 2DE and MS. The upper part represents the 2DE maps of proteins before and after treatment with CPLs at pH 7. The lower part is a Venn diagram of the number of proteins identified by LC-MS/MS before “a” and after “b” treatment with CPL. In spite of a relatively large number of proteins in common, the use of combinatorial peptide ligand library allowed evidencing the largest number of proteins that were not detectable before and hence considered as low-abundance species. Adapted from Fasoli et al. [82].

[92] (the latter targeted via affinity capture and comprising 13 grape and 15 yeast proteins, whereas the 106 species found in Recioto were all grape proteins!). Kwon [95] also reported a grand total of 20 proteins, but only five from grapes, twelve from yeast, two from bacteria and one from fungi. So it would appear that the booty obtained via CPL treatment is by far more abundant than with any other technique. Some interesting reviews on wine analysis have also been published [103–105].

An interesting example on the power of the CPL methodology comes from our work on the beer proteome [94]. With the help of CPLs, we could identify 20 different barley protein families (the most abundant ones being Z-serpins and lipid transfer proteins). Yet we could also detect two maize proteins. Indeed, it is stated by the producer that their beer is made with a mixture of two grains, barley and corn. But there is more to it: in addition to those, we could identify 40 unique gene products from *Saccharomyces cerevisiae*, one from *S. bayanus* and one from *S. pastorianus* as routinely used in the malting process for lager beer. Thus it is proven that CPLs permit to ascertain the brewing process and attest the genuineness of the beverage under analysis.

The natural extension of this work was to explore the trace proteome of nonalcoholic beverages, for at least two

reasons: (i) in order to certify the genuineness of such products and find out if they contain proteins of the vegetable extracts they have been prepared from; (ii) in order to screen for the presence of any potential allergen in such beverages. Indeed such analyses have been made on almond's milk [106] and coconut milk [107], as well as on a Cola drink [108] and even white-wine vinegar [109]. In the case of almond's milk [106], we have been able to identify 137 unique protein species, the deepest investigation so far of the almond proteome. In the case of orgeat syrup, a handful of proteins (just 13) have been detected, belonging to a bitter almond extract. In both cases, the genuineness of such products has been verified. On the contrary, cheap orgeat syrups produced by local supermarkets and sold as their own brands, were found not to contain any residual proteins, suggesting that they contained only synthetic aromas and no natural plant extracts. In the case of coconut milk [107], a grand total of 307 unique gene products could be listed, 200 discovered via CPL capture, 137 detected in the control, untreated material and 30 species in common between the two sets of data. This is by far the most extensive mapping of this nutritious beverage, in which, up to the present, only a dozen proteins were known, those belonging to the high- to very-high abundance class. This unique set of data could be the starting point

for nutritionists and researchers involved in nutraceuticals for enucleating some proteins responsible for some of the unique beneficial health effects attributed to coconut milk. An interesting detective story can here be told about the “invisible” proteome of a Cola drink [108], stated to be produced with a kola nut extract. Indeed, a few proteins in the Mr 15 to 20 kDa range could be identified by treating large beverage volumes (one liter) and performing the capture with CPLs at very acidic pH values (pH 2.2) under conditions mimicking reverse-phase adsorption. It turned out that the proteins identified were barely three: one belonging indeed to the kola nut, the other two identified as agave species. Indeed, in this beverage label it was stated that the Cola drink had been generously sprinkled with 6% agave syrup, honour saved, all around. Conversely, things did not go so well (for the producer) in the case of a ginger beverage [110]. Although in traces, we could confirm the presence of five grape proteins and one apple protein, but not even the faintest trace of any ginger root proteins. The first two findings are correct, as the producer stated that this beverage had been reinforced with 12% grape juice and 6% apple juice, but the absence of even traces of ginger proteins did not permit the classification of this beverage as a ginger extract on a proteomics scale. It was thus concluded that either the *Zingiber officinalis* was present in traces, or only its flavours had been added and not any root extract. Our cumulated data suggest that this could be the starting point for investigating the myriad of beverages that in the last decades have invaded the shelves of supermarkets the world over, whose genuineness and natural origin has never been properly assessed.

4 Concluding remarks

In the early morning, a taxi would pull up at Tiffany & Co. on Fifth Avenue in New York City and let Audrey Hepburn emerge (*Breakfast at Tiffany's*, Paramount, 1961). She would eat a pastry and drink coffee while standing outside the shop window and dream of diamonds and diadems, which would surely make her life much brighter than just gulping down a few (may be stale) cookies. On the same wavelength would surely be Marilyn Monroe as she sang (*Gentlemen Prefer Blondes*, Twentieth Century Fox, 1953):

“A kiss on the hand
May be quite continental,”
But diamonds are a girl's best friend.

But we, humble workers on the vineyard of our Lord, are just as happy drinking a glass of genuine wine and eating certified food, especially if authenticated via the CPL technique.

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