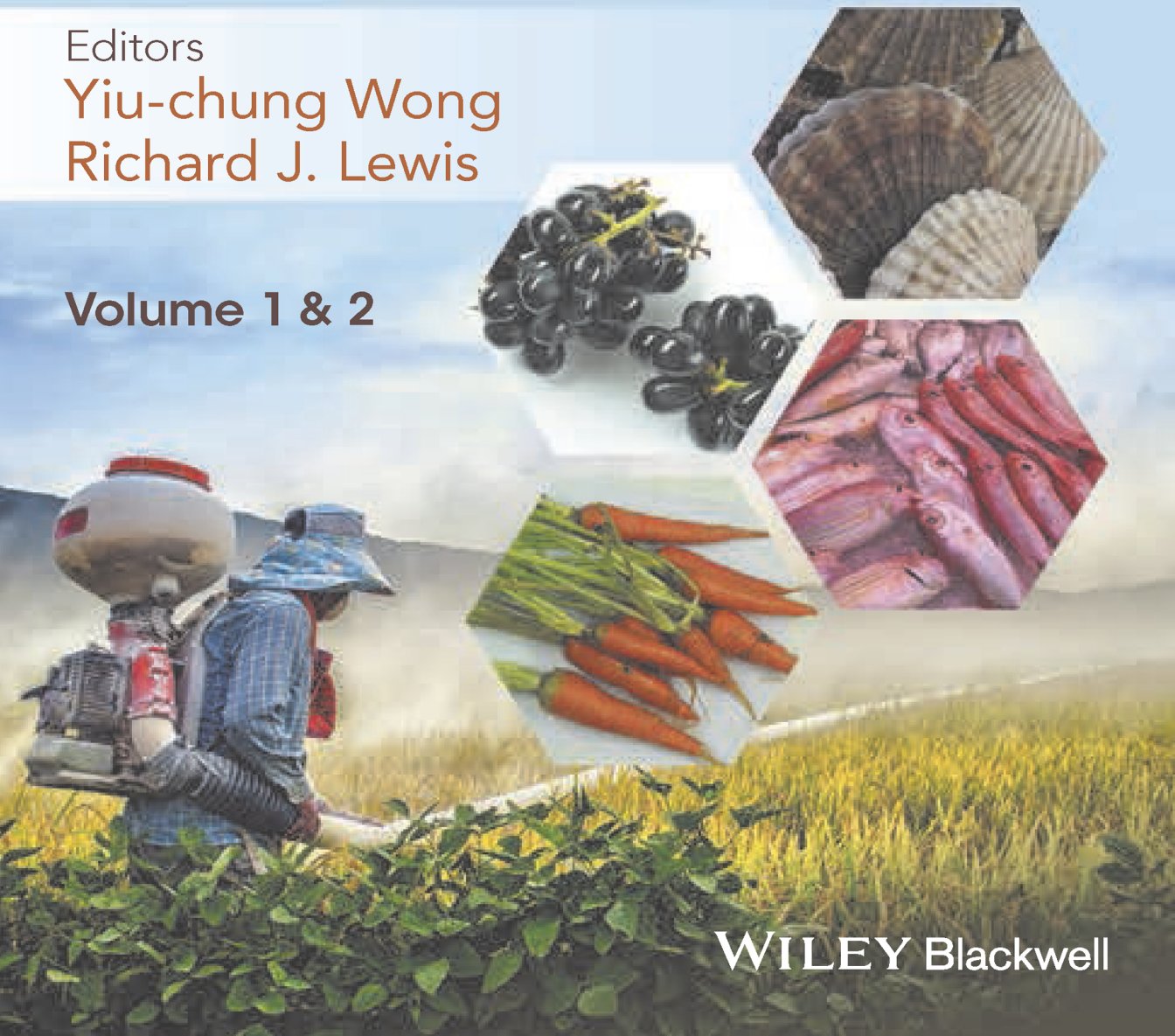
Analysis of Food Toxins and Toxicants

Editors
Yiu-chung Wong
Richard J. Lewis

Volume 1 & 2



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Foreword

I regard it an honour and privilege to have been asked to provide a preface for this significant monograph which surveys the recent and current developments in monitoring food toxins and toxicants which compromise human food safety. In the past, many incidents have caused profound impacts on people's health and on businesses in the food manufacture and distribution sectors. The awareness and knowledge of natural and synthetic toxins and toxicants in food has improved significantly in the last two decades via extensive worldwide collaborative research work and monitoring programmes.

The provision of reliable data from the use of advanced and versatile technologies is now a prerequisite for the investigation of the causes of food poisoning incidents, the prevention of such events and for the production of wholesome and safe food. Attention is drawn herein to the importance of quality assurance activities, as for any analytical measurements with potential as forensic evidential use, and to the need for relevant reference materials. The value of strategic risk assessments and data modelling are shown to be keys to the setting up of appropriate limits for toxin concentrations in foods and in food components. Hence this comprehensive review of such work, much of which has been undertaken with worldwide collaborations, to produce data using validated analytical methodologies is considered to be most timely.

The editors are to be congratulated for their selection of relevant and interesting topics. The various section authors have produced a readable, in-depth survey of the current position in the analysis of food toxins and toxicants and also have drawn attention to some important residual problems in certain areas concerning reference materials.

The volume is divided into five main sections: I (Chapters 1–4). Recent developments in analytical technology including sample pre-treatment and food additives; II (Chapters 5–10). Microbial and plant toxins, including plant pyrrolizidine alkaloids; III (Chapters 11–15). Marine toxins in fish and shellfish; IV (Chapters 16–19). Biogenic amines and common food toxicants, such as pesticides and heavy metals; V (Chapters 20–24). Quality assurance and recent developments in regulatory limits for toxins, toxicants and allergens, which includes discussions on laboratory accreditation and reference materials.

Due to the excellent editorial control all the chapters are easy to follow, coherent in layout and are comprehensively referenced, which most helpfully indicate the papers' contents by giving their titles in full.

This up-to-date set of accounts of analytical approaches available and the problems to be encountered in the detection and estimation for a variety of food toxins will be useful to

analytical chemists working in academic, manufacturing, distribution and regulatory food control laboratories.

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Preface

Food toxins and toxicants are widely discussed global issues, and given the threats they pose to human health, they represent one of the most important aspects of analytical chemistry. Over the past two decades, we have experienced various crises due to foodborne toxins causing profound impacts on human health and the food industry. Importantly, in countries where well-developed food management systems with reliable methods of measurement are not established, such outbreaks can lead to potentially life-threatening exposures and resource waste. Our knowledge and awareness of natural toxins and toxicants in foods have improved substantially through extensive research and worldwide networking programs in the field. Thanks to the continuous commitment and cooperation of various organizations, validated analytical methodologies can now detect sub-clinical levels of many food toxins and toxicants using advanced and versatile technologies. A comprehensive monitoring of food toxins and toxicants is a critical prerequisite to substantiate the causes of food poisonings and help prevent similar food catastrophes from taking place. The implementations of quality assurance to food toxin analysis, including the production of reference materials, strategic risk assessment and data modelling for toxin thresholds, are required to validate and strengthen the measurement applications. This book provides an up-to-date and comprehensive overview of the analytical approaches used to detect a range of food toxins. Contributions from more than 70 eminent food toxin scientists across the globe illustrate their expertise and experience to readers. We hope that it can provide useful guidance and instruction to analytical chemists and food scientists, both in industry and academia. In each chapter, the authors aim to provide a concise discussion on the latest methodology currently applied to measure a wide variety of food toxins and toxicants, including a detailed and illustrated overview of different separation and detection approaches used. Finally, we would like to express our sincere thanks to all of our renowned authors who contributed their invaluable time and their expertise to this book.

Yiu-chung Wong
Richard J. Lewis

Section I

Recent Analytical Technology for Food Pathogens and Toxins

1

Omic Analysis of Protein and Peptide Toxins in Food

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1.1 Introduction

The human body is continuously exposed to many substances potentially harmful to health, and therefore defined toxins. Toxins can be of exogenous or endogenous origin. Endogenous toxins are mainly represented by free oxygen radicals that are formed during the normal metabolic processes (cellular respiration, food digestion, excretion) and are waste products of metabolism itself. The level of endogenous toxins may increase in certain situations, that is, prolonged stress, very intense physical activity, large and high caloric meals. On the other side, exogenous toxins enter the body through ingestion, respiration, inhalation or skin adsorption. They may be chemical compound additives contained in many foods, waste products of drugs and heavy metals. These toxins may accumulate insidiously in the body, causing damage at various levels.

Most exogenous toxins derive from contaminated water, beverages and foods. They may contain a wide variety of xenobiotics, either naturally or in consequence of voluntary/involuntary addition. A large class of food toxins is that including those of protein and peptide nature. Well-known examples of toxic proteins are bolesatine and ricin. Bolesatine is a glycoprotein isolated from the mushroom *Boletus satanas* that causes serious gastroenteritis in humans. This lectin, at very low concentrations, has mitogenic activity on human lymphocytes, while at higher concentrations it inhibits protein synthesis (Ennamany *et al*. 1998). Ricin, a protein found in the seeds of the plant *Ricinus communis*, is a potent natural cytotoxin: it may cause cell death by blocking the protein synthesis activity on ribosomes. Because of their toxicity, accurate and sensitive methods for detection of protein toxins are needed. However the large complexity of these molecules (high molecular weight [HMW], presence of subunits, glycosylation, micro-heterogeneity) has made this task very difficult. In the last years, however, the application in food analysis of novel analytical 'omic' platforms, mostly based on mass spectrometry technologies, has made possible either qualitative and quantitative proteome or peptidome analysis. McGrath *et al*. (2011) have developed a sensitive and selective MS-based method to detect and quantify ricin in beverages, such as tap water, milk, apple juice, and orange juice, using isotope dilution mass spectrometry with a linear ion trap operating in product-ion-monitoring mode.

Extensive research in the last years has shown that data generated by the combined omic technologies represent a unique resource for food technology. The focus of this chapter is on

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Table 1.1 Peptide and protein toxins in foods and their reference analytical methods.

Toxins in foods	Analytical methods	Reference
Bacterial toxins	ELISA, HACCP, PCR	Patel (2012)
Fungal NRP toxins	HPLC–HRMS	Nielsen and Larsen (2015)
Mycotoxins and other fungal toxins	LC–MS/MS	Malachová *et al.* (2014)
Marine and cyanobacterial biotoxins	LC-MS/MS	Luckas, Erler and Krock (2015)
Phytotoxins	Omics technologies	Scognamiglio *et al.* (2015)
Food allergens	MS, GC, HPLC, CE	Zheng and Chen (2014)
Food additives	LC-Orbitrap-HRMS	Senyuva, Gökmen and Sarikaya (2015)
Food preservatives	LC-MS/MS	Robbins *et al.* (2015)
Food processing	LC-MS/MS	Nurit *et al.* (2015)

foods' peptide and protein toxins and on the most recent development in their methods of analysis. In particular, we shall see that 'omics' techniques constitute a potentially comprehensive class of methods for monitoring of food quality, allowing simultaneous qualitative and quantitative toxin measurement in a variety of food categories. The omic approach may provide as a global perspective of knowledge on biological systems, and this also includes foods, their evolution over time, and their impact on human health. Proteomics and metabolomics (along with their derived branches) are already mature - but still evolving - technologies capable of tackling composition and contamination of complex food matrices (Table 1.1). By these approaches, even low amounts of toxins in food samples can be rapidly detected also in the presence of interfering components (Boyer *et al.* 2011).

1.2 Methods of Food Toxin Analysis

The impressive increase in food production, processing and packaging amounts in the beginning of the new millennium to meet the food demand for a world population exceeding 9 billion people, has been paralleled by an increase of reported cases of food contamination with toxic substances, resulting in various outbreaks of human poisoning or intoxication.

The issue of food safety has an extremely high relevance for both human health and the food market economy and imposes the urgent need to improve the robustness of the available analytical methods for its assessment. The growing consumer awareness of food safety and quality, the increased demand for legal regulation and adequate labelling, together with the evolution of the deceptive strategies, are fuelling the development of up-to-date procedures of food control that have to be developed, standardized and validated.

Briefly, a food product can be contaminated if one of the food ingredients has been produced with contaminated or diseased organisms or when foodstuffs are incorrectly processed or packed. The presence of potentially harmful ingredients or contaminants has to be assessed by the detection of the target molecule(s) and by monitoring the biomolecular composition of the food.

Over the years, an arsenal of analytical methods, mainly based on morphological/anatomical analysis, organoleptic markers (odour, colour, texture) or chemical testing, have been developed

to check for food contaminants. In general, there are three basic detection strategies used for verifying a toxin contamination: i) demonstration of the presence of the toxin itself or of a surrogate marker; ii) indirect demonstration by verifying biological properties of the substance, e.g. agglutination or enzyme-linked immunosorbent assays (ELISA) positivity; iii) demonstration of an altered analytic profile compared to the uncontaminated food. Among these, the strategy of direct characterization of a toxin or of an appropriate surrogate marker is considered as the most reliable. For the above reasons, in the recent years new approaches have been developed to improve food characterization. Determination of a stable isotope ratio, especially on trace elements, provides a stable isotope signature useful to establish a close link between products and their environment.

In the case of pathogen contamination, most recent genomic and transcriptomic approaches specifically target RNA and/or DNA markers to detect foreign organisms in the final products derived from the contamination of the raw ingredients. DNA-based methods consist of the PCR amplification of DNA fragments arising from foreign organisms (Rodríguez *et al*. 2012). In this way specific DNA sequences can be identified and/or DNA fingerprints can be obtained. It is obvious that these methods are complicated when contaminants arising from several species, that often are taxonomically related, occur simultaneously. Furthermore, DNA-based analytical methods have a limited efficacy to establish the causes of contamination, for instance the use of noncompliant processed raw materials.

Although the detection of DNA markers benefits from having well-defined target analytes and the combined use of database analysis and experimental specificity minimize false positives, techniques relying on the phenotypic expression of specific protein or metabolite markers are less laborious and, in most cases, more reliable. The presence of the micro-organisms is not direct evidence that protein/peptide toxins actually have been produced. Conversely, for their intrinsic stability, toxins can remain in the food for long time after the microorganism itself is no longer detectable.

Monitoring of contaminant toxins generally relies on immunochemical assays. Commercially available tools are lateral flow devices or dipsticks, normally used for rapid screening, and ELISA, that also provide semi-quantitative determination (Singh *et al*. 2015). Typical limit of detection (LOD) of the tests based on ELISA kits is in the range of 0.1 to 5 ppm. Major concerns of the immunochemical methods consist of the fact that the targeted epitopes are usually not well characterized and that 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 antigenic sequences by altering the antibody reactivity. Many protein targets may be underestimated or even not detected by the most commonly used sandwich ELISA-based tests.

A wide array of chemical/biochemical techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), mono-dimensional (1D) or two-dimensional (2D) polyacrylamide gel electrophoresis (PAGE), and capillary electrophoresis (Figure 1.1) have also proved to be useful in component identification and adulterant detection in foods (Önal, 2007). Even though they have a relevant impact in contaminant detection and are extensively used for routine analysis, 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, appearance/disappearance/shift of electrophoretic bands or chromatographic peaks compared to a reference food cannot be considered for sure diagnostic of an instance of food contamination, as the variation of the band/peak could be due to normal food variability (false positive). On the other side, a contaminant might be masked by co-migration/co-elution

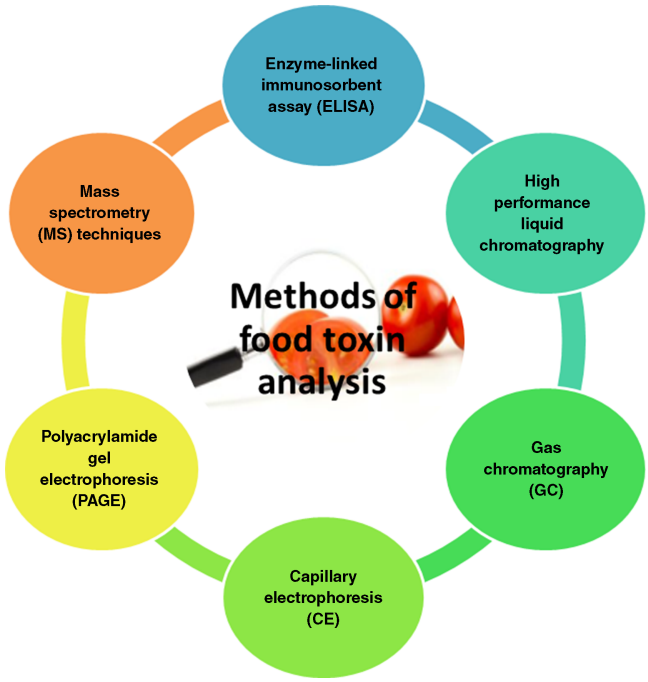

Figure 1.1 The array of chemical/biochemical analytical platforms used in food toxin analysis.

with a normal food constituent (false negative). In the light of this, 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 altered foods.

Given the limitations of the classically used methods, confirmatory strategies are also required to provide an unambiguous identification of markers of foreign food components. The proteomic approach can overcome these limitations. Proteomics is a branch of the omics technologies, a family of analytical techniques that rely on well-established analytical platforms, in particular on mass spectrometry (MS) techniques (Gallart-Ayala *et al.* 2015).

1.3 Analytical Techniques

1.3.1 MS-Based Proteomics

MS plays a fundamental role in the study of food (macro)molecules; this revolution has been triggered by the introduction in the 1990s of soft ionization techniques that allow very sensitive HMW molecules, such as electrospray (ESI) and matrix-assisted laser desorption ionization (MALDI). In protein analysis, using these techniques, it is possible to determine accurate mass of proteins and protein complexes, post-translational modifications (PTMs), correspondence of a protein sequence with that encoded in DNA, and *de novo* sequencing of peptides (Reinders *et al.* 2004). MS-based proteomics is based on two main experimental approaches: bottom-up and top-down proteomics. Both methods allow us to recognize the proteins present in a

biological sample, following two different strategies: the first approach is based on enzymatic protein digestion followed by MS identification of peptides produced by digestion (peptide mass fingerprint [PMF]), while the second approach provides for the recognition of the protein based solely on the molecular weight and by fragmentation of the undigested protein (Aebersold and Goodlett, 2001).

1.3.2 Bottom-up and Top-down Proteomics

Bottom-up proteomics allows the identification of protein based on information derived from the mass or the amino acid sequence of the peptides generated following digestion of the protein with an appropriate agent. The approach is based on the assumption that proteins which generate the same peptide map are characterized by the same primary structure and thus coincide; with this approach, it is possible to compare the sequence of a protein with that of a particular gene, to verify the sequence of synthetic proteins, and to detect PTMs. Protocols for the digestion of proteins separated using gel electrophoresis (in-gel digestion) have also been standardized (Dass, 2007). The determination of the molecular weight and amino acid sequence of the peptides produced by the digestion is carried out by MS/MS sequencing using LC-ESI-MS or MALDI-MS (Thiede *et al.* 2005). Bottom-up proteomics, therefore, allows fast and simple identification of a protein. However, a significant limitation of this approach is the quality of the results, which depends greatly on the purity of the protein treated. For this reasons, MS analysis is preceded and combined with appropriate chromatographic or electrophoretic techniques. Electrophoretic detection can be aided by use of appropriate immunochemical protocols. One example is the search for allergens which can be present only in trace amounts or be unexpected. Exemplary is the case of the discovery of a novel hazelnut allergen, which has been detected and characterized by combined immunological, electrophoretic, and MS/MS de novo sequencing (Nitride *et al.* 2013).

The top-down experimental approach for proteome analysis consists of the analysis of intact proteins (Figure 1.2). Protein identity is obtained by ESI or MALDI MW measurement and can be confirmed by MS/MS fragmentation of the intact protein. In top-down proteomics, a basic issue is instrumental resolution, and therefore the recent improvements in the technology of mass analysers have been important in its development. Fourier transform-ion cyclotron resonance (FT-ICR) and Orbitrap instruments are providing the higher resolution. Also, hybrid instruments (such as Q-TOF hybrid between quadrupole (Q) and time of flight mass analyser (TOF) that ensure a high resolving power and a fast scanning speed), which are easier to use, are able to provide adequate accuracy and resolution.

With the top-down approach, PTMs can be revealed for only relatively low molecular weight (LMW) proteins (10–20 kDa). Furthermore, when used for proteins contained in a complex sample, or in order to identify proteins present in very low concentration, a preliminary concentration or purification step is generally necessary. In fact, analysis of complex mixtures has two drawbacks: the first concerns the phenomenon of ion suppression due to the different ionization yield of proteins; the second is related to the limited dynamic range of MS, in particular MALDI, which does not allow obtaining valid signals for proteins present at low concentrations (Zhou *et al.* 2012).

In order to overcome the intrinsic limitations of both approaches, in recent years the intermediate 'middle-down proteomics' has been introduced, giving rise to the peptidomic branch of the omic family. It is based on limited peptide bond breakdown in order to obtain peptides with a greater number of amino acids (>20) compared to those produced in the bottom-up proteomics. This step is followed by determination of the amino acid sequence, which can provide information on protein isoforms and on PTMs. Moreover, in contrast to the

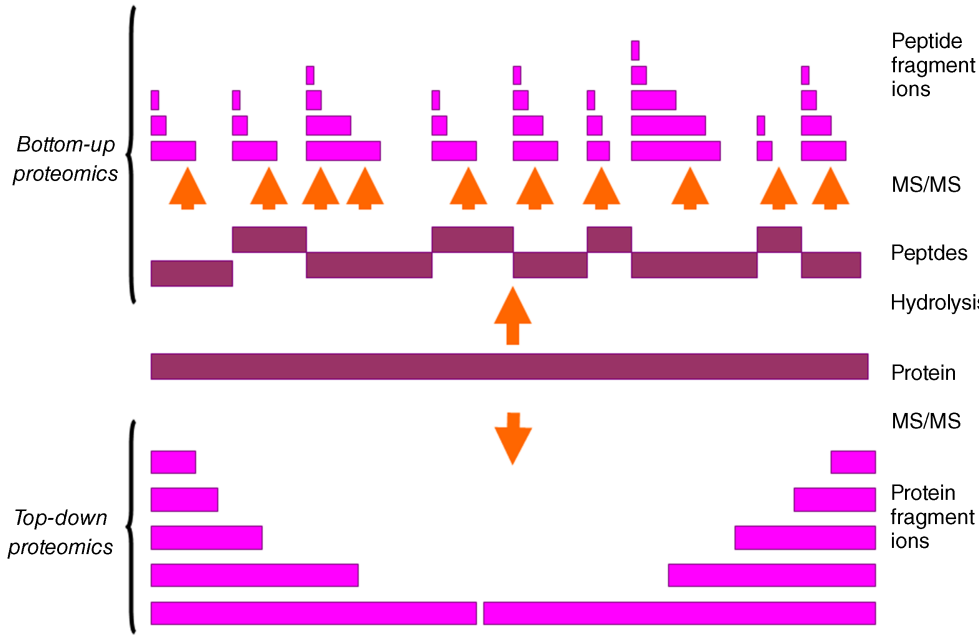

Figure 1.2 The flowchart of proteomic strategies in food toxin analysis.

top-down proteomics, which involves only the analysis of intact proteins, peptides considered in middle-down proteomics are easier to handle, ionize and fragment. These peptides, having a molecular weight characteristic of about 5–10 kDa, can be generated through an enzymatic or chemical digestion of the protein. The last step, as with all other approaches seen so far, is the comparison of experimental data with those of the literature for the identification of peptides/proteins.

Food matrices are extremely complex because they contain a large number of chemical species. This is actually the case of most food toxins, many of which are present in a concentration ranges (parts per million to parts per billion) hard to reveal with the routine analytical techniques, while others interfere with the analysis leading to unsatisfactory results. Furthermore, often food samples are also subject to rapid degradation and need to be stored under conditions of low temperature and in suitable packaging or containers that allow maintaining it unaltered. Also, a food sample can be altered in a more or less marked form by the reactants used or by the various treatments performed. This multiplies incredibly the complexity of a real food. For these reasons, the bottom-up proteomics approach is a valid and simple for single proteins, while it is quite difficult to obtain reliable results from matrices containing a greater number of species, which the usual occurrence in food analysis. This technique, therefore, requires that the food sample is effectively purified by chromatographic or electrophoretic methods; this inevitably causes an increase in the complexity and time required to perform the analysis. The top-down proteomics approach is substantially faster for the study of a complex sample, but the results are not as reliable, based solely on the molecular weight. Therefore, more data is required, such as those from MS/MS fragmentation, which, however, are difficult to obtain from an intact protein. Database screening can be helpful in the identification of the metabolite (i.e. a bacterial or a fungal peptide toxin), but only a limited number of sequences are already recorded at present. Databases are constantly being updated and enriched, and with time they will become more and more complete and reliable.