Chapter 24
The Role of Proteomics in the Discovery of Marker Proteins of Food Adulteration

Gianfranco Mamone, Gianluca Picariello, Chiara Nitride, Francesco Addeo, and Pasquale Ferranti

Abbreviations

1D	Mono-dimensional
2-D	Two-dimensional
AQUA	Absolute quantification
BLG	Beta-lactoglobulin
CE	Capillary electrophoresis
CID	Collision-induced dissociation
CML	Nε-carboxymethyl-lysine
CZE	Capillary zone electrophoresis
DIGE	Differential in-gel electrophoresis
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
GC	Gas chromatography
GMO	Genetically modified organism

G. Mamone • G. Picariello
Istituto di Scienze dell'Alimentazione (ISA) – CNR,
Via Roma 52 A/C, Avellino 83100, Italy

C. Nitride •
Dipartimento di Scienza degli Alimenti, University of Naples "Federico II",
Parco Gussone, Portici I 80055, Italy

P. Ferranti (✉) • F. Addeo
Istituto di Scienze dell'Alimentazione,
Consiglio and Pasquale Ferranti Nazionale delle Ricerche (CNR),
Via Roma 52 A/C, Avellino 83100, Italy

Dipartimento di Scienza degli Alimenti, University of Naples "Federico II",
Parco Gussone, Portici I 80055, Italy
e-mail: ferranti@unina.it

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HPLC	High-performance liquid chromatography
ICAT	Isotope-coded affinity tag
IEF	Isoelectric focusing
IEX	Ion exchange
IT	Ion trap
iTRAQ	Isobaric tags for relative and absolute quantitation
LAL	Lysinoalanine
LC	Liquid chromatography
LMW	Low molecular weight
LOD	Limit of detection
LOQ	Limit of quantification
MALDI	Matrix-assisted laser desorption ionization
MM	Mechanically recovered meat
MP	Milk powders
MRM	Multiple reaction monitoring
MS/MS and MS^n	Tandem mass spectrometry
MS	Mass spectrometry
MudPIT	Multidimensional protein identification technology
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDO	Protected denomination of origin: RP-HPLC: reverse-phase high-performance liquid chromatography
SDS	Sodium dodecyl-sulphate
SILAC	Stable isotope labeling with amino acids in cell culture
SIM	Single ion monitoring
SRM	Selected reaction monitoring
TOF	Time-of-flight
UPLC-FT-ICR-MS	Ultrahigh-pressure liquid chromatography high-resolution Fourier-transform ion cyclotron resonance mass spectrometry

24.1 Introduction

Adulteration in various consumer sectors is now commonly practiced. Raw materials and finished foods are not exempt, and according to several studies, this practice is increasing (French General Directorate for Fair Trading 2001). Adulterant detection and authenticity testing of foods, including dairy and meat products, eggs, cereals, legumes, beverages, and fruit products, are important for value assessment, to check unfair competition and, most important, to ensure consumer protection against fraudulent practices commonly observed in unscrupulous trade.

However, the consequences of food adulteration go very far beyond mere economical factors, and are much more complex. Actually, deceitful adulteration of food products is an objectionable practice also for health reasons, because consumption of products containing undeclared constituents may cause intoxication or problems such

as allergy and intolerance in sensitized individuals. For these reasons, the search for methods to exactly define the composition of the various products in order to distinguish them from similar but altered ones is among the most important issues in food and nutrition science. However, the definition of a food at the molecular level is not quite easy. Foods are, in general, complex matrices of very different chemical compound classes, reflecting the complexity of the animal or vegetal organism they derive from, with unique compositional and biochemical characteristics. This means that traditional analytical chemistry methods, traditionally and efficiently applied to discover gross food alterations (one for all, milk dilution by water addition) are inadequate to challenge the complexity of most food matrices at the molecular level.

Just as for any other biological system, the recently born "omic" sciences, intended as the systematic definition of subsets of specific biomolecules, also apply to foods. Thus, for instance, food proteomics include the systematic and complete cartography of the proteins, whereas metabolomics studies the complement of small molecules (low molecular weight arbitrarily assigned below 1–2 kDa) of a foodstuff. Phosphoproteomics, glycomics, and lipidomics concern specific classes of constituents of the systems under analysis. In particular, in consideration of the relevance for biological systems of lipids and sugars, lipidomics and glycomics are emerging as self-standing "omics" from the wider field of metabolomics.

The proteome is defined as the totality of proteins contained in a biological sample in a given state. Thus, in contrast to the static genome (with the exclusion of the epigenetic events), the proteome is highly dynamic, influenced not only by the genome but also by myriad external factors, such as metabolic state, treatments, and various interactions with other systems. Several recent papers have been focused on particular aspects of proteomics and of related "omic" sciences in the field of food analysis, dedicated to food composition and safety (Herrero et al. 2010; Davies 2010), to the search for marker proteins for food authentication, and to identification of food allergens (Picariello et al. 2011).

This chapter critically presents the newest exemplificative achievements of the "omic" sciences, with special emphasis on proteomics and peptidomics, generated by the synergistic efforts of researchers in the fields of analytical chemistry, in particular mass spectrometry, with those working in food technology and biotechnology.

24.2 Methods of Food Adulteration Detection

The impressive improvement of the last decades in food production, processing, and packaging has been paralleled by the escalation of deceitful practices of food adulteration.

The issue of food authenticity has an extremely high relevancy both for human health and for the food market economy and imposes the urgent need to improve the robustness of the available analytical methods for its assessment. Growing consumer awareness of food safety and quality, increased demand for legal regulation and adequate labeling, together with the evolution of the deceptive strategies are fueling

the development of up-to-date procedures of food control that have to be developed, standardized, and validated.

Briefly, a food product can be adulterated in the following ways: (1) a food component is replaced wholly or in part with a cheaper ingredient or with forbidden materials; (2) any constituent is wholly or in part subtracted from the food matrices; (3) foodstuffs are incorrectly processed or packed; (4) it has been produced with diseased vegetable or animal organisms; (5) in general, any of the quality parameters fall beyond the prescribed standards. Aside from the undeclared addition of preservatives or potentially harmful ingredients, that have to be assessed by the direct detection of the target molecule(s), the use of undeclared ingredients can be estimated by monitoring the biomolecular asset of the food commodity.

Over the years, an arsenal of analytical methods, many based on morphological/anatomical analysis, organoleptic markers (odor, color, texture), or chemical testing, has been developed to authenticate foods and to check for adulterants. In general, the three basic detection strategies used for demonstrating adulteration in food or agricultural commodity include: (1) demonstrating the presence of a foreign substance or a marker in the commodity, (2) demonstrating that one or more component levels deviate from normal, and (3) demonstrating that a profile is unlikely to occur. Among these, the strategy of detection of adulterans detection by the demonstration of the presence of foreign substances or a marker is considered as the most reliable and simplest (Wilhelmsen 2004, 2006).

For these reasons, in the last years, new emerging technologies are being applied to food authentication analysis. Determination of stable isotope ratio, especially on trace elements, provides a stable isotope signature useful to establish a close link between products and their environment (White et al. 1998). Most recent approaches specifically target proteins or DNA for discovering the signs of raw material derived from foreign organisms in food products. DNA-based methods consist in the polymerase chain reaction (PCR) amplification of DNA fragments arising from extraneous animal or vegetable organisms. DNA-based molecular tools have been proved useful for adulterant detection especially when the adulterants are biological substances (Lum and Hirsch 2006). In particular, it has been successful in species or variety identification as it can identify very subtle changes in the single base pair. In this way a specific DNA sequence can be identified and/or DNA fingerprints can be obtained. However, it is obvious that these methods are complicated when ingredients arising from several species, that often are taxonomically related, occur simultaneously. Furthermore, DNA-based analytical methods are of hardly any usefulness to establish the use of noncompliant processing of the raw material.

Although the detection of DNA markers benefits from having well-defined target analytes and the combined use of database analysis and experimental specificity should lower the chance of obtaining false positive, the techniques relying on the phenotypic expression of specific protein or metabolite markers are less laborious and, in most cases, more reliable.

Monitoring of protein markers generally relies on immunochemical techniques. Commercially available techniques of this type are lateral flow devices or dipsticks that are used for rapid screening and enzyme-linked immunosorbent assays (ELISA)

that also provide semiquantitative determinations. Typical limit of detections (LOD) of the tests based on ELISA kits are in the range of 1–5 ppm. Major concerns of the immunochemical methods consist in the fact that the epitopes, that are detected, are usually not well characterized and cross-reactivity with matrix components can result in false positive determinations. The reliability of the detection strongly depends on specificity and stability of the employed antibodies and can be affected by the changes induced on proteins by thermal or other technological treatments. Furthermore, food processing can modify both linear or conformational antigenic sequences, although altering the antibody reactivity. In addition, many protein targets may be underestimated or escape the most commonly utilized sandwich ELISA-based tests.

Chemical/biochemical techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), mono-dimensional (1D) or two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis, and so on, as well as ELISA have also been proved to be useful in component identification and adulterant detection in foods. However, although they are of considerable value in certain instances of adulterant detection and in routine analyses, these methods are merely descriptive as they compare a profile or a measured value with that expected for a given genuine product and therefore cannot explain the causes of the altered outcome at the molecular level. In other terms, the appearance/disappearance/shift of electrophoretic bands or chromatographic peaks compared to a reference food cannot be considered a definite diagnostic of an occurred food alteration, as the variation of the band/peak could be due to normal food variability (false positive). On the opposite side, an adulterant might be masked by comigration/coelution with a normal food constituent (false negative). In light of this, also conventional electrophoretic and chromatographic techniques alone, routinely used in this kind of analysis, in spite of the tremendous improvements in resolving power and sensitivity due to the technological advances, must be considered inadequate when facing the problem of describing the complex composition of natural or adulterated foods.

Given the limitations of classically used methods, it is clear that confirmatory strategies are also required to provide an unambiguous identification of markers of foreign food components. The proteomic approach can overcome these limitations. The core of proteomic science is the “omics” technologies that rely on well-established analytical platforms, in particular on mass spectrometry (MS) techniques.

24.3 Mass Spectrometry in Food Component Detection and Characterization

The MS-based proteomic/peptidomic approach can represent the ideal solution to such an analytical challenge. Major advantages of electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) MS techniques are the sensitivity and specificity of the analytical response in the detection of foreign proteins

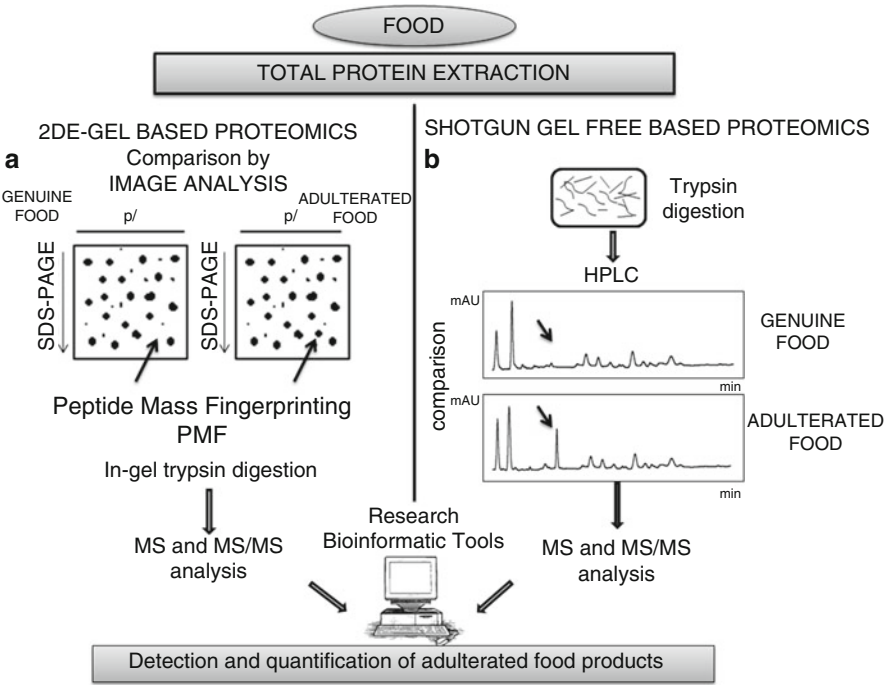

Fig. 24.1 Gel-free and gel-based proteomic approaches for the detection and the quantification of adulterants in food: (**a**) the gel-based approach allows detection of adulterated foods by comparison of protein profiles obtained by 2-D gel-electrophoresis separation followed by peptide mass fingerprinting for target proteins identification; (**b**) the gel-free approach analyzed by LC-MS/MS the total extracted protein digested with trypsin and followed by the identification of target proteins by online research in protein databases

or peptides. It is important to note that the MS response does not depend on the antibody specificity as in ELISA and is only marginally affected by interfering species.

As most foods are highly complex matrices, pre-fractionation of protein is required prior to MS analysis (Mamone et al. 2009); see Fig. 24.1. To date, two-dimensional electrophoresis (2DE) has proven to be a reliable and efficient method to separate in one step, a large number of proteins (Fig. 24.1a) (Gorg et al. 2005). An alternative to electrophoretic methods is liquid chromatography (LC) coupled with MS analysis to identify proteins directly from complex mixtures (Fig. 24.1b).

MS experiments can be addressed either to measure the molecular mass of a protein/derived-peptides (MS1) or to determine additional structural details such as the amino acid sequence and post-translational events, also including process-induced modifications, using tandem MS (MS/MS or MS^n).

The monitoring of intact proteins by the so-called "top-down" approaches is still of limited analytical throughput and efficiency, especially when complex foodstuffs are handled. Direct MALDI analysis of the protein extracts may provide fast and

accurate information on food composition and authenticity. Exemplary cases are the analysis of gluten residues in declared gluten-free foods and beverages, exploited by several studies (Hernando et al. 2008; Mamone et al. 2011), and the authenticity assessment of commercial fish species (Mazzeo et al. 2008).

Peptide-centric methodologies ("bottom-up" techniques) that consist in the analysis of proteolitically generated peptides introduce a series of advantages. The bottom-up "discovery-driven" proteomic experiments rely on either 2DE or gel-free procedures and can provide the almost complete protein suite of a system, largely covering the dynamic range of concentrations that usually occur in adulterated foods. In targeted experiments, when the nature of the adulterant is at least in part supposed, it can be defined as a subset of "signature" peptides specific to each foreign protein. These peptides are currently referred to as "proteotypic" peptides. Eligible "proteotypic" peptides are sequences specific to a given protein, generally obtainable through a tryptic digestion, with optimal molecular sizes (10–20 residues long) and high ionizability to be identified and sequenced by mass spectrometry. In addition, the ideal proteotypic peptide should exhibit the following properties: (1) high efficiency to ionize and to fragment; (2) uniqueness for the target protein(s); (3) absence of amino acid residues susceptible to chemical modifications such as Cys (reactive thiol group), Met (oxidable), Asn and Gln (deamidation), N-terminal Gln (pyroglutamate); (4) absence of post-translational modifications and of sequences that are prone to missed proteolytic cleavage (e.g., sequences such as KRXX or XXRK in the case of trypsin).

Once the panel of "proteotypic" peptides is defined, a single LC-ESI-MS/MS analysis of a tryptic digest of the protein fraction extracted from a complex food matrix can provide unambiguous identification of the protein target. The identification relies on three independent and convergent indications: chromatographic retention time, peptide mass, and sequence information (MS/MS experiments). Multiple reaction monitoring (MRM)–MS techniques can achieve sensitivities unparalleled by immunochemical techniques. The use of "proteotypic" peptides as "analytical substitutes" of the parent proteins enhances the potentiality of the MS-based methods, improving sensitivity and enabling the possibility of several confirmations through the monitoring of different peptides and related transitions (multianalyte methods). In addition, by designing appropriate internal standards by peptide synthesis (i.e., synthetic peptides that contain stable modifications, in order to introduce minimal molecular mass shift with respect to the target peptides without affecting ionizability) LC-ESI-MS/MS can provide robust and repeatable quantitative data.

The LC-ESI-MS/MS peptide-centric approach has recently been applied to detection of higher-value milk adulteration with cheaper bovine milk (Cuollo et al. 2010), as detailed in the following section, as well as to monitoring contaminating allergens in processed peanut products (Chassaigne et al. 2007). Also, taking advantage of the recently introduced high-resolution/wide dynamic range LTQ-Orbitrap instrument, coupled with 1D nanoflow RP-HPLC, 158 proteins have been recently identified in hen's egg white by shotgun proteomics, also including several known egg allergens (Mann and Mann 2011). It is almost superfluous to underline how the investigation methodology illustrated by these examples is revealing itself to be

invaluable in detecting either intentional or involuntary food contamination by allergenic ingredients.

In the shotgun approach (Fig. 24.1b), the information about the molecular weight of the intact proteins gets lost. However, a careful analysis of MS/MS spectra can turn out to be effective in discriminating foreign proteins. To reduce the complexity of the sample, multidimensional chromatography coupled with mass spectrometry (2-D LC-MS or MudPIT, multidimensional protein identification technology) can be employed. This approach provides a higher separation power than 1D chromatography, in addition to an extended measured dynamic concentration range to analyze complex proteomic samples. Various LC methods in the first dimension prior to RP-HPLC in the second dimension have been proposed. A preliminary ion-exchange (IEX) separation mode has become the most popular implementation (Washburn et al. 2001; Motoyama et al. 2007). At present, multidimensional chromatography-shotgun techniques are largely utilized in proteomics. Nevertheless, up to now only a few articles report on food contaminant identification using this approach. Among the methods related to these, a multiplexed chromatography consisting of sequential HILIC and TiO_2 enrichment and a chemical-based (hydrazide capture) strategy prior to nanoflow RP-HPLC-ESI-LTQ-Orbitrap MS/MS have been addressed to identify glycoproteins of wine potentially cross-reactive with plant allergens (Palmisano et al. 2010). With a slightly different approach, consisting of a preliminary chromatography step at the protein level, followed by RP-HPLC/MS/MS analysis at the peptide level, soybean proteins willingly or accidentally added to meat products could be detected (Leitner et al. 2006). Similarly, protein peanut allergens were identified in breakfast cereals by a 2-D chromatography method consisting of an affinity capture of proteins on immuno-beads followed by tryptic digestion and RP-HPLC-ESI-IT-MS/MS (Careri et al. 2008).

Also capillary electrophoresis mass spectrometry (CE-MS) is a promising methodology in proteomic applications because it is an alternative to 2DE and to chromatographic techniques for complex mixture analysis. It combines high efficiency separation of intact proteins with unique information of MS (Rob et al. 2007). The most recent applications of CE-MS to the analysis of proteins in food, which have been recently reviewed (Garcia-Canas and Cifuentes 2008) include the characterization of protein extracts to provide information about the protein composition with the aim of: (1) determining the origin of the ingredients, the technological process employed to produce a particular food, or the quality of a protein extract; (2) detecting high-quality products adulterated with cheaper products; and (3) monitoring and optimizing technological processes. In addition to these applications, some approaches are worth mentioning, such as those based on CE coupled with MS and developed to predict the amino acid composition of protein hydrolysates (Simó et al. 2005; Tessier et al. 2005), as well as the novel procedure that provides simplified 2-D mapping of CE-MS data in order to simplify the discovery and detection of specific markers (Erny and Cifuentes 2007).

Decisive support to all of the methods applied in proteomic analysis is provided by bioinformatic tools to manage the huge amount of MS/MS datasets. The identifications are usually administered automatically via powerful, commercially

accessible software programs such as Mascot and SEQUEST in combination with continually updated public databases such as those maintained by the NCBI and SwissProt (Johnson et al. 2005).

24.4 Proteomic-Based Methods for Food Protein Quantification

Because of its very low limits of detection and quantification (low-ppb range), MS analysis is the basis of modern proteomic methods for quantitative investigation of biological systems. Several conceptually different MS-based proteomic strategies, each with advantages and disadvantages have emerged in the last two decades.

Numerous MS-based methodologies for both relative and absolute quantification are widely used in proteomic studies, but all referable to two main approaches: in the first one, the analyte and its reference standard are the intact proteins; in the second one, the analyte is a peptide arising from protein digestion by proteolytic enzymes.

At the beginning of the "proteomic era," the first established method applied for differentially expressed proteins was based on 2DE fractionation followed by MS analysis (Pietrogrande et al. 2006) (Fig. 24.1a). Classical 2DE employs a two-step separation whereby denatured proteins are first separated according their isoelectric point (isoelectric focusing, IEF) followed by separation as a function of molecular mass (sodium dodecyl-sulphate–polyacrylamide gel electrophoresis, SDS-PAGE). After gel staining, comparison of sample control and case proteomes allows detection of differentially expressed proteins. The gel image analysis requires the use of computer programs that are continuously being developed and improved (i.e., Melanie Software (Amersham Biosciences), PDQuest (Bio-Rad), Progenesis (Nonlinear Dynamics)). The spots of interest are then excised and in-gel digested with trypsin prior to MS (peptide mass fingerprint) or MS/MS analysis in order to determine the protein identity.

2DE represents an excellent tool for mapping differences in protein expression, but certain drawbacks limit its use. First, the gel staining methods are either not sensitive enough or have a limited linearity (Westermeier and Marouga 2005). Second, proteins characterized by extreme pI or MW values can escape detection. Third, the intrinsic gel-to-gel variation of 2DE compromises the quantitative comparison of protein expression levels. (Van den Bergh and Arckens 2004). These drawbacks have been in part circumvented with the introduction of differential in-gel electrophoresis (DIGE) developed by Amersham Biosciences (Ünlü et al. 1997). To identify specific protein components, electrophoresis-based separations, in either the one- or two-dimensional version, coupled with MS analysis may be integrated with immunoblotting detection with antisera raised against specific proteins, as illustrated in the case of milk proteins (Chianese et al. 2010). This approach has been used to characterize milk variants in individual or bulk milks, or to monitor the evolution of dairy products such as yogurt and ripened cheese during maturation (Addeo et al. 1995) by analyzing the modification of milk protein and peptide patterns over time. Today, electrophoretic analysis is still the basis of the official methods in the

EU regulation to assess adulteration of high-value milk with cheaper cow milk (Addeo et al. 2009). Therefore, the implementation of well-established analytical procedures with proteomic platforms for objective molecular marker identification is of greatest promise in this field.

Gel-free MS-based quantitative proteomics is a promising technology capable of bridging the gaps of the 2DE-based analysis (Fig. 24.1b). This approach was made possible by the development of high-resolution mass analyzers and multistage MS instruments (i.e., Q-TOF, orbitrap, Fourier transform ion cyclotron resonance).

In the direct quantification of intact proteins, according to a "top-down" approach, the intensity of analyte multicharged ions is compared with that of internal or external standards. To quantify cow's milk allergens in mixed fruit-juice samples (Kuppannan et al. 2011) and whey drink (Huber et al. 1999), several multiple-charged ions of whey proteins were simultaneously monitored. In these specific cases an external calibration was carried out. With a similar approach, but utilizing beta-lactoglobulin (BLG) from species different from bovine as an internal standard, Czerwenka et al. quantified cow BLG in different milk-derived products (Czerwenka et al. 2007).

Nevertheless, the sensitivity of top-down mass spectrometry methods is limited by the wide distribution of protein charge states. Therefore, the analytical strategies named "shot-gun" proteomics, is being adopted in a number of research laboratories for the quantitative analysis of a number of analytes (Washburn et al. 2001). This approach is built on the direct analysis by high-resolution LC-MS/MS of peptide derived from in-solution digestion of protein complex mixtures.

The use of bottom-up methodologies for quantitative analysis in proteomics is steadily increasing. Quantitative methodologies for large-scale quantification such as stable isotope labeling with amino acids in cell culture (SILAC), isotope-coded affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ), or label-free approaches are capable of exploring the dynamics of whole proteomes through the relative quantification among multiplexed samples (Wu et al. 2007; Gingras et al. 2007). However, the above strategies were originally developed to determine differentially expressed proteins. For this reason, these quantitative methods have started to be addressed to the quantification of food adulterants only recently.

The selected reaction monitoring (SRM) MS approaches have emerged as tools for the precise quantitative determination of targeted proteins in complex samples (Picotti et al. 2010). The most common applications of SRM in proteomics rely on the principles of stable isotope dilution (SID) methods (Mayya and Han 2006). Conceptually, SRM and SID are not new techniques, as they were introduced as early as the late 1970s for the quantitative analysis of small molecules by GC-MS. Their integration in proteomic platforms is finding exponentially increasing application so that they can be now considered the gold standard for absolute quantification. Multiple reaction monitoring (MRM) represents an improvement of SRM in terms of selectivity and sensitivity. Both techniques have been broadly adapted to QQQ-based instruments (QQQ, QQQ-IT, QQQ-TOF), in which the first quadrupole (Q1) mass analyzer is set to filter only the preselected peptide precursor ion(s) of

choice and other ions are excluded. The second quadrupole (Q2) is a collision cell that fragments the precursor by collision-induced dissociation (CID) and transmits fragment ions to the third quadrupole (Q3). Q3, in turn, transmits to the detector only fragment ion(s) with pre-set *m/z*, whereas all the remaining fragments are excluded (Careri et al. 2007). MS is not intrinsically a quantitative analytical technique, because the ionization efficiency of the protein/peptide is affected by a wide range of physicochemical properties (size, charge, hydrophobicity). For accurate quantification the protein/peptide amount must be referred to that of a suitable standard.

The typical workflow for this methodology is (1) isotopic labeling of protein sample; (2) digestion of combined labeled protein samples to obtain peptide mixtures; (3) chromatographic fractionation of mixed peptide samples; (4) analysis of the separated peptides by LC-ESI- MS/MS; and (5) processing of the MS results to obtain relative protein abundance as well as protein identification by database searching.

The core of quantitative proteomic analysis is the isotopic labeling (i.e., 12C/13C, 14N/15N and 1H/2H) of proteins that permits direct comparison of two proteome states in a single analysis. In this way, the same peptides generated from the hydrolysis of different samples have a similar chemical performance, but a difference in mass detectable by MS techniques. Peptide peak intensities can be used for relative quantification of these peptides.

The purpose of quantification was successfully gained with the introduction of absolute quantification (AQUA) strategy based on the use of specifically designed internal standards. AQUA standard peptides are synthetic copies of proteotypic peptides, which are isotopically labeled to one or more amino acid positions in order to shift molecular mass, without appreciably influencing retention time and ionization properties. Hydrolyzed protein samples are spiked with known amounts of standard AQUA peptides and both native and surrogate peptides are monitored by LC–MS operating in MRM mode. The absolute amount of peptide(s) is determined by the ratio of the ion intensities of AQUA peptide and its native cognate (Gerber et al. 2003; Mallick et al. 2007). Applications to food contamination studies are still scarce but recently increasing in number. By this strategy, allergens in commercial soybean (Abdel Rahman et al. 2011), tomato sauce (Brambilla et al. 2009), and industrial crab production (Abdel Rahman et al. 2010; Barre et al. 2008) have been evaluated.

An alternative to SRM for less complex samples, when an extremely high specificity is not required, could be the single ion monitoring (SIM) which is commonly performed on a single quadrupole. During this process, the MS analysis time is focused only on analytes with a specific mass corresponding to the ion of interest, whereas all others are excluded. SIM analysis often requires a high-resolution separation in order to minimize interference from other species.

The protein extraction from the food matrix is a crucial step; interference by nonprotein compounds, such as tannins, should be carefully prevented. The choice of proteotypic peptides and ion transitions is also decisive and, although predictive software have been developed (Alves et al. 2011; Gallien et al. 2011), they need to be defined experimentally through a prior full-scan LC/MS analysis of the proteolyzed

protein extract of the original food matrix (targeted analysis). The possible occurrence of isoforms of protein markers should be considered by selecting distinctive proteotypic peptides if markers are willing to be individually quantified.

A further critical step in large-scale SRM is the control of the conditions and the reproducibility of the HPLC separations. To overcome possible pitfalls in this sense and to assure reproducibility, the efficiency of the stationary phase, the high quality of eluents, and a strict temperature control are mandatory. These precautions also allow us to narrow down the time window used to monitor the transitions of a specific peptide, in order that the LC–MS run can be divided into a higher number of time segments, thus dramatically increasing the number of peptides analyzed during the entire LC–MS run. In this manner sensitivity can be increased at subfemtomole levels enabling the detection and quantification of low ppb of analyte.

A new and promising alternative for quantifying protein abundance changes in shotgun proteomic analyses is the so-called label-free comparative proteomics which avoid any chemical sample alteration (Ono et al. 2006). The label-free approach has been designed to simplify the analytical procedure avoiding use of stable isotopes. Quantification is based on the measurement of signal intensity or of the so-defined mass spectral counting (Liu et al. 2004; Choi et al. 2008), both parameters linked to the protein abundance.

The label-free approach to the analysis of food adulteration is in its early stages and the number of applications is still limited. Using the distinctive peptides as protein markers, a label-free method has been set up for quantifying genetic variants of α_{s1}-casein content in bulk goat milk, which is an important parameter to evaluate the cheese-making aptitude of caprine milk (Picariello et al. 2009a). The targeted monitoring of mass and transitions of selected proteotypic peptides, overcomes the bias due to the presence of a large number of dominant components. These procedures are obviously strictly demanding in terms of chromatographic resolution and mass spectrometry performance, but their relatively low cost for standards and progress in the newest generation of MS instruments and in software development have made them quite attractive at least for semi-quantification.

24.5 Raw Materials and Ingredients: The Proteomic Approach to Quality and Authenticity Assessment

The application of proteomic technologies in food science is eventually aimed at defining the entire and detailed (bio)chemical composition of a food and its modification along the production process to correctly evaluate its nutritional, toxicological, and functional properties.

As a matter of principle, all the macrocomponents of a food may be used to trace its history. Thus, both glycomics and lipidomics strategies have been addressed to this purpose. Lipidomic procedures have been successfully applied to assess, for instance, the authenticity of composition of edible oils and butters (Picariello et al. 2007) as well as to trace the modifications related to heat treatments to reveal the improper

or illegal use of frying oils (Picariello et al. 2009b). However, more than any other class of food components, the proteome reflects very closely the (bio)chemical processes a system undergoes. The proteins of a food are, at the same time, a passive source of amino acids but also an active food component giving a food its textural, functional, and sensory characteristics. Proteins also play an active crucial role in inducing food modifications during production, maturation, and storage, by catalysis of processes such as oxidation, proteolysis, or lipolysis. In doing so, proteins are largely responsible for the overall characteristics of many food preparations. Because of the potential to generate a systematic view of protein composition and biological as well as chemical interactions, the application of proteomic analysis in food science is steadily growing.

The aim of these studies is eventually to define a complete database of the protein composition "fingerprint" of a given food. This has also provided important outcomes in the field of food authentication. The European Food Safety Authority has recently established a comprehensive system of authentication and traceability of food and feed in to order to ensure food safety for human consumption at every stage of production. The EC regulation No. 509/2006 (EC Council Regulation 2006) protects consumers through a system of effective and impartial controls that define the safeguard of the Protected Designation of Origin (PDO). From the legislative point of view, quality standards have been established through the requirement of quality labels that specify the chemical composition of each product and in most cases also geographical origin and production methods. This requirement has prompted an increased interest in developing analytical methods to assess quality and authenticity of food products.

The issue of food authentication, particularly urgent in the case of PDO food products, also requires efficient traceability systems, to ensure the correct application of the procedures throughout the productive processes and during distribution. The sensitivity, specificity, and speed in the analytical response candidate MS-based techniques are among the most accurate and versatile strategies to face the challenging tasks in this field. Moreover, proteins encrypt highly distinctive information about the identity and history of a specific product. Thus, it is not surprising that targeted MS-analysis of both proteins and proteolytic peptides have been largely developed for food authentication purposes. Of great relevance are the studies aimed to fingerprint the PDO typical products in terms of their peculiar metabolomic pattern, in order to differentiate them from non-PDO imitation products. One such example is Napoli-type salami, a ripened sausage originated in Campania (South Italy) but now produced in any region of Italy. A consortium of producers in Campania has applied to the EU for the PDO label in order to protect the artisanal product from industrial imitations from other geographical areas. A way to distinguish the PDO product from the industrial resides in the proteomic as well in the metabolomic profile (the overall metabolomic approach is illustrated in Fig. 24.2). In Fig. 24.3 the volatile profile of the two products is compared from where differences emerge and also to find a molecular rationale in the different ingredients and technology employed.

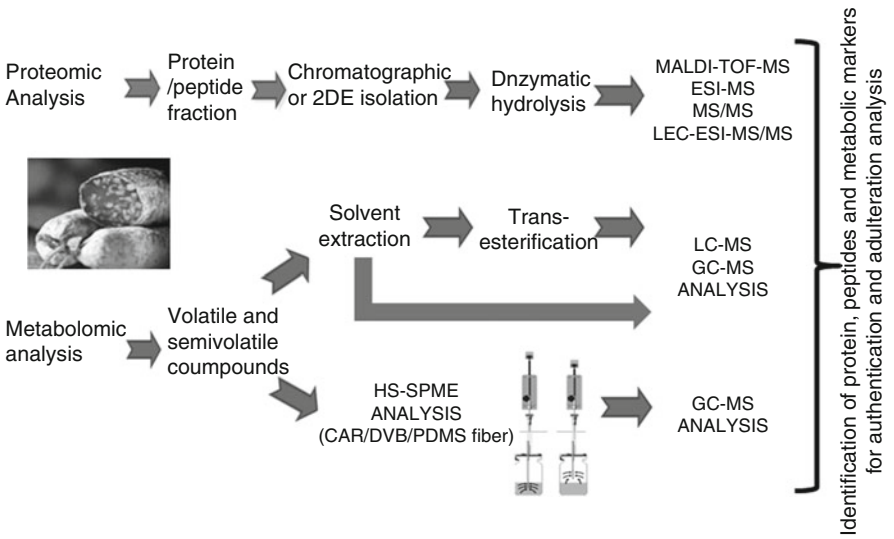

Fig. 24.2 Analytical proteomic and metabolomic approach for fingerprinting of typical products, exemplified by the case of Naples-type salami

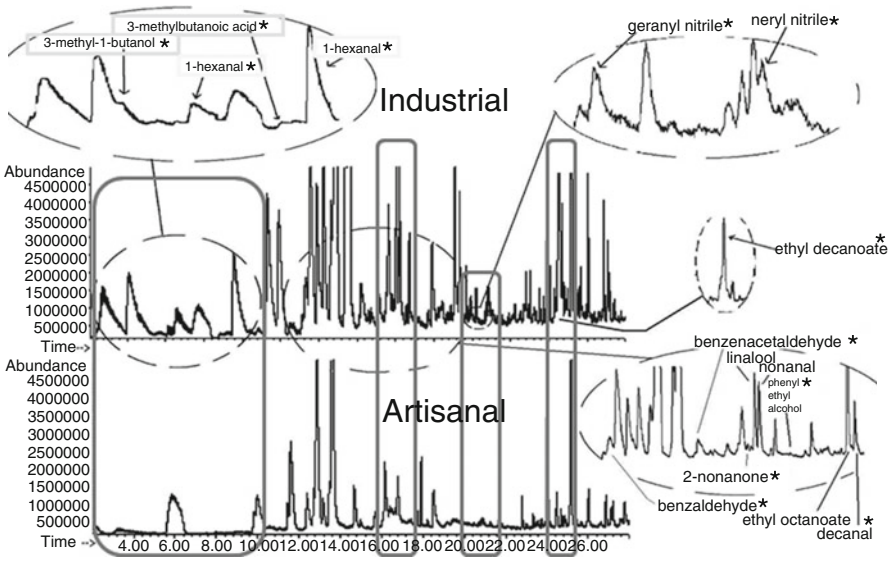

Fig. 24.3 Metabolomic analysis reveals molecular differences and identifies molecular markers to differentiate artisanal products from industrial imitations

The wide-ranging contribution and potentialities of MS to assess the quality of milk-based products has recently been reviewed (Guy and Fenaille 2006). Several MS-based procedures have been developed to authenticate "raw materials" used in manufacturing dairy products. As already mentioned, addition of milk of higher commercial value with cheaper bovine milk is a quite frequent adulteration practice in cheese-making. Taking advantage of the species-specific amino acid substitutions along the homologous protein chains that affect molecular weight of both caseins and whey proteins, it is possible to define fingerprinting profiles of milk from different species either by electrospray or MALDI–TOF–MS.

Angeletti et al. (1998) demonstrated the efficiency of MALDI–TOF–MS in the detection of bovine caseins in water buffalo mozzarella cheese. Analogously, adulteration by bovine milk was detected in ewe cheese (Fanton et al. 1998). On the basis of the identification of a water buffalo-specific β-casein by CE followed by offline MS, an improved strategy to distinguish bovine and buffalo milk by monitoring the products of plasmin hydrolysis has recently been developed (Somma et al. 2008). Selecting the whey proteins as biomarkers, Cozzolino et al. (2002) identified the fraudulent addition of bovine milk to buffalo and ewe dairy products at levels as low as 2%. Similarly, the detection of bovine BLG in goat milk by means of LC/ESI–MS has been reported (Chen et al. 2004).

A CE-MS-based approach was proposed by Muller et al. (2008) for the detection of adulteration of caprine and ovine milk, with lower-value bovine milk. A low-pH electrolyte was selected in order to minimize the adsorption of the proteins onto the inner capillary wall. By analyzing the whey protein fraction from milk, the method allowed the detection of added bovine milk into the nonbovine ones within the concentration range of 5–95%.

As shown by the above example, in CE-MS special attention has to be paid to the selection of MS-compatible volatile separation buffers and the selection of the adequate strategy when capillary coating is required to avoid MS signal suppression (Simpson and Smith 2005). Recently CE-MS of proteins has been studied using both online and offline detection (Haselberg et al. 2007). A comprehensive study of the use of CE volatile separation buffers compatible with ESI-MS detection was carried out in order to obtain as much information as possible from the zein protein fraction from maize (Erny et al. 2007). A coating with ethylpyrrolidine methacrylate- N,N-dimethylacrylamide copolymer was used in order to avoid protein adsorption into the inner capillary wall. It was observed that the use of ammonium gradient during CE separation significantly enhanced the analysis of the zein fraction giving rise to efficiency improvements of up to twentyfold. These findings were next applied in the analysis by CE-ESI- MS of the zein fraction of different maize cultivars using two different mass analyzers, that is, TOF and Ion Trap (Erny et al. 2008). Although both instruments provided good results in terms of sensitivity and repeatability, CE-ESI-TOF/MS allowed the identification of a higher number of proteins.

The molecular profiling performed by MALDI-TOF-MS is emerging as a general tool for the discovery of biomarkers also applied to food authentication. In meat and fish authentication, and in food-borne bacterial identification MS-based techniques have the capability to substitute or complement other available strategies,

such as the multiplex PCR assay. In one study (Mazzeo et al. 2008) an innovative method based on MALDI-TOF mass spectrometry has been developed and successfully applied to fish authentication. Highly specific mass spectrometric profiles from 25 different fish species were obtained. Signals generated from proteins with molecular weights of about 11 kDa have been selected as specific biomarkers for unambiguous discrimination. This method is also suitable for verifying commercial product authenticity and to rapidly discriminate species subjected to fraudulent substitutions, such as those belonging to Gadidae and Pleuronectiformes. For example, biomarkers for fillets of sole, European plaice, and Greenland halibut were defined in parvalbumins, differentiated on the basis of the different molecular weight. Proteome analysis has also been applied with the aim of finding new marker proteins suitable for seafood authentication (Pineiro et al. 2003). Using gel-based methods, a polypeptide has been identified as a good candidate for the differentiation of high-value hake species from other, less valuable species of the same fish. Furthermore, marker proteins were suggested to test for shelf life and freshness of seafood as well as for the differentiation between fresh and frozen fish.

24.6 Food Authenticity: Finding Markers of the Technological Process

A relatively new application of proteomic/peptidomic technologies for food authentication is in the discovery of process markers, informative molecular markers indicative of both the use of specific raw ingredients and of specific industrial process. Food production in general requires a series of technological steps involving physical and chemical processes: thermal treatments, spray-drying, cooking, extrusion, gel or dough formation, chemical or enzymatic hydrolysis, cross-linking, and oxidation, just to mention a few, all of which may induce deep and different structural changes in the food constituents. For this reason, although in raw food materials the characterization of protein constituents, still in a relatively "native" state, can be considered quite standardized at present, proteomics of processed foods remains a challenging task and requires properly designed approaches in order to verify conformity of food components and characteristics with those declared and to reveal adulteration.

For example, the skim milk powders (MP) are largely utilized as feed or as ingredients for the manufacture of products ranging from industrial cheeses to fermented meat products, in which they are added as emulsifier, texturizers, and for their high nutritional value. Soybean proteins are also frequently added to a variety of food products not only for economic reasons, as in the case of MP, but also because of their interesting functional properties during the processing of food. The addition of plant proteins to MP is, however, an illegal practice; therefore the detection and identification of adulterations in MP, typically by addition of low levels of cheap plant proteins (legume, cereal proteins) is an important issue to assess genuineness of several widespread industrial food preparation categories.

Because of the complexity of the matrices (both milk and vegetal protein sources), the detection with traditional analytical methods (HPLC, electrophoresis) is impracticable at the very low amounts of adulteration frequently used. Also, the official EU reference capillary zone electrophoresis (CZE) method currently used to prove fraudulent addition of plant proteins to MP has limited reliability and accuracy. For these reasons, the application of proteomic methodologies, based on combined chromatographic and MS methods, opens up new possibilities in the routine quality control of MP. Two LC/ESI–MS/MS methods (Luykx et al. 2007; Cordewener et al. 2009) were successful in detecting peptides originated from the major seed proteins of soy (glycinin, β-conglycinin) and pea (legumin, vicilin) in MP. They share the use of LC/ESI–Q–TOF–MS/MS aimed at targeting specific tryptic peptides from legume proteins. The first one included a pre-fractionation step of the (adulterated) MP samples. The second more general approach is based on an untargeted analysis combined with statistical PCA analysis. This method also allows us to distinguish MP samples from different manufacturers or produced according to different process conditions (milk protein composition, heat treatment).

A further example is that of mechanically recovered meat (MM). MM is industrially obtained by recovering residual raw meat from animal bones or poultry carcasses from which the bulk of the meat has been already removed mechanically. MM has the appearance of finely comminuted meat and is incorporated in a wide range of meat products, as an inexpensive source of meat. Although MM has a similar chemical composition to authentic or hand deboned meat (HDM), it has a lower commercial value. This has led to the exclusion of MM from the EU definition of meat (Directive of the European Parliament N. 101/2001). As a consequence analytical procedures are needed to differentiate it from hand-deboned meat. A very recent study has utilized a proteomic approach to find potential markers for the detection of chicken mechanically recovered meat. Intact proteins were extracted from raw meat and then analyzed with off-gel electrophoresis followed by SDS-PAGE and identification of potential markers by nano-LC-MS/MS. By this analytical workflow it was possible to extract, separate, and identify key proteins from processed meat material and to define chicken MM markers (Surowiec et al. 2011).

As a second example, a proteomic-based method has been developed for the detection of chicken meat within mixed meat preparations (Sentandreu et al. 2010). The procedure is robust and simple, comprising the extraction of myofibrillar proteins, enrichment of target proteins using off-gel isoelectric focusing, in-solution trypsin digestion of myosin light chain 3, and analysis of the generated peptides by LC-MS/MS. Using this approach, it was possible, for example, to detect 0.5% contaminating chicken in pork meat with high confidence. Quantitative detection of chicken meat was done by using AQUA stable isotope peptides synthesized on the basis of the sequence of previously selected species specific peptide biomarkers. Linearity was observed between the amount of the peptide biomarker and the amount of chicken present in the mixture; further independent replication is required now to validate the method. In spite of its simplicity, this approach can be used effectively for the detection of both raw and cooked

meat. It is amenable to highly processed foods, which can be particularly problematic as the tertiary protein structure is often affected in processed food precluding immunoassays. In addition, proteomic analysis allowed identification of definitive discriminatory peptide sequences, unlike the DNA/PCR-based methods used presently. Interestingly, the study also envisaged the translation of the technology to routine MS equipment, making the methodology suitable for public inspection laboratories.

Other limiting factors to proteomic analysis of processed foods are: (1) the increased protein complexity (e.g., production of oxidized protein families or of mixtures of hydrolytic fragments) and (2) the interaction of protein with other proteins or with the other molecules within the food matrix (e.g., in the dough network formation or in the case of the condensation products between carbohydrates and proteins in the early stages of the Maillard reaction). However, these reactions not only constitute an analytical complication but, on the other hand, may also be precious markers of the type of process and of the modifications of food quality. For this reason, recent procedures of protein chemistry, sometimes borrowed by classical biochemistry protocols, have been developed or adapted to obtain efficient protein extraction and characterization from processed foods.

In meat products, soy proteins serve as emulsifiers to avoid fat coalescence during heat treatment. However, regulatory authorities in some countries have forbidden soy protein or limited the amount that can be added, and if such threshold levels for soybean protein content in foodstuffs have been established, appropriate analytical techniques are needed for the reliable detection and/or quantitation of soybean proteins (soy protein isolate, SPI) in meat products. Currently, there are only a limited number of methods available for the (semi)quantitative determination of soybean proteins in processed meat products. The AOAC Official Method (AOAC Official Method of Analysis 988.10) based on an ELISA, has been criticized by regulatory authorities (e.g., the U.S. Food and Drug Administration) because of reliability and accuracy. In addition, highly sensitive assays would be desirable to be able to detect low ppm amounts of soybean proteins due to their allergenic potential (Koppelman et al. 2004). Liquid chromatographic assays which have been proposed are only applicable to raw meats. A recent proteomic study applied multidimensional HPLC-MS/MS to identify adulteration with SPI on heat-processed meat products from bovine, pork, chicken, and turkey (Leitner et al. 2006). Pre-fractionation by perfusion chromatography was needed to isolate peaks of interest from meat products containing SPI. After enzymatic digestion using trypsin, the collected fractions were analyzed by nanoflow LC-MS/MS. Several variants and subunits of the major seed proteins, glycinin and conglycinin, were identified in SPI, along with two other proteins. In soybean-protein-containing meat samples, different glycinin A subunits could be identified from the peak discriminating between samples with and without soybean proteins added. Among those, glycinin G4 subunit A4 was consistently found in all samples. Consequently, this protein (subunit) can be used as a target for new analytical techniques in the course of identifying the addition of soybean protein to processed meat products.

Heat treatments preserve or improve organoleptic properties and nutritional value of foodstuffs. Prior to being eaten, packaged, or stored, many foods require thermal processing such as pasteurization, cooking, and roasting. Thermal treatment results in enhanced food safety and quality but at same time may induce some undesirable reactions that result in a loss of nutritional factors or in the formation of mutagenic and carcinogenic molecules (van Boekel et al. 2010; Seal et al. 2008).

Proteins are among the main targets of food thermal damage. Proteins are strictly related to identity and history (in most cases also to geographical origin and to production methods), therefore monitoring of modified protein as a consequence of a heat treatment assesses quality and authenticity of each food product.

The most prominent modifications of food when subjected to heat treatments, concentration, or drying is the nonenzymatic reaction known as the Maillard reaction (Friedman 1996). The early stage of this reaction consists of interaction between amino acid residues and the carbonyl group of the reducing sugar, to give a Schiff base that is subsequently converted to the Amadori product (Chichester 1986). Maillard products are extremely important in food preparation for developing desirable flavor, odor, or color in food such as coffee, bakery products, and roasted meat or for improving the physicochemical properties of milk whey proteins (van Boekel MAJS 2006). On the other hand, an uncontrolled Maillard reaction (in consequence of incorrect or undeclared heat treatment), yields advanced glycation end products resulting in a decreasing of bio-availability of several essential amino acids, (Birlouez-Aragon et al. 2004; Hewedi et al. 1994), decreasing of protein digestibility, and formation of some undesirable compounds (Taylor et al. 2003; Uribarri and Tuttle 2006). Owing to sensitivity, specificity, and speed in the analytical response, MS represents an excellent analytical tool to face the challenge of characterizing Maillard product (Mamone et al. 2009). In the last decades MS has been largely applied for determining Maillard products in complex food matrix.

Malt and beer are an example of food and beverages in which glycated proteins are produced during the malting process. Malt and beer proteins may undergo extensive reactions with glucose generated from starch degradation. The degree of the protein glycation influences the quality and the properties of the beer foam. Glycated forms of lipid transfer protein (LTP) in malt and beer have been identified by MS in combination with of gel-electrophoresis (Jégou et al. 2001) or monolithic chromatographic media (Bobalova and Chmelik 2007). More recently an interesting gel-free MS-based approach (2-D HPLC coupled to MALDI-TOF/TOF), allowed the characterization of barley glycated proteins during brewing, and identification of potential glycation markers for fast and efficient monitoring of the correctness of the malting conversion. (Petry-Podgórska et al. 2010; Zídková et al. 2010)

Because of their high lactose content, milk and dairy products are matrices where the potentiality of MS-based techniques have been better applied to study heat treatment-induced modifications. Based on the 324-Da molecular weight increase, lactosylation protein can be easily detected (Siciliano et al. 2000). Lactosylation of intact milk proteins has been evaluated by LC-ESI-MS analysis within commercial whey-based products (Hau and Bovetto (2001)) and milk (liquid and powdered)

samples. (Siciliano et al. 2000; Scaloni et al. 2002). MALDI-TOF-MS has been also applied for characterization of lactosylation of whey proteins in model milks (Meltretter et al. 2007; Catinella et al. 1996), in heat treated milk (Meltretter et al. 2008), and commercially available dairy products (Meltretter et al. 2009).

BLG and α-lactalbumin have been identified as main targets of the Maillard reaction (Siciliano et al. 2000). Whey protein lactosylation accounted for almost 3%, 30%, and 70% of the BLG content in pasteurized, UHT, and dry infant formula samples, respectively. Selective lactosylation upon different thermal processing demonstrated that whey proteins can be exploited as indicators to trace the quality and thermal history of milk (Siciliano et al. 2000; Morgan et al. 1997; Jones et al. 1998; Czerwenka et al. 2006; Losito et al. 2007; Monaci and van Hengel 2007).

MS/MS techniques have been successfully used to localize the main amino acid sites of lactosylated BLG (Leonil et al. 1997; Fogliano et al. 1998), casein (Scaloni et al. 2002), and lysozyme (Yeboah et al. 2004). Identification of minor lactosylated species has required the use of more specific LC-MS/MS technique such as "neutral loss" or "precursor ion scanning" (Molle et al. 1998; Marvin et al. 2002; Fenaille et al. 2004).

In some cases, glycated protein pre-separation alone is insufficient for dealing with Maillard reaction complexity. Therefore, the most abundant casein and whey protein may have to be depleted from the sample. A selective depletion procedure was developed for identification of lactosylated sites in milk and in powdered milk infant formulas (Arena et al. 2010) using a combination of proteomic techniques based on analyte capture by combinatorial peptide ligand libraries, selective trapping of lactosylated peptides by m-aminophenylboronic acid-agarose chromatography, and two different fragmentation MS/MS modes. By this approach 271 nonredundant modification sites in 33 milk proteins have been characterized.

A proteomic enrichment protocol has been recently developed to identify lactosylated casein phosphopeptides in milk samples based on hydroxyapatite chromatography and MALDI-TOF-MS. The amount of modified peptides varied with heat treatment severity. With a 10% limit of detection of UHT milk addition to raw or pasteurized milk, casein phosphopeptides (CPP) were used as markers of thermal treatment in milks heated at various intensities (Pinto et al. 2012). On the other side, proteomic techniques have been scarcely applied to detection of the advanced glycation end products (Henle et al. 1997; Hegele et al. 2008; Hasenkopf et al. 2001) in food samples. Among these products lactulosyl-lysine, Nε-carboxymethyl-lysine (CML), lysine aldehyde, methionine sulfoxide, cyclization of N-terminal glutamic acid, and oxidation of cysteine and tryptophan modification have been screened as the major heat-induced markers of alterations of whey milk proteins by MALDI-TOF-MS analysis (Birlouez-Aragon et al. 2004). A more detailed presentation concerning the distribution of free and protein-bound glycation-induced lysine modifications in raw and processed cow milk has been described (Hegele et al. 2008). More recently a method based on LC-MS/MS using a stable isotope dilution assay has been proposed for quantitative analysis of CML in various dairy products. The method, based on enzymatic digestion

and LC–MS/MS analysis, estimates a limit of detection for CML of 8-ng CML per mg protein and the limit of quantification of 27-ng CML per mg protein (Delatour et al. 2009).

Other protein markers of heat treatment, not generated by reaction with lactose, such as lysinoalanine, histidinoalanine, N-terminal pyrrolidone, or partial protein hydrolysis may also occur in milk (Friedman 1999). The most interesting analytically are the heat-susceptible phosphorylated serine and threonine residues of caseins yielding dehydroalanine and methyl-dehydroalanine, respectively. The conjugated carbon–carbon double bond reacting with nucleophilic amino- (Lys), imidazole- (His), or thiol-(Cys) group forms lysinoalanine (LAL), histidinoalanine, and lanthionine residues, respectively. In particular, LAL has been determined in milk and cheese products by LC/ESI/MS in positive selected ion monitoring (Calabrese et al. 2009). The method encompasses acid protein hydrolysis to amino acids prior to LC–MS/MS analysis. This approach has been usefully to demonstrate that LAL is not present in raw milk or some dairy products such as Mozzarella cheese; in contrast, high amounts of LAL are present in calcium caseinate and milk powder, materials frequently used for fraudulent PDO cheeses adulteration. Because of the high specificity, LAL has been proposed as a useful marker to demonstrate either uncorrected heat treatments or the occurrence of heated milk/milk powders that could have been added to fresh milks for cheese adulteration.

The peptide composition dynamically changes in a food as the result of proteolysis, modifications induced by technological treatments, and interactions with other components. They decisively affect both biological as well as functional properties of food products. Thus, food peptidomics, intended as the global characterization of the peptidome of a given food product, embraces a wide research area that aims to establish the origin of the peptidome, its evolution, the impact on the sensorial properties, and its beneficial or adverse effects on human health. Food peptidomics also encompasses the development of analytical strategies to study the food peptidome.

Peptides retain memory of the history a food has undergone. Therefore, peptides are much more than just convenient analytical substitutes of parent proteins in many of the proteomics workflows; peptidome indeed provides matchless information about a given food product, for instance in the case of fermented foods.

The change in peptide composition of cheeses during ripening is a striking example. The peptide profile indicates the origin and evolution of the protein fraction. During the ripening period, the cheese chemical components undergo important chemical, physical, and enzymatic modifications: proteolysis and other reactions, such as lipolysis and lactic and propionic acid fermentation, influence the organoleptic properties of the final product. Proteolysis directly contributes to flavor (release of peptides and amino acids) and off-flavors (bitter hydrophobic peptides), also liberating substrates for other reactions. Thus, for the development of an acceptable cheese flavor, a well-balanced breakdown of the protein (i.e., casein) into small peptides and amino acids is necessary. On the other hand, recently there has been great interest in the protection of typical food products from adulteration,

sophistication, and falsification. A large use of MS techniques has been made in the past to trace the proteolytic events that occur in the ripening of Parmigiano-Reggiano, Emmenthal, or Cheddar cheeses (Piraino et al. 2007). The peptidome profile also indicates origin, authenticity, and typicality of PDO cheese. The proteolysis as a function of the rennet typology has been assessed in Fiore Sardo ovine PDO cheese (Pirisi et al. 2007).

A peptidomic approach based on capillary electrophoresis-MS allowed us to identify specific β-casein fragments, referred to as γ_4-casein, in buffalo milk arising from plasmin hydrolysis (Somma et al. 2008). This polypeptide, being unique to water buffalo milk, might be conveniently used as a marker of this species milk.

Although to a minor extent, proteolysis also occurs on frozen foods. This is the case of the γ-caseins produced even during the cold storage of buffalo curd (Di Luccia et al. 2009). Interestingly, peptidomics changes have also been observed in ice-stored fish, in addition to the expected changes due to cooking (Bauchart et al. 2007). The proteolytic peptides specifically generated are molecular targets to distinguish fresh from cold-stored materials, and therefore to assess the fraudulent use of disallowed refrigerated or frozen ingredients.

The biochemical changes occurring during the processing of dry-cured ham have been studied with peptidomics techniques relying on MS-approaches. The intense action of endogenous proteolytic enzymes that are activated in the early post-mortem phase induces extensive degradation of muscle proteins. The recent identification of a large number of small peptides released from enzymes belonging to the glycolytic pathway has confirmed that muscle sarcoplasmic proteins are relevant substrates for proteolysis (Mora et al. 2011a). Generally in real foods, as in this case, the identification of the released peptides is made difficult by the lack of a cleavage specificity. Nevertheless, the multistage MS-based peptide sequencing can furnish information about the activity of early (calpains) as well as the medium- and long-term acting (cathepsins) endogenous proteases. The identification of oligopeptides characterized by the consecutive loss of terminal amino acids has demonstrated an additional intense secondary proteolysis in dry-curing hams that is due to both amino- and carboxy-peptidases (Mora et al. 2009).

The MS-based identification of large- and small-sized fragments of myosin (heavy and light chains) and actin has demonstrated that muscle myofibrillar proteins undergo extensive proteolysis as well, in the maturing of dry-cured ham (Mora et al. 2011b; Di Luccia et al. 2005; Sentandreu et al. 2007).

The proteolytic machinery of lactic acid bacteria concurs to a more extensive proteolysis of fermented sausages (Picariello et al. 2006). However, a few peptidomic investigations have been published related to these products. The peptidomic definition of proteolytic patterns could be helpful in assessing or predicting the suitability of specific strains of lactic acid bacteria as starter cultures for dry fermented sausages. The MS identification of species-specific peptide biomarkers has been proposed as a tool for determining the authenticity of meat-derived products (Sentandreu and Sentandreu 2011).

24.7 Proteomics, Peptidomics, and Metabolomics in Food Maturation, Preservation, and Safety

From the metabolomic perspective, most foods can be considered as complex chemical mixtures consisting of various metabolites and chemical additives in a solid, semi-solid, or liquid matrix. Some foods consist of just a few different compounds (artificial energy drinks, soft drinks, purified vegetable oils) whereas other foods consist of hundreds of compounds (milk, cheese) and still others may have thousands of compounds (fruits, meats, and most prepared foods). These compounds may constitute a fingerprint of the food itself and of its history. In food technology, detection of the metabolomic profile is gaining great importance for several fields of applications. These include tracing the evolution of a product from the starting materials to finished foods; evaluating the effects of either desired or undesired microbial action on food quality; monitoring the production or persistence of functional and bioactive compounds; and, in general, measuring the action of technological treatments (physical, chemical, enzymatic) on the development of rheological and sensory characteristics. Examples in dairy, cereal, wine, or meat products depict the metabolomic fingerprint of these products. Wine metabolome analysis by ultrahigh-pressure liquid chromatography high-resolution Fourier transform ion cyclotron resonance mass spectrometry (UPLC-FT-ICR-MS) in combination with multivariate statistical data processing has been proven able to discriminate the wines according to cultivar, vintage, provenance, maturation, and quality, opening the way to its extensive use in authentication studies (Cuadros-Inostroza et al. 2010; Liger-Belair et al. 2009; Gougeon et al. 2009), at present still based on subjective sensory analysis or analysis of a limited number of compounds. The capability to distinguish among high- and low-quality products on the basis of a comprehensive metabolomic identification of measurable objective biomarkers or molecular pattern related to (sensory) quality is providing new powerful tools for the defense of PDO products from adulteration (the case of salami in Fig. 24.3).

Because of the globalized nature of food production and processing, along with the growing concerns over food safety and food quality, more stringent and precise food monitoring systems are being set up. Proteomic and metabolomic approaches are therefore also being applied to food safety control.

Milk and dairy product quality can be affected by mastitis, which is rather frequent in intensive dairy farming (especially of high-milk yielding cows such as Holstein Friesian). The legal parameter used to evaluate the level of contamination of mastitic milk is the somatic cell count, that is, the mammary gland cell released in the milk by infected animals. However, this parameter is only an indirect indicator of the milk status. Consequently, adulteration of healthy with mastitic milk or forbidden use of mastitic milk is a complex task. A direct evaluation could be provided by identification of specific protein markers, as proposed by studies showing a different degree of milk protein hydrolysis in mastitic milk compared to healthy milk (Hogarth et al. 2004). These proteomic approaches not only provide clues for

evaluating technological as well as nutritional losses due to infection, but identification of protein markers may be the basis for the development of analytical methods to evaluate illegal use of poor-quality mastitic milk in bulk milks.

A further issue is the evaluation of proteins specifically expressed in mastitic milks, aimed to evaluate the efficacy of adjunctive therapies for the treatment of inflammation associated with coliform mastitis in order to facilitate new veterinary drug approvals. At present, these studies are accomplished using ELISA tests. However, because a relatively limited number of bovine-specific antibodies are commercially available, reliance on antibodies can be very limiting for biomarker discovery. Proteomic approaches have instead the capability to analyze a large number of protein targets in a single experiment, independent of antibody availability. Proteomic approaches based on LC-MS/MS, recently reviewed (Boehmer et al. 2010), have been used as a means to characterize proteins in various bovine milk fractions, both under normal physiological conditions as well as during clinical mastitis. Temporal expression patterns generated using spectral counts, an LC-MS/MS label-free quantification strategy, corresponded well with ELISA data for acute phase proteins with commercially available antibodies. Combined, the capability to identify low abundance proteins, and the potential to generate temporal expression profiles, indicate the advantages of using proteomics as a screening tool in biomarker discovery analyses to assess biologically relevant proteins modulated during mastitic disease, including previously uncharacterized targets.

The analysis of pathogenic microorganisms is also of particular interest, as the risks associated to their contamination are not limited to their living presence and capacity of infectivity, but they can generally release protein/peptide toxins able to survive for long time even in foods after bacterial cell contamination has been removed, as it happens for many of the microbes that cause food-borne diseases (*L. monocytogenes*, *S. aureus*, *E. coli*, *C. botulinum*, and various *Salmonella* species). These pathogens release protein virulence factors into extracellular medium and to the cell surface that have essential roles in the colonization and insurrection of the host cells, and thus reflect the degree of bacterial pathogenicity. These toxins, being heat-stable and resistant to food processing, can be a danger to consumer health. Bacterial protein toxins have been involved in a number of infectious and food-borne diseases. Chromatographic methods coupled with MS have been carried out to define the toxin contamination levels of milk (Sospedra et al. 2011) and two ripened PDO Italian cheeses, Grana Padano and Pecorino Romano (Schlosser et al. 2007; Ferranti 2005). A procedure combining proteomic approaches with immunochemical, chromatographic, and electrophoretic techniques and MS/MS analyses was developed to monitor production and levels of enterotoxin A and B of *S. aureus* and Shiga-like toxins produced by *E. coli* O157:H7. By producing cheese samples using milk willingly contaminated with bacteria it was possible to monitor 10–100 ppb contamination level, and analysis of market samples allowed to exclude toxin contamination in the two cheese types.

The development of fast and accurate methods for species differentiation of food pathogenic and spoilage bacteria is another important issue to ensure food quality and safety and to detect use of forbidden contaminated materials. MALDI-TOF-MS

has been applied to species identification of microorganisms, proving to be a rapid and cost-effective technique and allowing species differentiation due to the highly specific spectral profiles obtained (Bohme et al. 2010). The analysis of cell extracts by MALDI-TOF MS was also applied to create a mass spectral library of the main pathogenic and spoilage bacteria potentially present in seafood and was demonstrated to be a rapid and accurate method for microbial species differentiation, as well as for the classification of unknown strains isolated from seafood.

Among contaminants along the food chain, algal toxins produced by cyanobacteria are another special concern. Cyanobacteria (blue-green algae), present worldwide within water blooms in eutrophic lakes and drinking water reservoirs, producing several different biotoxins. Among these, potent peptide hepatotoxins such as microcystins, cyanopeptolins, and microviridins may be released in the water environment. The occurrence of hepatotoxins-containing algal blooms in freshwaters and subsequently in cultivated fish and seafoods has been implicated in several animal and human poisoning outbreaks worldwide. Also, algae species such as Spirulina are finding large application as ingredients of food, feed, and vitamin integrators. Therefore, methods to monitor the presence of hepatotoxins in water and animal tissues is of growing interest for surveillance agencies, because they can contaminate drinking water reservoirs and the food chain. Proteomic approaches based on MALDI-TOF-MS and Q-TOF/MS were revealed as useful for fast screening and accurate quantitative determination (LOD <0.1 ppb, limit of quantification (LOQ) 1 ppb) in water (Ferranti et al. 2011; Gallo et al. 2009; Ferranti et al. 2008, 2009). The analysis was then extended to the set-up of a method for quantitative toxin determination in food and feed integrators for men and animals and also to detect protein markers to discover the illegal use of unsafe water in fish and seafood aquaculture. It can be foreseen that these methods are being integrated to design sensitive sensors on a microchip surface for automated detection.

24.8 Genetically Modified Foods

A genetically modified organism (GMO) is an organism whose genome has been modified by genetic engineering with the introduction or deletion of specific genes in order to create a new gene set. The constant search for agriculture development led to the introduction of GMOs in food technologies. The main modifications consist in (1) induction of higher resistance to environmental stress, pathogens, and parasitic plants; (2) increase of organoleptic, nutritional, and technological values, as for instance rice containing higher protein and riboflavin A levels or tomato with inhibitors of the enzymes responsible for pectin degradation or soy with greater amounts of lysine; and (3) reduction/inactivation of antinutritional factors. GM food world production is mainly concentrated in the United States, Brazil, Argentina, Canada, and China. Because of the on-going debate on possible hidden adverse effects on humans, GM plant cultivation has expanded quite slowly in Europe,

where, to date, only two GM vegetables can be grown: maize Mon810 (1998) and potato Amflora (2010) (http://www.efsa.europa.eu/it).

The gene alteration implies the modification of only a limited group of target compounds, for example, the modification of already existing proteins or the generation of novel proteins. Potential disadvantages of this technique can be the underexpression of the total seed storage protein or the production of unexpected proteins with potential allergic and/or toxic character (Finnie et al. 2004).

Current legislation imposes the monitoring of all the production steps from cultivation to transformation and commercialization, the traceability of raw transgenic materials, and the labeling of the final GM products. These aspects raise the needs for inexpensive and fast techniques for one-step detection of GM contamination in food products.

Furthermore, in several cases the presence of GMOs in food products can be due to an accidental cross-contamination, and the legislation admits the commercialization of products containing levels of GMO-derived materials with an amount <0.5% (Petit et al. 2007).

However, GM raw materials can be also intentionally introduced in the food processing chain following deceptive adulteration, aimed to improve the quality of final products or to reduce costs of production. In all these cases the GM raw materials occur in a much lower amount if compared to the raw material used, and this makes the detection of contamination difficult. Another obstacle to assessment of GM material contamination is the intrinsic high complexity and variability of seed storage proteins and the high influence of soil and climate factors on protein expression.

The GM material assessment is generally carried out by DNA profiling based on PCR methods. However, although the detection of DNA markers benefits from having a well-defined target gene and the combined use of database analysis and experimental specificity reduce false positives, it has to be underlined that the presence of a transgene in a food product does not guarantee the phenotypic expression of the transgene traits.

Therefore, gene products have to consider more reliable target analytes than their gene counterparts. The typical approach for GM protein detection relies on the detection of recombinant proteins by immunochemical analysis and quantification by ELISA. These techniques are easy, inexpensive, and suitable to screen a large number of samples. In general, the set-up of these assays requires identification and characterization of the transgenic protein to raise specific antibodies against the target (Farid 2004). For instance, the lateral flow strip protocol is a useful commercial tool for the detection of RUR, Cry3Bb, and Cry1Ab proteins in corn and of CP4 EPSPS, that is a transgenic protein that confers to several crops, including soybean and maize, the resistance to the herbicide glyphosate. This technique is very fast and sensitive as confirmed by a detection limit of 1 seed out of 1,000. As a relevant limitation, this strategy necessitates that the target protein is in its native and immunoactive form, thus implying that proteins in treated foodstuffs might escape detection. Furthermore, in several processed foods linear epitopes or toxic sequences can be hydrolyzed and released from the parent protein, although retaining their harmful

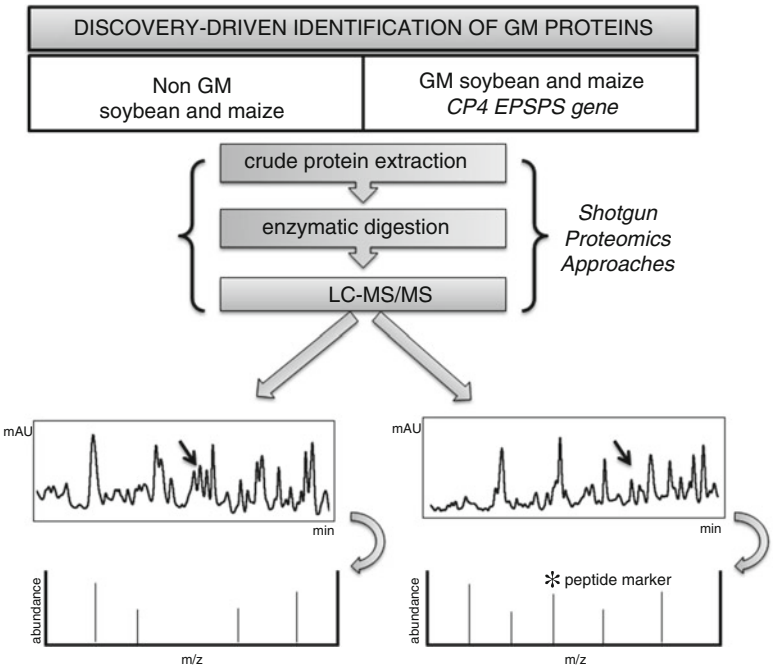

Fig. 24.4 The typical shotgun approach to GM biomarker detection and identification in food matrix. This kind of approach can be applied for the detection of any kind of peptide marker

potential. These sequences tend to be underestimated by the most commonly utilized sandwich ELISA-based tests.

Only in the last years, however, first attempts have been made to apply advanced proteomic techniques for comparison of protein profiles of transgenic material and control wild-type lines in order to detect peculiar modifications of proteins and peptides to be used as molecular markers of GM contamination (a classical analytical scheme in Fig. 24.4). Comparison of 2DE maps of proteins extracted from a wild-type and a hybrid tomato, in which a virus-resistance gene trait, showed, however, that this modification is not detectable by the proteomic approach because the hybridization did not affect protein expression appreciably (Corpillo et al. 2004). Similar results were obtained by Careri et al. (2003) who compared proteins extracted from transgenic potatoes with a control line and by Finnie et al. (2004) who, comparing the protein maps of the Golden Promise barley cultivar with different transgenic barley lines, restricted the potential alteration of the transgenic lines only to the pattern of post-translational modifications.

On the other hand, the combination of 2DE, image analysis, and MS-based approaches has been demonstrated to be very effective for the comparison of GM and non-GM products when the modification interests an encoding gene trait. It has been shown that the introduction of a gene that induces increase of the amount of

low molecular weight (LMW) glutenins in the genome of a bread wheat cultivar, caused a positive improvement of dough viscoelastic properties. 2DE-PAGE profile of proteins showed a significant over-expression of the LMW fraction and the down-regulation of the remaining seed storage proteins (Scossa et al. 2008).

One of the main drawbacks of the 2DE-based techniques is the gel-to-gel variation that complicates the image analysis. However, this limit can be overcome by 2DE DIGE analysis. According to this technique proteins from GM and non-GM materials, after being labeled with two different ultrahigh-sensitive fluorescent dyes with different light emission frequency, were separated in a single gel. As described by Islam et al. this technique associated with peptide mass fingerprinting, helped detection of 66 discriminating gene products that were characteristic of transgenic pea lines, properly designed to express α-amylase inhibitor typical of common beans.

Only a few studies published up to now propose MALDI-TOF-MS and LC-MS/MS analysis as additional useful tools for GM food traceability. Among these, Simó et al. (2010) described the comparison of hydrolyzed proteins from GM soybeans versus control lines by a shotgun proteomic approach exploiting CE-ESI-TOF-MS. Remarkably, no detectable changes were identified between the two profiles, thereby demonstrating that the process of GM soy production affects protein expression only to a minor degree. Differently, Fernandez Ocana et al. (2007, 2009) demonstrated that the LC-MS approach may be diagnostic for the detection of the potential target CP4 EPSPS, with a detection limit of 0.9%. These contrasting reports indicate that much extra work in the identification of suitable protein markers is still needed to make the proteomic/peptidomic approach as much versatile as the genomic one. On the other side, these studies are accumulating a huge set of information from which compositional GM material databases are being constructed to direct the upcoming development of proteomic methods.

24.9 Conclusions and Perspectives

Proteomics and the related “omic” sciences are ideally positioned to be used in many areas of food science and nutrition research and to address a multiplicity of aspects, including component analysis, quality/authenticity assessment, consumption monitoring, and study of the physiological impact in nutritional intervention studies. Nevertheless, the number of reports on food adulteration is relatively low when compared with other consolidated techniques of investigation. Despite their steadily increasing use and their enormous potential, marker-assisted proteomic strategies have not yet delivered the expected benefits in the context of the fight against food adulteration. One of the primary limiting factors is related undoubtedly to the instrumentation and maintenance costs. Taking into account the advantages deriving from the short operative time required for analyses, these drawbacks could be at least in part overcome by the establishment of unified monitoring centers and by an adequate policy of resource rationalization. The development of up-to-date

databases of food protein components also plods along to balance instrumental advances, actually restraining the informative level of the "omic"-based procedures. A general view displays a scenario where the technological platforms and methodical prerequisites are now available, so that proteomics can massively breach into food science with special focus on quality assessment, whereas the operative procedures are not standardized and the process of data mining still appears to remain a challenge that hampers routine control programs. Thus, it emerges that significant improvements need to be made to optimize and automate the monitoring of target compounds and to render the assessment flowchart less dependent on the skills of the operators. In the view of the evolution toward automation the new trends in sample pre-treatment or pre-fractioning have also to be opportunely comprised. To this purpose, it has to be also underlined that comprehensive maps of validated markers of specific food traits are still missing. The extended high-throughput application of the omic methodologies is already now enlarging the panel of the "probes" to be monitored and is going to provide the means to standardize and validate the procedures for routine analyses and food authentication studies. It can be reasonably expected that in the future descriptive proteomic and omic studies will accelerate the annotation of food components and the definition of the (bio)chemical events induced by food processing, also including the complex pathways of biomolecular interactions that are emerging as key issues of the system biology in general, and of food and nutrition in this special case. As a conclusive stage of the entire process, legislative efforts would be required to progressively introduce innovative regulations, in order to parallel the analytical advances at an applicative level, with the ultimate aim of concretely translating the scientific achievements in terms of human health and wellness.

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