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Review

Proteomics and its applications for food authentication and food-technology research

José Manuel Gallardo [a], Ignacio Ortea [a,b], Mónica Carrera [a,c,*]

[a] Department of Food Technology, Marine Research Institute (IIM), Spanish National Research Council (CSIC), Vigo, Pontevedra, Spain
[b] Present address: Institute for Reference Materials and Measurements (EC-JRC-IRMM), Geel, Belgium
[c] Proteomics Group, Institute of Molecular Systems Biology (IMSB), ETH Zürich, Wolfgang-Pauli-Str. 16, 8093 Zürich, Switzerland

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A B S T R A C T

This review is a critical overview of advances in proteomics applied in food technology, which may be classified into two main topics: (i) authentication of food components as a tool to comply with food-labeling regulations and policies; and, (ii) food-technology research, mainly for the development of fast, reliable methods to detect and to identify spoilage and/or pathogenic microorganisms in food and for the study of changes in food components as a consequence of food processing.

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1. Introduction

Technological advances in the food industry offer substantial benefits to consumers in the global food market. Consumers demand products that meet their nutritional preferences and are extremely vulnerable to food-safety issues. Both academia and the food-science industry face a new challenge: the need to develop strategies and products that are not only safe but also contribute to the maintenance of good health and that may even prevent the development of specific disease-risk factors. In light of this, the recent successes of proteomics methodologies make them a promising strategy to address these concerns.

Proteomics is defined as the large-scale analysis of proteins in a particular biological system at a certain time [1]. Proteomics includes not only the structural and functional knowledge of

* Corresponding author Present address: Proteomics Group, Institute of Molecular Systems Biology (IMSB), ETH Zürich, Wolfgang-Pauli-Str. 16, 8093 Zürich, Switzerland. Tel.: +41 44 633 67 09; Fax: +41 44 633 10 51.

E-mail address: carrera@imsb.biol.ethz.ch (M. Carrera).

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proteins but also the study of their modifications, interactions, localization and quantification. Proteomics is emerging as a powerful tool for food-technology research [2,3] because it is helping to address the major challenges faced by food analysts and researchers:

- the development of simple, fast methodologies for routine use;
- the analysis of complex or highly processed food matrices; and,
- the quantification of trace levels of analytes with a high degree of selectivity.

This review illustrates up-to-date applications for and the new trends in proteomics methodologies for food-technology research, classified into two main topics:

(1) the authentication of food components, where proteomics is used as a tool to comply with food-labeling regulations and policies; and,
(2) the food-technology area, mainly in the development of fast, reliable methods to detect and to identify spoilage and/or pathogenic microorganisms in foods and in the study of the changes in food components as a consequence of food processing.

2. Proteomics

Proteomics studies are usually divided in three main areas, as follows.

2.1. Protein identification and characterization

Mass spectrometry (MS) is the method of choice for characterization and identification of proteins. The analysis of a proteome usually relies on one or several separation steps followed by MS analysis. The general approach consists of comparing MS experimental data with calculated mass values obtained from a sequence database using a search engine, such as Mascot [4].

In an approach known as peptide-mass fingerprinting (PMF) [5], a technique, such as two-dimensional gel electrophoresis (2-DE), is used to isolate an unknown protein, which is then enzymatically digested into peptides and subjected to MS. Another approach, usually known as peptide-fragmentation fingerprinting (PFF), uses tandem MS (MS/MS) to produce fragment-ion data from one or more peptides from the protein to identify the protein unambiguously [6]. Alternatively, MS/MS fragment-ion data from the entire protein can be used in a similar way for an approach known as top-down proteomics. For all these approaches, it is necessary for the corresponding protein to be present in the database. If the unknown protein is not present in the database, the best match will probably be the entry with the closest homology, usually a related protein from a related species. If the sequence similarity within protein databases is too low, peptides must be sequenced *de novo* [7], meaning that the MS/MS spectrum must be interpreted manually or through computer-assisted identification of the fragment ions with mass differences corresponding to the masses of the individual amino acids (AAs).

More than 300 different types of post-translational modification (PTM) occur on proteins in response to a wide range of intracellular and extracellular signals [8]. PTMs play crucial roles in protein function because they may alter protein activity, localization or stability. The mass shift in the modified AA with respect to the unmodified residue is the basis of the detection and the characterization of PTMs by MS. However, because PTMs are usually present in very low stoichiometry, modification-specific enrichment techniques are needed [8].

2.2. Differential proteomics

Quantitative information at the protein level, such as the relative abundance of a specific protein among different samples or the absolute amount of the protein, is very helpful when determining differences between different conditions (control *vs.* case). Relative quantification can be achieved with different methodologies, which may be classified as gel-based, label-based, and label-free approaches.

Gel-based methods consist of comparing the signal of an electrophoretically-isolated spot among different samples.

For label-based methods, proteins or peptides are labeled using a mass tag that is introduced metabolically, enzymatically or chemically, and relative quantification is obtained from the MS read-out. Quantification is based on the ratio of heavy/light peptide pairs.

Label-free approaches avoid the use of labeling with stable isotopes. The protein amount is calculated based on the MS-derived ion-current signal of the peptides or proteins or on the number of identified MS/MS spectra (spectral counts) for the protein.

However, for the absolute quantification of proteins, isotopically-labeled synthetic peptides are needed as internal standards for each target protein. For a more detailed description of all these quantitative proteomics approaches, we refer the reader to Panchaud et al. [9].

2.3. Functional proteomics

Most proteins function closely with other proteins. Functional proteomics studies address the integrated analysis of the functional interactions among different proteins and the networks thereof. In this sense, interactomics is defined as the study of the interactions between a specific protein and others and the consequences of these interactions [10–12]. For this transition from structural to functional proteomics, different platforms are currently being developed, but they are not necessarily MS-based; some of these techniques include affinity purification, yeast two-hybrid assays, protein microarrays, activity-based proteomics, phage display and capture-compound MS.

2.4. Proteomics workflows

A challenge for MS technology is the complex nature and large dynamic range of proteomes. Partial purification, depletion of high-abundance proteins, and selective enrichment are some of the methods used when working with complex samples [13]. After one of these techniques, further separation is performed at the protein and/or peptide level, typically based on gel electrophoresis and/or liquid chromatography (LC), before analysis by MS. Depending on how proteins will be analyzed in the mass spectrometer, two different proteomics workflows can be followed: bottom-up or top-down approaches (Fig. 1).

In the most common workflow, referred as a bottom-up or peptide-based approach, the protein/s of interest are converted into peptides using enzymes, such as trypsin, and the resulting peptide fragments are then analyzed by MS [1]. Bottom-up approaches can be further divided depending on whether the fractionation step is performed before (at the protein level) or after the enzymatic digestion (at the peptide level). A typical method for the former strategy utilizes a 2-DE gel-based approach, wherein proteins are separated based on their isoelectric point (p*I*) and molecular weight (*Mr*), so they can be individually excised from the gel and digested into peptides that are analyzed by MS. In the latter approach, also referred as shotgun proteomics, the protein mixture is enzymatically digested without prior fractionation, and the resulting peptides are analyzed by LC–MS. When the peak capacity

of one chromatographic separation is insufficient, multidimensional LC is widely used, usually with a combination of strong cation-exchange and reversed-phase (RP) columns.

By contrast, top-down approaches allow the characterization of the peptides produced by the fragmentation of intact proteins directly inside the mass spectrometer, which avoids the enzymatic digestion step. This approach is now possible due to the high mass accuracy of the new high-resolution mass spectrometers, although these machines display limited performance due to instrumental constraints [14].

2.5. MS analysis

MS and bioinformatics are the two tools that have revolutionized proteomics studies, making possible the high-throughput analysis of thousands of proteins in one experiment. Briefly, MS consists of the ionization of analytes at an ion source [electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI)] and the separation of the analyte ions depending on their mass-to-charge (m/z) ratio at one or several mass analyzers [quadrupole (Q), ion trap (IT), time-of-flight (TOF) or Fourier-transform ion-cyclotron resonance (FTICR)]. Subsequently, a detector registers the ion current released from the analyzers, producing the corresponding mass spectrum.

Currently, hybrid mass spectrometers, which combine different types of analyzers, are common (Q-TOF, QQQ, TOF–TOF and Q-IT). The combination of several mass analyzers allows users to perform two (MS/MS) or even several (MS^n) stages of MS. Different fragmentation mechanisms [collision-induced dissociation (CID)-related fragmentation modes (CID or HCD) and electron-transfer dissociation (ETD)] are currently available. The most significant advances in proteomics-based MS instrumentation are summarized elsewhere [15].

Data-dependent analysis (DDA) is the most popular acquisition mode when the objective is to study the highest possible number of proteins present in a sample. This method consists of MS/MS fragmentation of the most abundant ions detected during a survey MS scan. This is the approach followed for discovery approaches. Quantitative data can also be obtained using appropriate methodologies [9].

When information about the m/z of the proteins or peptides under study is available, data can be obtained using several acquisition modes included under the term "targeted proteomics". These experiments focus on specific ions selected by the operator, which increase the reproducibility, the sensitivity and the selectivity of the analysis. These techniques can be used to identify, to monitor and to quantify low-abundance peptides not detected by DDA analyses. In the acquisition modes known as selected ion monitoring (SIM) or selected MS/MS ion monitoring (SMIM), only the selected m/z values are detected or fragmented [16]. When the m/z of the precursor ion and one or several product ions are known, targeted proteomics analysis can be performed using selected-reaction monitoring (SRM) or multiple-reaction monitoring (MRM) acquisition modes [17]. These highly-sensitive targeted acquisition modes are the gold standards for the quantification of proteins and the monitoring of candidate-biomarker proteins in hypothesis-driven studies [18].

3. Proteomics in food authenticity

Food authentication is one of the major areas involved in food quality and safety. Several regulations have been implemented to assure correct information and to avoid species substitutions [19]. Food-species identification has traditionally relied on morphological or anatomical analysis. However, this is a difficult task

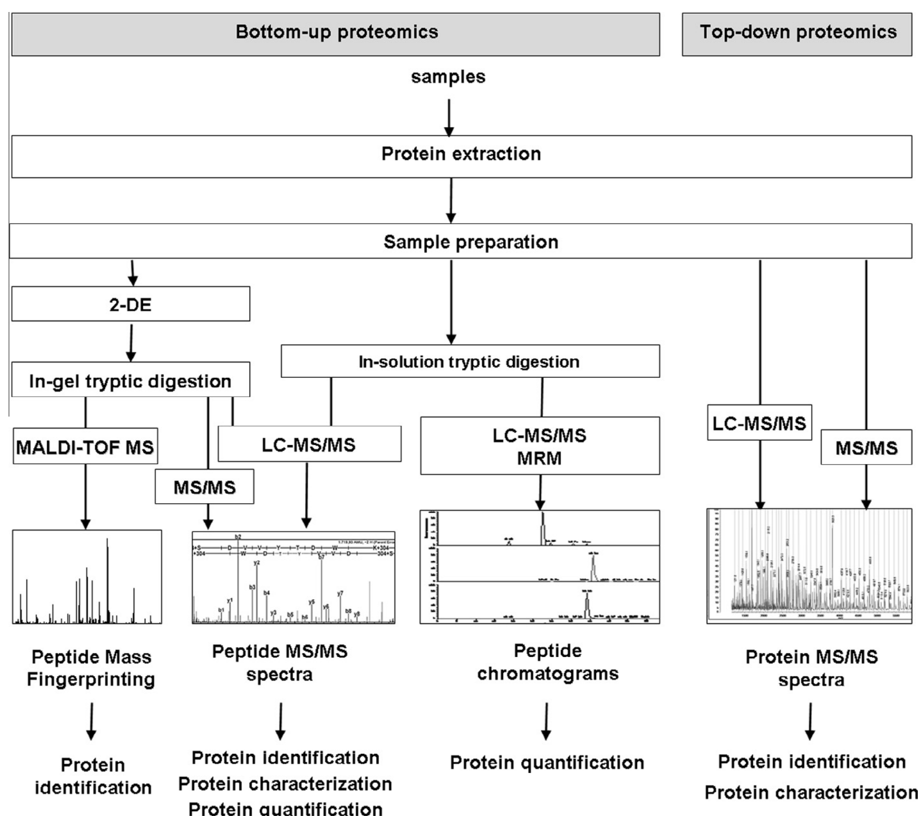

Fig. 1. Proteomics workflows commonly used for food-technology research.

in the case of closely-related species, especially for those products that have been subjected to processing. There is therefore a strong need for fast, reliable molecular identification methods that provide authorities and food industries with the tools needed to comply with labeling and traceability requirements, thus ensuring product quality and protection of the consumer.

Over the past two decades, several electrophoretic, immunological and DNA techniques have been developed for authentication purposes [20]. Limitations of these classical protein-based procedures include the lack of stability of some proteins during food processing and the labor and the time required for these procedures. Moreover, DNA-based procedures are not exempted from some important limitations. During the processing of food products, disruption of cellular integrity can occur, causing the release of hydrolytic enzymes. A combination of these enzymes with heat treatment and an acidic environment can negatively affect DNA integrity, reducing the length of fragments to be amplified and consequently increasing the chances of having non-specific identifications.

Proteomics methodologies have recently been proposed as promising strategies for food authentication [21–24]. Proteomics tools take advantage of the high-throughput capacity of MS to achieve fast, robust and sensitive protein and peptide characterization, detection, and quantification. These methodologies can be applied to species that are poorly characterized in genomic databases, avoiding the time-consuming steps of DNA amplification and sequencing. Moreover, proteomics-based methodologies can be automated to produce fast, reproducible results that allow the high-throughput analysis of foodstuffs. A compilation of proteomics-based studies applied to date for food-species authentication was collected in Gallardo et al. [25]. New trends and potential directions are discussed in the following paragraphs.

Fig. 2 illustrates the proteomics pipeline (discovery phase and target-driven phase) used in our laboratory for seafood authentication [26,27].

3.1. Discovery phase

The goal of the discovery phase is to explore a particular proteome comprehensively, using reference samples, to identify potential species-specific peptide biomarkers (Fig. 2). The identification and the characterization of these diagnostic peptides is commonly performed using bottom-up proteomics. Species-specific peptides from parvalbumin (PRVB) [28] and arginine kinase (AK) proteins [29] have been demonstrated to be good markers for the identification of fish and shellfish species, respectively. The characterization of these peptide biomarkers is the first step toward their subsequent use as sensitive, diagnostic targets in the next step of the pipeline (Fig. 2).

3.2. Target-driven phase

In this second phase, a targeted proteomics approach is used to monitor the species-specific peptide biomarkers previously char-

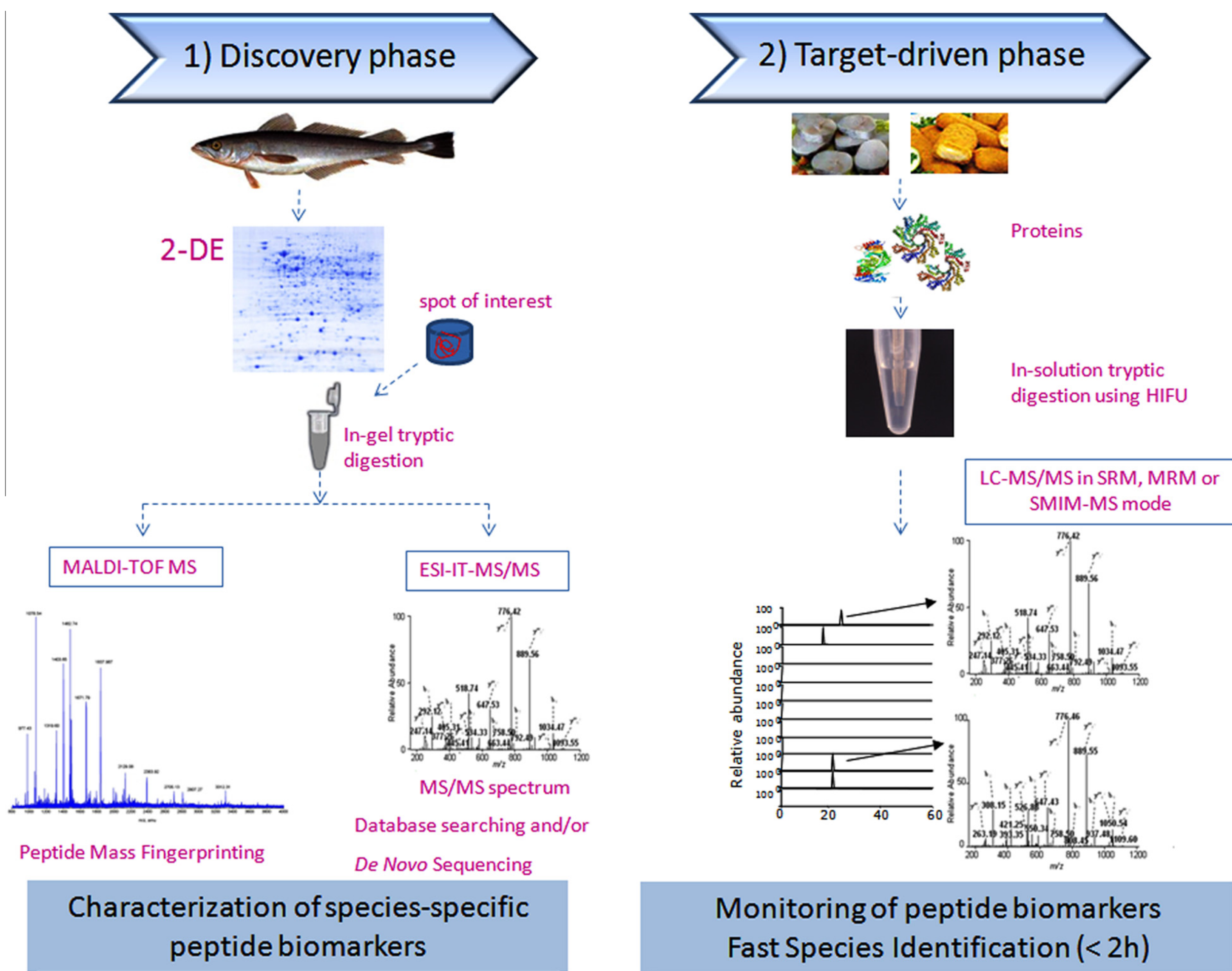

Fig. 2. Proteomics pipeline used for identification, characterization and detection of species-specific peptide biomarkers for food authentication.

acterized (Fig. 2). As was described previously, for species without sequenced genomes, the selection of species-specific peptides requires a preceding exhaustive *de novo* MS sequencing analysis [7]. Once the species-specific peptides are collected, the MS analyzer is centered on analyzing one or several peptides by SRM, MRM or SMIM MS modes [16,17]. Monitoring transitions (suitable pairs of precursor and fragment ion *m/z*) is a common assay to identify peptide biomarkers. The advantages of this targeted proteomics approach are its high specificity and sensitivity, which permit the quantification of proteins in complex samples at concentrations below the ng/mL range [17]. In addition, in one study, species-specific peptides for beef, pork, chicken and turkey were used to detect less than 0.5% w/w chicken contamination of pork meat, even after cooking. This study is comparable with other DNA methods [30].

A fast strategy for monitoring species-specific peptides was recently described by our group (Fig. 2). This method combines fast sample preparation using high-intensity focused ultrasound (HIFU) trypsin digestion and the peptide-detection ability of MS in SMIM scanning mode [26,27]. Because PRVBs are thermostable proteins, the workflow also identifies fish species even in processed and precooked products. This method has been validated using different real commercial samples. This workflow constitutes the fastest method for peptide-biomarker monitoring (less than 2 h), and its application to food-quality control provides authorities with a rapid, effective method for food authentication and traceability in any foodstuff, which guarantees quality and safety to consumers [26].

In contrast to the increasing use of proteomics for the authentication of food samples, quantitative analysis of food samples by proteomics techniques has been limited to date. Numerous quantitative MS-based methodologies offer excellent strategies for both relative and absolute quantification [9]. However, the above strategies recently started to be used for the quantification of food adulterants. Thus, LC–MS/MS operating in MRM mode combined with the use of standard stable isotope synthesized peptides (i.e. AQUA peptides) has been evaluated for relative and absolute quantitative analysis of chicken meat in meat mixes, tomato sauce and industrial crab production [30]. Also, a label-free quantitative approach has been established using distinctive peptides for quantifying the content of genetic variants of casein in bulk goat milk [31].

Peptidomes may be considered as novel sources of information for food traceability [32]. Peptidomics is defined as the discipline that studies and analyzes the composition, interactions and properties of the entire endogenous peptide pool of a determined biological system. For example, peptidomics studies have been used to determine the origin of cheese and to detect soybean or milk proteins in other commercial protein preparations [33]. We expect that the use of the new powerful MS fragmentation modes, such as ETD and HCD, will expand the in-depth analysis of peptidomes to identify new potential biomarkers for food authentication.

Current legislation also imposes monitoring of the traceability of raw transgenic materials and the labeling of genetically-modified-organism (GMO) products [34]. A modified gene implies modification of endogenous proteins or generation of novel proteins. Although GMO detection is generally performed using DNA profiling, the presence of a transgene in a food product does not guarantee the expression of the transgenic product. Detection of transgenic proteins is therefore a more realistic strategy. In this sense, we consider that the use of a targeted proteomics approach will be useful for detection of GMOs in foodstuffs.

4. Proteomics in food technology

4.1. Proteomics in food spoilage and microbiology

Food spoilage is a process caused by different biochemical changes due to microbial activities. These alterations depend on inherent and non-inherent microflora and on growth conditions, such as temperature, pH and a_w. Contamination incidents during food processing are responsible for significant economic losses for the food industry and serious food-borne diseases. More than 250 known pathogens, mostly microbes and their toxins, are known to cause food-borne illness. Although the identification and the classification of microorganisms are based on morphological, biochemical and DNA approaches, proteomics methodologies are being introduced to assist in the identification of food-borne pathogens and microorganisms responsible for food spoilage.

In the context of food technology, MALDI-TOF–MS analysis of intact bacterial cells or protein extracts was successfully applied to detect and identify different food-borne pathogens and food-spoilage bacteria in food products (Fig. 3). Remarkably, this method identified: 146 strains of *Listeria* spp. in meat, poultry, dairy and vegetables [35]; 126 strains of *Salmonella* spp. in chicken, turkey, swine, and cattle [36]; 26 gram-negative and 52 gram-positive bacteria in seafood products [37,38], and, biogenic amine-producing bacteria involved in food poisoning [39].

Also, different commercial databases have been created for bacterial identification by MALDI-TOF–MS, such as SARAMIS (AnagnosTec GmbH, Zossen, Germany), the MicrobeLynx bacterial identification system (Waters Corporation, Manchester, UK), and MALDI Biotyper (Bruker Daltonics Inc, Billerica, MA, USA).

Recently, a new public reference library, Spectrabank (http://www.spectrabank.org), was created [40]. This library contains the mass spectral fingerprints of the main spoilage-related and pathogenic bacteria species from seafood, and includes 120 species of interest in the food sector.

Regarding new potential directions and applications, we consider that the use of quantitative targeted MS proteomics approaches (i.e. absolute protein abundance by SRM) or the imple-

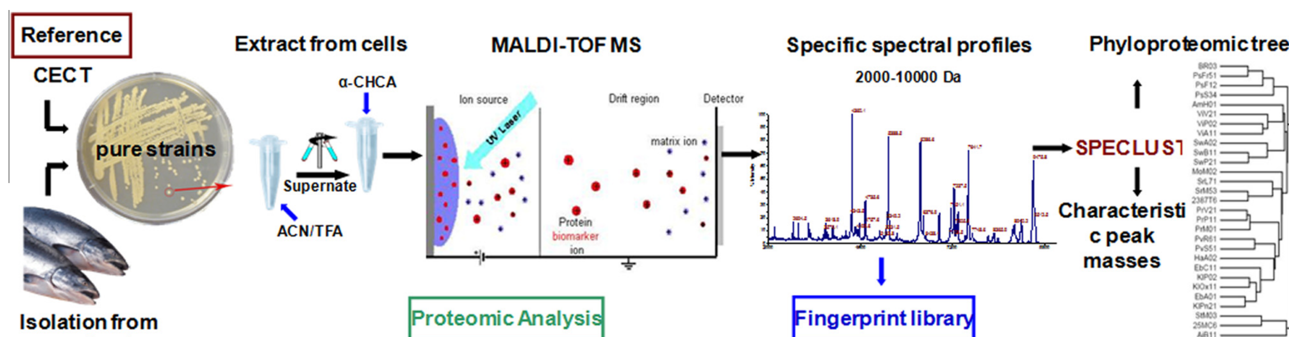

Fig. 3. Analytical scheme for identification of bacteria using MALDI-TOF–MS. {Reprinted with permission from [37]. ©2010, American Chemical Society}.

mentation of new fast platforms, such as lab-on-chips, nanoproteomics or protein arrays [41], will be very useful tools for rapid detection, identification and quantification of microorganisms in any foodstuff. In addition, the emerging development of the new top-down proteomics approaches offers new options for the identification of bacteria in food samples based on monitoring intact species-specific proteins ($\leqslant$40 kDa).

4.2. Proteomics in food processing

Among the different processing treatments used in the food-manufacturing industry, thermal processing (refrigeration, freezing, cooking, blanching, spray-drying, pasteurization, and sterilization) is one of the most widely used. These procedures are employed to improve the safety and the organoleptic and nutritional characteristics of food and to extend the shelf life of foodstuffs. However, due to the variability of products and processes used, chemical reactions can take place among the main components (proteins, lipids and carbohydrates), and, depending on the severity and time of the treatment, effects detrimental to the quality of foodstuffs can occur. A comprehensive overview of proteomics-based studies applied to date for food processing is compiled in Gallardo et al. [25]. A brief review concerning the effects of thermal processes on changes and modifications of food proteins using different proteomics methodologies is presented in this article.

Oxidation modifications, such as carbonylation, thiol oxidation and aromatic hydroxylation, and Maillard glycation (the reaction of sugars with AA side chains) are the protein modifications most frequently reported in foodstuffs that have been subjected to thermal processing. However, condensations and eliminations of side chains or peptide backbone breakdown have also been described [42].

Carbonylation occurs by oxidation of some AA side chains into ketone or aldehyde derivatives by reactions with compounds of lipid oxidation or by glycoxidation with reducing sugars. These protein-carbonyl compounds are markers of protein oxidation, and, recently, several carbonylated proteins and protein oxidation sites in milk [43], meat [44] and fishes [45] were identified using a classical bottom-up proteomics approach based on 2-DE and MS/MS. Specific labeling of protein carbonyls using fluorescein-5-thiosemicarbazide (FTSC) was developed and combined with 2-DE and MALDI-TOF/TOF to study the carbonylation of sarcoplasmic and myofibrillar proteins from fish subjected to metal-catalyzed oxidation [45].

Maillard glycation is another of the most-investigated protein modifications and occurs mainly during heating and storage of milk and dairy products. Maillard glycation, a non-enzymatic reaction between the amino groups of proteins and reduced sugars (lactose), produces glycoconjugate-condensation products, such as lactosylated proteins [46]. Diverse proteomics strategies based on bottom-up methods have been used to study Maillard reactions in milk and dairy products [47]. These strategies use native size-exclusion chromatography (SEC) coupled online with ESI-MS/MS for rapid identification and characterization of lactosylated proteins in thermally-processed milk products [47]. Recently, the results of new investigations suggest that the ETD–MS-fragmentation mode is an excellent, powerful technique for the analysis of non-enzymatic glycated peptides. The combination of the selective enrichment of lactosylated peptides, ETD–MS and peptide-ligand libraries has been applied for the identification of lactosylated proteins in thermally processed milk [48]. Using this methodology, 271 non-redundant modification sites on 33 milk proteins were identified.

Bioinformatics tools play an important role in the identification and the prediction of protein-modification sites. Within bioinformatics software, there are several programs designed to optimize the detection of protein modifications from MS data, including PTMProphet, ModifiComb and Peptoscope [49]. We expect that the availability of new repositories and databases, such as RedoxDB [50], the use of new specific labeling and enrichment methods, the use of the powerful new MS fragmentation modes, such as ETD and HCD, and the mapping and discovery of protein modifications by top-down proteomics will expand the applications for proteomics in the identification and the characterization of protein modifications in further food-related projects.

5. Concluding remarks and future direction

As is reported in this review, proteomics methodologies are increasingly helpful to address the major challenges in food authentication and food-technology research:

(1) the identification of food components;
(2) the development of fast and reliable methods for the detection and identification of spoilage and/or pathogenic microorganisms; and,
(3) the study of changes in food proteins as a consequence of food processing.

Two consecutive phases of the proteomics pipeline used in our laboratory (discovery and target-driven phases) allow the identification and the characterization of several peptide biomarkers, which can be rapidly monitored by MS using an innovative, rapid strategy based on the use of HIFU protein digestion. This novel targeted proteomics approach allows the unequivocal, fast authentication of food species in less than 2 h. This pipeline is currently being implemented for the rapid, reliable identification of microorganisms in different foodstuffs. Also, we believe that the extensive use of this new targeted proteomics strategy will find a relevant position in food technology.

In addition, different proteomics technologies based on the high-throughput analysis of protein modifications are being used to study the effects caused by thermal processing and storing of food proteins in foodstuffs.

Nevertheless, although proteomics currently contributes enormously to the development of food technology, new applications and developments offer promising proposals for new food-related projects. The quantitation of food products, the analysis of the peptidome, the identification of GMOs, the availability of repositories and new protein data in public food-related databases, the use of specific labeling and enrichment methods for food-protein modifications, new advances on ETD and HCD MS fragmentation modes, top-down proteomics, nanoproteomics and the extensive use of the fast, targeted proteomics approach are some of the new potential food-related directions proposed in this work. We therefore expect that proteomics technologies will offer interesting new opportunities within the field of food-technology research.

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