**New approaches based on comparative proteomics for the assessment of food
quality**

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21 **Abstract**

22 During the last decade, the use of integrated ‘omics’ including genomics, transcriptomics,
23 metabolomics and proteomics has provided a better knowledge of food systems. In fact,
24 technical improvements achieved in mass spectrometry instruments together with the last
25 developments of bioinformatics tools and hardware have allowed proteomics to be the
26 method of choice for food control through the identification of quality biomarkers and
27 the study of the proteome expression data. In this context, modern comparative
28 proteomics is the basis for establishing quantitative differences in the expressed proteome
29 of a certain sample by comparison with a reference through the relative and absolute
30 quantification of proteins and peptides.

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45 **Introduction**

46 The first step towards a better control and characterisation of foods is based on
47 understanding organism systems from a biological point of view. In this regard, the use
48 of ‘omics’ strategies based on the study of genome through genomics (i.e. identification
49 of mutations), transcriptome through transcriptomics (i.e. elucidating the structure and
50 levels), proteome through proteomics (identifying and quantifying the expressed proteins)
51 and metabolome through metabolomics (i.e. characterizing the metabolites present in the
52 system) are necessary for a better knowledge of food systems [1**].

53 During the last decade, proteomics has been the method of choice to develop
54 methodologies for quality control in food production processes as well as in food safety
55 [2, 3]. In fact, the knowledge of structure and function of proteins present in raw food
56 materials and final food products is essential for the optimisation of processing
57 methodologies as proteins are main figures in quality parameters such as texture, colour,
58 crops yield, or changes derived from the response to stress. What is more, technical
59 improvements achieved in mass spectrometry (MS) instruments by increasing their
60 sensitivity and accuracy together with the last developments of potent bioinformatics
61 tools and hardware have allowed the development of more sophisticated methodologies
62 for the identification of quality biomarkers and study the proteome expression data. In
63 this context, comparative proteomics is the basis for establishing quantitative differences
64 in the expressed proteome of a certain sample compared to a control or sample of
65 reference and it allows predicting or identifying potential biomarkers of interest through
66 the relative and absolute quantification of proteins and peptides.

In this review, main methodologies used in comparative proteomics and the latest approaches for the assessment of food control through the characterisation of processes and final product have been described.

Main methodologies used in comparative proteomics

Comparative proteomics can be approached from both a qualitative point of view and under a quantitative perspective. The identification of protein profiles is usually done using Peptide Mass Fingerprint (PMF) strategy that includes a first separation of proteins using two-dimensional gel electrophoresis (2-DE). So, proteins are first separated based on their isoelectric point (pI) and later separated based on their molecular weight. Then, bands containing differential proteins are excised, digested by trypsin, and the generated peptides are extracted and analysed by MS [4] (**Figure 1**). Numerous software have been specifically developed for the detection of differences between samples in the 2-DE. After separation, the obtained protein mapping can be analysed by comparing the expressed proteins between samples and some of these software even permit the relative quantification of the identified bands.

The technical evolution of mass spectrometers occurred during the last decade as well as the development of bioinformatics data analysis platforms have led to a great advance in quantitative proteomics techniques [5], being comparative proteomics one of the main methods of choice used in the assessment of food quality and safety (**Figure 2**). In this sense, protein/peptide quantitative differences between samples can be measured using two main approaches: the use of labelling techniques that involve stable isotopes and the use of label-free methodologies. Labelling techniques are considered to be the most accurate in quantifying protein abundances whereas label-free methods are considered to be simple as do not require extra sample preparation, versatile as require low amount of sample, less time-consuming, and cost-effective when compared to labelled

methodologies [6]. One of the most used label-free approaches is the measurement of the ion areas through the extracted ion chromatograms (EIC) for the relative quantification of proteins and peptides. This methodology has been used in foods to compare protein abundances between two or more sets of samples. On the other hand, Multiple Reaction Monitoring (MRM) is the most used method for the absolute quantification of peptides in complex mixtures. MRM is a very sensitive labelling method that can selectively detect and quantify small and low abundant peptides based on the screening of specific transitions from precursor peptides to product ions.

Characterisation of processes through comparative proteomic approaches

The processing of foods including cooking, pasteurisation, drying, fermentation, or modern techniques such as pulsed electrical fields, microwaves or high pressure treatments influences to a great extent the quality of the final product. In this regard, comparative proteomics has been used to analyse proteome changes that occur in food during its processing [7**].

Bottom-up approaches based on comparative proteomics are the most common methods used for the characterisation of proteome changes as well as amino acid modifications when applying thermal treatments [8, 9]. As an example, **Figure 3** shows a schematic diagram illustrating the experimental approach used for shotgun proteomic analysis of heat-dependent milk protein modifications. So, cooked meat proteome changes have also been investigated using 2-DE coupled to image analysis and MS to evaluate protein modifications between raw pork meat and cooked ham [10]. Also changes in proteins mainly due to Maillard reactions and redox modifications resulted from the thermal treatment of milk and dairy products have been analysed by MS in tandem. A MRM-based methodology has been recently used to quantify the amount of cross-linked proteins derived via Maillard reaction after heat treatment as well as the glycooxidation product

Nε-carboxymethyllysine (CML) in a model system [11], showing the efficiency of the MRM method for the quantification of low-abundance post-translational modified proteins.

Differences in proteome expression has also been studied in cereals, fruits and vegetable products during the development or ripening on the plant [12-14] and during post-harvest treatments such as drying [15, 16] and long storage times [17-20].

The optimisation of processing conditions of lactic acid bacteria used for the manufacture of fermented dairy, sourdough, meat, and vegetable foods or used as probiotics is being studied using proteomics to elucidate the proteome diversity as well as the mechanisms of regulation and adaptation of *Lactobacillus* strains [21*-23]. Moreover, changes during processing such as environmental stress due to extreme temperatures in rice crops or aquaculture [24, 25], differences in CO₂ or N₂ air composition in vegetables [26] and cereals [27, 28], effect of altitude in fruits [29], presence of high concentrations of salt, copper or iron in soybean [30] and wheat grain [31], water stress in cereals [19] and pulses [32], and differences in the photoperiod in maize [33] can affect the crop yield. Also animal post-mortem meat changes have been studied using different approaches of comparative proteomics to detect differences in muscle proteome subjected to ante-mortem stress conditions and to determine potential biomarkers of stress [34, 35].

Characterisation of the final food product through comparative proteomic approaches

The first step in the production of any food is the control of raw material characteristics as this largely determines the final food product. Meat quality attributes can be influenced by many different parameters including animal breed, feeding regime, muscle type, protein/peptide/lipid profiles, meat ageing, and consumer preferences. In fact, colour, flavour and texture are key factors for the acceptance of meat products by consumers.

Thus, post-mortem changes in glycolytic enzymes, enzymes involved in tricarboxylic acid cycle and redox reactions have been recently related to colour stability in lamb samples [36, 37]. In this regard, Yu et al. (2017) [38] used a label-free strategy based on comparative proteomics to elucidate muscle-specific beef colour stability of Holstein cattle during post-mortem storage. The tenderisation of meat occurred after rigor mortis period can be influenced by a wide variety of factors such as genetics, pre-slaughtering individual environment or post-slaughtering conditions, although sarcoplasmic proteins and proteolytic enzymes are the main responsible. Several authors review meat tenderisation from a proteomic perspective describing changes in the structural proteins actin, myosin, troponin, tropomyosin, titin, desmin and nebulin as well as the proteolytic enzymes calpains, cathepsins, proteasome and caspases [39-40*].

The profile of expressed proteins studied through the comparison between breeds using different proteomics approaches plays an important role in hybrid selection. Li et al. (2016) [41] indicated that crossbreeding a swamp buffalo with a river buffalo has a notable effect on the protein content and composition that may be exploited for producing high-quality raw milk in food technology applications and dairy food production. On the other hand, Rodrigues et al. (2016) [42] studied muscle proteomic and phosphoproteomic profile of Angus and Nellore cattle, relating main differences with the quality of their meat. Similar approaches have also been described in fish species by comparing the proteomic profile of *Chamelea gallina* and *Tapes philippinarum* species [43].

The combination of 2-DE with MS has been applied in meat quality research to understand proteome changes resulting from growth and development, post-mortem metabolism, and tenderness, as well as to identify potential protein biomarkers. Comparative proteomic approaches have also been used in the determination of muscle tissue defects such as the differentiation between DFD and PSE meat, which could affect

the final quality of meat or meat-derived products [44, 45*]. The information derived from these studies as well as its application in biomarker research is very important in the use of raw meat for the most adequate purposes in meat industry. Specific biomarkers have also been recently described for the characterisation of freshness of buffalo milk quality. Among the identified markers, some were indicative of the action of various proteolytic enzymes and the resulting occurrence of specific defence components with physiological role to limit bacterial/virus content in buffalo milk [46]. A spectral counting label-free approach has been recently applied to characterise differences in the peptide profile of raw and pasteurised ovine milk cheese and to relate it with the bioactive potential [47]. Also a label-free quantification method has been used to determine differences in the peptide profile of Spanish Teruel, Italian Parma and Belgian dry-cured ham and its potential bioactivity [48]. Despite some studies reflect the increase in quantitative data of bioactive peptides, there is still an important challenge to overcome when analysing complex matrices where the peptides have been generated with no control on the enzymatic action. In this sense, the main difficulty is the optimisation of MRM parameters to perform a sensitive and accurate quantification without the interferences of other peptides or signal suppression.

Post-translational modifications that affect structural and functional properties of proteins have also been studied through the combination of liquid chromatography and 2-DE with advanced MS in stored milk and processed milk of bovine samples [49, 50].

Conclusions and future remarks

Proteomics constitutes a fundamental tool to develop methodologies for quality control in food production processes as well as in food safety. In fact, the knowledge of proteins present in raw food materials and final food products is essential for the optimisation of

processing methodologies as proteins are main figures in quality parameters such as texture, colour, crops yield, or changes derived from the response to stress. Due to the increasing demand for foods in terms of amount, quality and safety that is expected for the coming years, novel approaches based on comparative proteomics will continue being developed, probably through the use of modern MS applications such as MALDI-imaging, or the incorporation of separation techniques such as ion mobility or capillary electrophoresis to the analysis. Food proteomics is still very young in comparison with other life science disciplines and proteomic databases for many species are still incomplete. However, as methodologies and applications continue to develop, food proteomics will be an increasingly important analytical tool for many different aspects in the assessment of food quality.

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385 **Figure 1.** Scheme of traditional proteomic approach for the identification of proteins
386 from a food matrix.

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388 Figure 1

Figure 2. Overview of comparative proteomics application to the assessment of food quality and safety.

Figure 3. Schematic diagram illustrating the experimental approach used for shotgun proteomic analysis of heat-dependent milk protein modifications. PB chrom, phenylboronate chromatography. Whey proteins added with amounts of counterparts from corresponding skimmed milk was used to prepare soluble components that were further subjected to peptide ligand library-based enrichment; this mixed material is named in the figure as equalized whey. Reproduced with permission from Arena S, Renzone G, D'Ambrosio C, Salzano A.M, Scaloni A: Dairy products and the Maillard reaction: a promising future for extensive food characterization by integrated proteomics studies. Food Chem. 2017, 219: 477-489.