Food allergens are a series of agents, mainly proteins, which cause various unpleasant and often clinical symptoms in humans through consumption of foods. In recent years, the fields of molecular biology and immunology have offered alternative approaches to food allergen testing. Molecular and immunochemical techniques are most widely used to detect allergenic products at trace levels, for example through screening or multiple-allergen detection (the development of methods in which several allergens or allergic compounds can be detected simultaneously). Both techniques are useful in ensuring that food allergens are monitored and accounted for throughout the food production process. Recently, chromatographic techniques have also started to be used for food allergen testing, offering researchers a variety of novel ways to detect allergen contamination.

Food Allergen Testing – Molecular, Immunochemical and Chromatographic Techniques is an in-depth review of the current scientific knowledge on food allergens, covering the major methodologies and techniques used in validated analytical approaches. Bringing together a host of international experts, this book provides food scientists and technicians with a contemporary and much-needed tool to assist them in testing foods for the presence of allergens and contaminants.

This book will be required reading in academic settings where courses on food technology and related fields are offered. It will also be valued by research institutions that are engaged in providing food analysis services or are carrying out innovative research on food testing techniques.

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Food Allergen Testing
Food Allergen Testing

Molecular, Immunochemical and Chromatographic Techniques

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Introduction

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1.1 Adverse reactions to food

Allergy is a disorder of the immune system caused by a variety of substances, in the majority harmless, which are present in the environment (dust, pollen or latex), animals (venom of stinging insects), foods, or medications such as aspirin and antibiotics such as penicillin. Allergic diseases may adversely affect the quality of life of a person, influencing various aspects of his or her physical, psychological, social and economic well-being. For some individuals allergy may be fatal or life threatening, depending on the severity of the adverse reaction. Food allergy has become an important food safety issue worldwide due to the increase of allergic incidents after food consumption. The important health and economic impact of this issue has led to the development of various legislative and technical actions from corresponding official bodies in the last two decades in order to manage food allergy.

An adverse reaction to food is a general term that includes a variety of clinical manifestations induced in an individual by ingestion, inhalation or contact of a food or a food additive. A first attempt to define further the term resulted in a classification of adverse reactions into either food allergy (hypersensitivity) or food intolerance, depending on the involvement or not of an immunological mechanism [1,2]. A modified classification was proposed by the European Academy of Allergology and Clinical Immunology (EAACI) subcommittee, categorizing the adverse reactions to food as either toxic or nontoxic depending on whether the abnormal clinical response relies upon the food itself (provided that the relevant dose is high enough to produce an adverse reaction), or upon the individual’s susceptibility to a certain food, respectively [3]. Nontoxic adverse reactions are either immune mediated or non-immune-mediated. The term food allergy (food hypersensitivity) refers specifically to an immunological reaction involving the
immunoglobulin E (IgE) mechanism. However, cell-mediated responses related or not to IgE-mediated mechanisms may lead to food allergy. The term food intolerance (nonallergic food hypersensitivity) is used only in non-immune-mediated reactions, describing an abnormal physiological response, of enzymatic, pharmacological, idiosyncratic or undefined nature, of the individual [4–6].

A food allergen is an antigenic molecule, principally of protein nature, that induces an immunologic response [2]. An allergen may exist in multiple forms (isoallergens) in one species. Isoallergens share high amino acid sequence identity and immunological cross-reactivity. If the sequence identity between allergens is greater than 90%, these are referred to as isoforms or variants (polymorphic variants) of the same allergen. The allergen nomenclature has been defined by the Allergen Nomenclature Subcommittee of the World Health Organization and the International Union of Immunological Societies (WHO–IUIS) [7] and revised by the EAACI nomenclature task force [3].

1.2 Manifestation mechanisms and symptoms of food allergy

Adverse reactions induced by food ingestion, inhalation or contact affect one or more target organs such as the skin, the respiratory and gastrointestinal tracts, and the cardiovascular system [4].

Allergic (food hypersensitivity) reactions may be IgE or non-IgE-mediated, or may include both types of mechanism. The IgE-mediated food allergic reactions can be described as generalized (involving anaphylaxis and food-dependent exercise-induced anaphylaxis), cutaneous (such as urticaria and flushing), gastrointestinal (including the oral allergy syndrome (OAS), pollen food allergy syndrome and gastrointestinal anaphylaxis) and respiratory reactions (such as broncho- and laryngo-spasms, or rhinoconjunctivitis) [8–10]. Non-IgE-mediated reactions include contact dermatitis, food-protein-induced enteropathy and celiac disease, whereas examples of mixed-type reactions (IgE and non-IgE-mediated) are atopic dermatitis, gastroenteritis and asthma [10–12].

Food-intolerance adverse reactions can be described as a physiological (non-immunologic) response of the individual to the ingested food. They can be further classified as toxic or pharmacological (include poisoning–intoxication, e.g. bacterial or heavy metal, and reactions caused by specific food substances, e.g. caffeine or various amines) and nontoxic food intolerance, which includes adverse reactions such as lactase deficiency, gastroesophageal reflux and anorexia nervosa [2,6].

The above types of reaction can be clinically manifested by a plethora of symptoms ranging from moderate (pruritus, urticaria and various types of oedema) and more intense (bronchospasm, abdominal cramps, nausea, vomiting, diarrhoea), to serious and severe symptoms such as asthma, cardiac arrhythmias, hypotension, shock and coma [8–12].
The minimal allergen doses able to elicit an adverse reaction after ingestion, inhalation or contact are difficult to define. Allergen threshold doses below which individuals will not manifest an allergenic response (lowest observed adverse-effect level, LOAEL), may be very low, can show variability from a certain individual to another and could be affected by various factors such as exercise, stress and general health condition [13,14]. The establishment of threshold doses is determined by use of specific food challenges. A food challenge test is a progressive introduction of small amounts of the suspected allergen to the body, through an oral, respiratory or other route. The food challenge used for threshold dose establishment is known as the double-blind placebo-controlled food challenge (DBPCFC). The LOAEL may sometimes be difficult to determine due to the differences in the procedure of DBPCFC followed [15].

Current legislation does not define threshold doses for food allergens; however, future action on this matter is under discussion. The US Food and Drug Administration (FDA) has recently posted an announcement regarding the establishment of threshold doses, requesting information and data on whether threshold doses for major food allergens can be safely established. The relevant questionnaire included points for discussion on matters such as how clinical dose distribution data should be used, what approaches exist for using biological markers or other factors related to the severity of allergic responses, what data and information exist on dietary exposure patterns for individuals on allergen avoidance diets in a threshold risk assessment and what data or other information exists on current levels of exposure associated with the consumption of undeclared major food allergens in packaged foods.

1.3 Diagnosis and treatment of food allergy

Diagnosis of allergic reactions to certain foods beneath physical examination or medical and case history recording is performed with in vitro determination of IgEs, and in vivo specific skin prick tests (SPTs) and positive-controlled oral food challenges (with either fresh or dehydrated food) such as DBPCFC and open food challenges (OFCs) [16]. In vitro diagnostic tests together with SPTs are used to scan for specific IgEs and thus confirm sensitization to a certain food; however, they do not establish the diagnosis of the allergy. The latter is achieved with an oral challenge. OFC is normally used after a negative SPT or in order to establish the end of an elimination diet for a certain food. Oral DBPCFC is considered so far the best type of oral challenge performed, since it introduces double blinding and placebo incorporation (neither the patient nor the medic is aware of the content of the trial), eliminating in this way subjective characterization of the results and bias.

Because the nature of the allergic responses to food is quite complex (immune or cell-dependent mechanisms, immune cross-reactivity (recognition of multiple antigens by antibodies of single specificity) for different allergens, genetic
no general treatment for food-allergy cure has been established, yet. Strict exclusion of the offending foods from the individual’s diet has proved to be the only effective way to avoid food allergy, together with standard rescue medical treatment (antihistamines, glucocorticoids, epinephrine (adrenaline)) for control of allergic symptoms due to accidental exposure.

With the aim to act on the cause and not just downregulate the symptoms of allergy, allergen immunotherapy has been developed as the alternative approach to deal with the problem [17,18]. The aim is to induce immunologic tolerance to the offending allergen through repeated administration of the allergenic products or other immuno-triggering agents (e.g. monoclonal anti-IgE antibodies) via different oral and cutaneous administrations. Despite the partial efficacy of certain types of food immunotherapy [17,18], still there are various issues to be resolved, including large-scale studies on long-term efficacy, investigation and registry of side effects, as well as discussion of various ethical and regulatory issues, in order to suggest a valid immunotherapy approach for treatment of food allergy.

1.4 Food allergy prevalence

The prevalence of adverse food reactions cannot be defined clearly due to the great number of allergic events of minor intensity that happen to individuals and remain undeclared. Food allergy appears to affect nearly 2.0% of the adult population [19], though this percentage is increased in young children less than 3 years old, reaching 6–8% [20]. However, there is a change observed in both the overall and specific food allergy prevalence with respect to age, due to the development of oral tolerance to specific foods from childhood to adulthood, and appearance of specific allergies such as pollinosis, which is most frequent in adults [20]. Other factors, such as the geographical location [13,21,22], the extent of industrialization of a society, the genetic background and the cultural and dietary habits of a population [22,23], play an important role in the determination of the prevalence of specific allergies.

1.5 Allergenic foods: an increasing list

Food allergic reactions are induced by a variety of allergens present in foods of either animal or plant origin [5]. The majority of the allergic reactions caused by animal-originated allergens are due to the consumption of certain foods such as milk, eggs, fish, crustaceans (shrimp, lobster, crab and crayfish) and molluscs (clam, scallop, oyster). Main allergens of plant origin are present in certain categories of foods such as legumes including peanut, soybean and lupin, cereals containing gluten such as wheat, rye and barley, a great variety of tree nuts including almond, hazelnut, walnut and many others, various vegetables or vegetable seeds such as
celery, mustard or sesame, and fruits such as apple and peach. The phylogenetic conservation and redundancy of various proteins between species, and the stochastic (and in some cases unpredictable) nature of the individual’s immunological response to any chemical substance, are two factors that could contribute in the a priori characterization of any food as ‘potentially allergenic’ for an individual. The report of case studies on rare allergic responses to certain food may of course generate an increasing list of food allergens.

The above foods are considered (either as a category or individually) main allergenic foods according to legislations issued from continental (EU, Codex Alimentarius Commission) or country (US, Japan, UK, Australia etc.) legislation bodies, and the majority of them require labelling declaration on food products. However, country legislative adaptations may extend or narrow the list of mandatory declared allergenic foods (e.g. buckwheat in Japan, various types of nut in US and Australia), and compounds present in foods (e.g. sulfur dioxide and sulfites in EU and Canada) [24].

The allergenic proteins contained in the specific foods or food categories described above are categorized in a limited number of protein families. Plant allergens are members of the cupin, prolamin and cystein protease superfamilies as well as of various pathogen-related protein families: profilin, lectin and other protein families. The main allergenic proteins of animal origin are α-lactalbumin and β-lactoglobulin (from the whey fraction), and various caseins from milk, egg-white ovomucoid, ovalbumin and ovotransferrin, egg-yolk albumin, tropomyosins and parvalbumins from seafood [25]. As of May 2013 there have been 995 allergens reported, which belong to 186 protein families, as described in the AllFam database.

Profound information can be retrieved from a considerable number of specialized allergen databases (Table 1.1), freely available on the Internet. These databases provide molecular data and biomedical and clinical information on allergens, together with tools for sequence manipulation and allergenicity prediction via use of specific algorithms [27,37,38]. The need for proper characterization of allergen databases and analytical recourses (full description of database contents, criteria for information included and database update, description of bioinformatics tool algorithms and parameters used) has been also reported, pointing out the need for a centralized allergen reference database and the use of validated bioinformatics algorithms.

1.6 Methods for food allergen detection

There are various analytical methods currently used, either for food allergen in vitro study or food allergen detection in food matrices. These methods – with respect to their principal concept – had already been applied in clinical research for the study and monitoring of the patients’ responses to a pathological cause, or had been used in other fields of basic and applied research [39]. The need for the development of
### Table 1.1 Web available allergy databases and allergenicity prediction sites.

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<td>AAS</td>
<td>[28]</td>
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<td>AAS</td>
<td>[36]</td>
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</tbody>
</table>

MD, molecular data; BCI, biomedical and/or clinical information; AAS, allergenicity assessment site; N, nomenclature – allergen official names; GI, general information.
analytical tests in order to monitor food allergens before reaching to the allergenic individual has led to the adaptation or evolution of these methods in the last 15 years.

There are many challenges that should be faced in order to develop an analytical method for food allergen determination in food products. The necessity for high specificity, accuracy, precision and sensitivity is apparent. Furthermore, the method should perform well with a variety of matrices, food products that contain different ingredients or that have undergone various types of food processing. Detection of the allergen is not always feasible, since this may often be present in trace amounts or may be masked by the food matrix. In addition, it is of importance – for the allergen concentration determination per se in an offending food and subsequently on concluding on its allergenic potency – to know about any alterations of the allergen’s behaviour during food processing [30,40]. Moreover, factors such as ease of use and low cost should be taken in consideration when developing an analytical method.

The methods employed so far are targeting either the allergen itself (protein or glycoprotein) or a marker (specific protein or DNA fragment) that indicates the presence of the offending food [39].

The majority of the allergenic protein-targeting assays are immunoassays, though non-immuno-based assays have been developed to a great extent as well. Immunoassays are based on the use of specific antibodies for the detection of the allergenic proteins shortly after or during their separation in a suitable matrix. The antibodies used are either IgE from sera of allergic individuals, polyclonal antibodies produced after immunization of a variety of animals such as rabbit, goat, sheep or chicken, and monoclonal antibodies produced in vitro by hybridoma cells. To what extent an immunoassay will be successful enough depends mainly on the quality of the antibodies used. The optimization of the immunization schedule as well as a careful characterization, purification and selection of the best-performing antibodies will improve both the affinity and the specificity of each method.

The immunoassays that are being used for allergen detection include double immunodiffusion (Ouchterlony), the radioallergosorbent test (RAST), which is commonly used in clinical diagnosis and for qualitative allergen detection in food, immunoblotting used either after separation of the protein samples in one-dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and less frequently by two-dimensional (2D) gel electrophoresis or no separation at all (dot blot), biosensor-based assays (use of specific antibodies in combination with surface plasmon resonance), microarray immunoassays and the enzyme-linked immunosorbent assay (ELISA), which comes in various formats (sandwich, enhanced and competitive ELISA) or type variations such as dipstick and lateral-flow device (LFD) qualitative or semi quantitative assays [39].

Chromatographic analysis coupled to mass spectrometry (MS) is a protein-targeted approximation for food allergen determination, though alternative to immunoassays since no antibodies are used. Methods such as liquid chromatography (LC) coupled with electrospray ionization tandem MS (ESI-MS/MS) and
multiple-reaction monitoring (MRM) offer the means for determination of multiple allergens in a single analysis [41].

The study of food allergens at a proteome level is a combinatorial approach principally used to characterize the whole allergenic potential of an offending food product, rather than to detect and quantify a single food allergen. Proteomics as a term is used to describe the study of the protein content of a sample (whole species, organ, tissue or food product in this particular case) using a range of methods, including multidimensional separation of the proteins via electrophoresis, LC coupled to MS, arrays to study protein–protein interactions and bioinformatics.

The DNA-targeted analytical methods for food allergen determination are based on the detection of specific DNA fragments via the polymerase chain reaction (PCR), real-time PCR for both detection and quantification, and capillary electrophoresis. The generation of multiple copies of a precisely selected DNA fragment of the species of interest makes PCR a very sensitive technique. The optimization of the PCR comes through the careful in silico design of a specific set of primers and fine adjustment of the methods parameters, as well as with improvement of the DNA extraction procedures [39].

A combination of PCR and ELISA techniques (PCR-ELISA) results in an approximation using amplification of the DNA target at the first step, and a subsequent amplification of the signal via detection of the amplicon with ELISA, using antibodies that bind to molecules attached to nucleotides [42].

Both protein- and DNA-based methods have their advantages and drawbacks concerning their applicability for specific detection and quantification of food allergens. However, the nature and complexity of the food matrix or product to be tested and the extent of processing during food production are important factors to determine, as well the choice of method to be used.

The validation of methods for food allergen detection and quantification is a prerequisite for reliable and accurate results that will ensure compliance with food labelling standards and guarantee a higher level of protection for the consumer. Three ISO standards have been developed from CEN (European Committee for Standardization). For immunochemical methods CEN developed the EN 15633-1:2009 standard, Foodstuffs – Detection of Food Allergens by Immunochemical Methods – Part 1: General Considerations [43]. The standard is based on an ELISA technique, which is the most common for food allergen testing and is able to detect all 14 main food allergens except celery, as referred to in EU Regulation 1169/2011. A second CEN standard, EN 15634-1:2009, Foodstuffs – Detection of Food Allergens by Molecular Biology Methods – Part 1: General Considerations [44], has been developed for food allergen testing by PCR. A third standard, EN 15842:2010, Foodstuffs – Detection of Food Allergens – General Considerations and Validation of Methods [45], has been developed for validation of the methods. Although there is no standard published by CEN for food allergen determination by chromatographic methods, various private entities have developed LC-MS/MS methods to determine food allergens. Though the number of immunochemical and molecular assays developed for food allergen determination
is increasing day by day, there are few method comparison and validation data available.

This book provides information on the methods used currently for food allergen detection. In Chapters 2–4 an overview of the principles of immunological, molecular and chromatographic methods that have been developed is presented. Chapter 5 presents the main food allergens of animal origin (egg, milk, fish, crustacean and molluscan allergens) and reports on the specific assays that have been developed for their detection and quantification in foodstuffs. In Chapter 6 a similar presentation of plant-origin food allergens is made, and the assays for legume, cereal, vegetable, fruit and tree nut allergen determination in foods are described. Chapter 7 provides a deeper insight on cereal gluten allergens, since these are present only in monocot plants and cereals constitute the basis for human alimentation worldwide. The detection methods applied for gluten determination in foods are presented and a report on gluten determination in nonfood products such as pharmaceuticals and cosmetics is also made. Chapter 8 gives an overview on testing of allergens such as sulfites, lysozyme, casein, egg or caffeine in alcoholic and nonalcoholic beverages. In Chapter 9 a presentation of the current status on legislations for food allergen testing and labelling is made. Chapter 10 gives information on the regulatory environment for food allergens and the reference materials used for food allergen testing. Finally, in Chapter 11 a report on the proficiency schemes followed for validation of allergen-testing methods is presented.

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The importance of food-induced allergic responses has increased over the last decade. One can debate if this rise in importance is due to an increased awareness or an increase in sensitivity [1]. However, since true immediate hypersensitivity reactions to more than 160 different foods have been noted [2], sensitivity to food can be problematic for an allergic individual and hazardous and life threatening for some individuals. Some of this reactivity, such as seen in coeliac disease, is food intolerance rather than true food allergy (IgE mediated), but the outcome for the patient is similar: an unacceptable reactivity to ingested food. It is estimated that 90% of all food hypersensitivity is caused by eight major foods. These include peanuts, tree nuts, milk, eggs, wheat, fish, shellfish and soybeans [3]. The importance of these major allergens is also evidenced by the type of testing that is requested at allergen testing laboratories. Figure 2.1 illustrates the allergen testing requested and completed (as a percentage of total by allergen type) at the Food Allergy Research and Resource Program (FARRP, Lincoln, NE) during fiscal year 2012 (1 July 2011 to 30 June 2012) [4].

Although avoidance of these allergenic foods might be a method to protect against an allergenic event, this methodology poses some major hurdles. For many individuals, the presence of minute traces of the offending allergen can trigger a dramatic response, and therefore knowledge of the presence of a food allergen is critical.

Hidden allergens are a major concern for both the allergenic consumer and the food manufacturer. Ingestion of food containing a hidden allergen such as peanut has been documented to be the cause of fatality in severely allergic individuals [5]. These allergens gain access to food products through either unintentional or unknown contamination of raw materials or cross-contamination during the manufacturing process, including contamination due to processing aids, reworked product or allergen carry-over from shared equipment [6]. In order to ensure that risk of potential
allergenicity is minimized, food manufacturers have two choices: they must either
label food as having a potential risk if it was produced in a facility that may contain
known allergens, thus limiting consumer choices by default, or they must ensure that
adequate testing including raw material testing and environmental monitoring [7] is
performed to confirm that unwanted proteins are not inadvertently present in the food
and to validate the effectiveness of the manufacturing control measures. Therefore, for
any food manufacturer, it is essential that sensitive, accurate, reliable and rapid
methodologies are available to analyse raw materials, manufacturing equipment and
environments, and food matrices to ensure that allergenic components are not present.

2.1 Immunoassays for detection of food allergens

There are several different analytical methods available for the detection of food
allergens, and the choice of testing method is driven not only by the needs of the end
user but also by the characteristics of both the testing methodology and the sample
to be analysed.

- An analytical assay utilized for the detection of allergenic components must be
  sensitive enough to detect minute amounts of the allergen even in the presence of
  other components, which may inhibit reactivity. In order to safeguard the
  consumer, an ideal assay would never generate a false negative result at the
desired limit of detection (LOD). Although a threshold for each allergen has not been established, it is accepted that assays should detect the presence of the allergen at concentrations as low as 5 ppm for peanut, since this has been found to be the lowest dose that produces an adverse effect (LOAEL) in clinical testing [8].

- An analytical assay utilized for the detection of allergenic components must be specific and have limited cross-reactivity to ensure that false positive results are not obtained. High rates of false positives are costly for the food manufacturer and cause waste of otherwise acceptable food ingredients.

- An analytical assay utilized for the detection of allergenic components must be reliable and robust for use in the manufacturing environment. The assay must deliver repeatable results and must be able to withstand differences in the testing environment, including but not limited to temperature, humidity and light.

- An analytical assay utilized for the detection of allergenic components must be cost effective for the end user. This means that the assay utilized must not require highly trained personnel to perform the assay, nor must the method require the use of high-cost equipment.

Based on these criteria, the most commonly utilized analytical methods for detection of food allergens are immunoassays. Immunoassays are chosen because of their ease of use and cost effectiveness. Additionally, many of these assays are highly sensitive and specific, providing an accurate analysis of the test sample [9]. There are several different assay forms that utilize antibody for detection of allergenic components, including western immunoblot, radioallergosorbent test (RAST), enzyme-linked immunosorbent assay (ELISA) and lateral-flow devices (LFDs). ELISA and LFDs are the most commonly utilized immunoassays, since they are readily formatted as ready-to-use kits and therefore allow for rapid and robust testing. Western immunoblot and RAST methods are effective; however, they are technically challenging, have multiple steps and for western immunoblot assays require specialized equipment. Of the two most commonly utilized immunoassays, ELISA and LFDs, ELISAs are usually more sensitive than LFDs and allow for quantification at very low LODs. The ELISA format requires higher level of technical expertise, equipment and a longer time to result. LFDs are rapid and easy to perform and require no equipment for a semiquantitative or qualitative result.

2.2 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a very useful tool for the detection of allergenic substances in food, and because of its precision and quantification capabilities it is the most commonly utilized method for analysis of food allergens [10]. In an ELISA, the targeted protein binds to a specific antibody, which is enzyme labelled [11]. A substrate specific to the enzyme produces a colorimetric reaction that can be detected. The ELISA can be either a direct
sandwich format or competitive format. Both formats allow for a quantitative value based on comparison with a standard curve. In a direct sandwich format the allergen of interest is ‘sandwiched’ between two antibodies, a capture antibody and a detector antibody, which is tagged with an enzyme. In the presence of the allergen, colour is generated from the antibody:allergen:antibody complex and the amount of colour generated is directly proportional to the amount of allergen present (Figure 2.2).

In a competitive ELISA, the antibody is immobilized to a solid surface. The enzyme-labelled antigen is mixed with the sample and then exposed to the surface containing the immobilized antibody. If the allergen of interest is present in the sample, it will bind to the antibody and block the binding of the labelled antigen to the antibody immobilized on the plate. In a competitive format the amount of colour generated is inversely proportional to the amount of allergen in the sample, as the allergen in the sample and the labelled antigen compete for binding to the antibody immobilized on the plate (Figure 2.3). Competitive ELISAs are most commonly used to detect small molecules and are less commonly used for detection of larger protein molecules, which are the primary target for food allergen analysis. However, for some smaller allergens or hydrolysed peptide fragments it is not possible to bind two immunoglobulin
molecules to produce an antibody:allergen:antibody sandwich [12]. In these situations, the competitive format allows for the use of one specific antibody for analysis.

2.3 Lateral-flow devices (LFDs)

LFDs exist in both sandwich and competitive formats, although sandwich-format LFDs are most common in food allergen testing. These assays are qualitative or semi-quantitative, and in most instances provide a presence or absence result and not a specific concentration. Commercially, there are two different types of sandwich LFD utilized: those with an overload line and those without an overload line. The sandwich-format LFD functions as follows (Figure 2.4): An extracted sample is