DISSERTATION

Development of immunochemical tests for the detection of peanut and hazelnut proteins in food

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Kurzfassung

Nahrungsmittelallergien stellen vor allem in Industrieländern ein bedeutendes Gesundheitsproblem dar. Erdnüsse und Nüsse wie z.B. Haselnüsse sind verantwortlich für einen Großteil der durch Lebensmittel hervorgerufenen anaphylaktischen Schocks. Für sensibilisierte Personen stellen deshalb nicht deklarierte Allergene in Lebensmitteln ein ernsthaftes Risiko dar. Erdnüsse und Haselnüsse finden breite Anwendung in der Lebensmittelindustrie, vor allem bei der Herstellung von Fertigprodukten und Süßwaren. Aufgrund von „Kontaminationen“ potentiell allergen-freier Lebensmittel kommt es relativ häufig zu unbeabsichtigter Konsumation geringer Mengen von allergenen Proteinen, den sogenannten „versteckten Allergenen“. Sensitive, sowie schnell und einfach durchzuführende Analysemethoden für den Nachweis von kleinsten Mengen dieser allergenen Proteine sind notwendig um den Konsumenten Sicherheit und umfassende Produktinformation bieten zu können, und um die von der EU geforderte Kennzeichnungspflicht zu erfüllen. Zu dieser Thematik hat die Europäische Kommission in den Jahren 2000-2005 das Projekt „AllergenTest“ (QLRT-2000-01151) zur Entwicklung von schnellen und benutzerfreundlichen immunologischen Testmethoden zum Nachweis von Erdnuss- und Haselnussproteinen in Nahrungsmitteln finanziert, das Projekt wurde vom Analytikzentrum des IFA-Tulln koordiniert. Zwei „enzyme linked immunosorbent assays“ (ELISAs) und zwei „lateral flow devices“ (LFDs) wurden im Rahmen des Projekts entwickelt.

Der erste Teil dieser Arbeit berichtet über die Entwicklung weiterer Testmethoden, Dipsticks (Streifentests) und Microarrays zum Nachweis von Erdnuss und Haselnuss in Lebensmitteln mithilfe von monoklonalen Antikörpern und Eidotterantikörpern. Ziel dieser Arbeit war es die Eigenschaften der Testformate und der verschiedenen Antikörper zu vergleichen.
Die Microarray Technologie ist an sich ein relativ neues Forschungsgebiet, vor allem die Anwendung von Microarrays für die quantitative Analyse komplexer Proteinlösungen stellt bis dato Neuland dar. In dieser Arbeit wurde erstmals versucht, Microarrays zum Nachweis von Erdnuss und Haselnuss in Lebensmitteln einzusetzen. Obwohl Eidotterantikörper eine kostengünstige, tierfreundliche Alternative zu Gewinnung von Antikörpern aus Säugetieren darstellen, haben sie bei der Entwicklung von Immuntests bisher relativ selten Anwendung gefunden. Diese Arbeit ist eine der ersten bei der Eidotterantikörper in einem Schnelltest zum Nachweis von versteckten Allergenen in Lebensmitteln getestet wurden. Allerdings führte die Verwendung von Eidotterantikörper zu einer vergleichsweise geringen Sensitivität bei beiden Testsystemen. Deshalb wurden die Tests mit monoklonalen Antikörpern weiter entwickelt. Das Microarray Format war das sensitivere System mit einem Detektionslimit von 1-2.5 ppm für Erdnuss in verschiedenen Lebensmittelmatrices, verglichen mit einem Detektionslimit von 30-100 ppm für die entwickelten Erdnuss und Haselnuss Dipsticks. Allerdings erfüllte das Dipstick Format aufgrund der kürzeren Inkubationszeiten und der einfacheren Handhabung eher die Anforderungen für einen schnellen, benutzerfreundlichen Test.

Der zweite Teil dieser Arbeit berichtet über die Organisation und Durchführung eines Ringversuches mit 8 europäischen Teilnehmern zur externen Validierung der, im Rahmen des EU-Projekts entwickelten ELISA und LFD Testsysteme. Neun verschiedene, zum Teil komplexe Lebensmittelmatrices wurden mit unterschiedlichen Konzentrationen an Erdnuss und Haselnuss versetzt. Insgesamt wurden 25 verschiedene Blindproben mit jedem der vier Tests vermessen, und die Ergebnisse statistisch ausgewertet.

Der Haselnuss ELISA konnte für die folgenden 5 Lebensmittelmatrices erfolgreich validiert werden: Dunkle Schokolade, Speiseeis, Salami, Suppenpulver, Cornflakes. Die ermittelten relativen Standardabweichungen bewegten sich zwischen 2%-10% (RSDr) und 12%-50% (RSDR), die Wiederfindungen lagen zwischen 108%-215%.

Aufgrund massiver Probleme mit der Stabilität des Erdnussgehalts in den Proben, war eine externe Validierung des Erdnuss ELISA im Rahmen des Ringversuches nicht möglich.

Mit dem Erdnuss LFD konnten die Erdnusskonzentrationen in folgenden Lebensmittelmatrices erfolgreich nachgewiesen werden: Dunkle Schokolade, Milchschokolade, Kekse, Speiseeis, Cornflakes. Die Sensitivität dieser Messungen betrug 82.7%, die Spezifität betrug 100%.

Mit dem Haselnuss LFD konnten die Haselnusskonzentrationen folgender Lebensmittelmatrices erfolgreich nachgewiesen werden: Dunkle Schokolade, Kekse, Speiseeis, Salami, Suppenpulver, Cornflakes. Die Sensitivität der Messungen betrug 69.4%, die Spezifität betrug 100%.

Für die tatsächlich positiven Proben betrug die Übereinstimmung der Messungen innerhalb der Labors 75.95% mit dem Erdnuss LFD und 64.79% mit dem Haselnuss LFD, die Übereinstimmung der Messungen zwischen den Labors 70.28% mit dem Erdnuss LFD und 55.88% mit dem Haselnuss LFD. Für die tatsächlich negativen Proben betrug die Übereinstimmung beider Werte mit beiden Tests je 100%.

Die 4 neu entwickelten Testsysteme wurden zusätzlich in einer internationalen Lebensmittelstudie eingesetzt. 40 verpackte Lebensmittel aus 11 verschiedenen Ländern wurden auf ihren Gehalt an Erdnuss und Haselnuss untersucht. Die Messergebnisse wurden mit der Kennzeichnung auf den Verpackungen verglichen. 25.3% aller Produkte enthielten Erdnuss und / oder Haselnuss ohne entsprechende Kennzeichnung. 12% aller Produkte sollten laut Kennzeichnung Erdnuss / Haselnuss enthalten, dies konnte aber durch die Messungen nicht bestätigt werden.

Aufgrund der Ergebnisse die mit den 4 neuen Testkits während der externen Validierung, im Ringversuch und in der Lebensmittelstudie erzielt wurden, kann man darauf schließen, dass die Testkits für die Detektion von Spuren von Erdnuss und Haselnuss in Lebensmitteln geeignet sind. Obwohl bereits zahlreiche ähnliche Testsysteme entwickelt wurden, sind bis jetzt nur wenige davon vollständig validiert. Der beschriebene Ringversuch stellt den ersten erfolgreichen Versuch dar, Schnelltests zur Detektion von Allergenen für eine größere Auswahl an Lebensmittelmatrices im Rahmen einer internationalen Vergleichsstudie zu validieren.

Abstract

Food allergy is an important public health problem especially in industrial countries.
Peanuts and tree nuts, such as hazelnuts are responsible for the most food related anaphylactic reactions. Hence undeclared allergenic components in food products pose a major risk for sensitized persons. Peanuts and hazelnuts are widely distributed in food industry, especially in processed food and confectionary products and accidental ingestion of trace amounts of allergenic protein is quite common due to contamination of putative safe food, so called “hidden allergens”. Accurate, rapid and sensitive methods for the determination of traces of allergenic proteins are an essential requirement to allow for consumer information and protection and to meet EU food labelling directives. From 2000-2005 the European Commission supported the project “AllergenTest” (QLRT-2000-01151), which dealt with the development of rapid easy to use immunochemical tests for the detection of peanut and hazelnut proteins in food, the project was coordinated by the Center for Analytical Chemistry, IFA-Tulln.
Two ELISA (enzyme linked immunosorbent assay) and two LFD (lateral flow device) test kits have been developed in the course of the project.

The first part of this study reports on the development of additional immunochemical tests, dipstick assays and microarrays for the detection of peanut and hazelnut in food employing monoclonal and egg yolk antibodies.
The aim of this work was to compare the performance of the assay formats and the different types of antibodies. The microarray technology is a rather new field of research in general, application of antibody microarrays for the quantitative analysis of complex protein solutions is still quite uncommon. This work was the first attempt to use antibody microarrays for the detection of peanut and hazelnut proteins in food. Although egg yolk antibodies are an inexpensive, convenient and animal friendly alternative to antibodies derived from mammals, they are rarely employed in the development of fast immunoassays and immunoassays in general. This study was one of the first efforts to apply egg yolk antibodies for the detection of hidden allergens in food. Hence the application of egg yolk antibodies lead to a reduced sensitivity in the performance of both assay types compared with the monoclonal antibodies. Therefore assay formats employing monoclonal antibodies exclusively were developed. The microarray format proofed to be more sensitive with a limit of detection between 1-2.5 ppm, when detecting peanut in various food matrices compared to detection limits ranging from 30 – 100 ppm for the peanut and hazelnut dipsticks. However the dipstick format required distinctly shorter incubation times, with a user-friendly performance independent of laboratory equipment and therefore rather meets the requirements of a rapid and easy-to-use immunoassay.

The second part of this thesis reports on the accomplishment and results of a collaborative trial, involving 8 European laboratories, that was organised for the external validation of the new ELISA and LFD test kits developed in the EU-project. Nine, in part complex food matrices, were spiked with various amounts of milled peanut or hazelnut powder. In total 25 blind samples where analysed with each test kit and results evaluated statistically.

The hazelnut ELISA was successfully validated for the 5 food matrices dark chocolate, ice cream, salami, instant soup and cornflakes. Relative standard deviations for the validated matrices varied from 2%-10% (RSDr) and 12%-50% (RSDR) respectively, the recoveries ranged from 108%-215%.

External validation of the peanut ELISA in the collaborative trial was not possible due to instability of the spiking value in the sample material.

With the peanut LFD peanut content in the food matrices dark chocolate, milk chocolate, cookies, ice cream, and cornflakes, was successfully detected, the sensitivity of measurement was 82.7%, the specificity of measurement was 100%.

The hazelnut LFD proofed capable to detect hazelnut in the food matrices dark chocolate, cookies, ice cream, salami, instant soup, and cornflakes, the sensitivity of measurement was 69.4% and the specificity was 100%. For the true positive samples the accordance of measurements (agreement within laboratories) was 75.95% for the peanut LFD and 64.79% for the hazelnut LFD, the concordance of measurements (agreement between laboratories) was 70.28% for the peanut LFD and 55.88% for the hazelnut LFD. For the true negative samples the accordance and concordance of measurements was 100% for both LFDs.

Additionally the developed ELISA and LFD kits were employed in a food survey. 40 pre-packaged food samples from 11 different countries were analysed regarding their content of peanut and hazelnut respectively. Results were compared with the declarations on the packages. 25.3% of total products contained peanut and / or hazelnut without declaration. 12% of products in total were declared to contain peanut / hazelnut but did not.

Concluding, the results of the internal validation of test kits, the collaborative trial and the food survey indicated that the 4 new developed test kits represent valuable tools for the detection of traces of peanut and hazelnut protein in various complex food matrices. Although similar test systems already exist, few of them are fully validated, further the collaborative trial performed in this work represents one of the first attempts where rapid assays for the detection of allergens were validated for such a wide range of different food matrices in an international study.

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I THEORETICAL PART

1 INTRODUCTION AND OBJECTIVES

Food allergy is an important public health problem especially in industrial countries [Besler, 2001]. Peanuts and tree nuts, such as hazelnuts are responsible for the most food related anaphylactic reactions [Sampson 1998 and 2003]. At the moment the only treatment for food allergy is avoidance of the causal food protein. Indeed accidental ingestion of trace amounts of allergenic protein is quite common due to contamination of putative safe food, so called “hidden allergens” [Brett, 1998]. In order to improve consumer information and protection, the EU legislation has recently been modified regarding the declaration of ingredients in food labelling for pre-packaged foods. A set of allergenic ingredients and products thereof have been mandatory included in the list of ingredients on food labelling, among them peanuts and tree nuts [European Commission, 2003].
To meet the mentioned demands, powerful analytical tools are required to enable potentially contaminated food products to be surveyed on the market. Immunoassays are sensitive, specific and inexpensive methods for the detection and quantification of complex biological molecules like proteins.

From 2000-2005 the European Comission founded the project “AllergenTest” (QLRT-2000-01151) for the development of rapid, easy-to-use immunochemical tests for the detection of proteins with allergenic potential in food. The project should focus on the detection of the highly relevant peanut and hazelnut proteins. Novel assay designs should be developed including dip-sticks and lateral flow devices, an important step towards the development of these easy to use systems are laboratory based ELISAs.
Eight institutions from 6 different EU-member states participated in the project:
IFA-Tulln, Center for analytical chemistry (A)
RIKILT (NL)
CSL (UK)
TU München (D)
r-Biopharm (D)
Uni Milan (I)
Deco / Proteste (PT)
Central Manchester University Hospitals (UK)
Monoclonal and polyclonal antibodies for the test development were provided by RIKILT and CSL, egg yolk antibodies were prepared at IFA-Tulln.
The German partner r-Biopharm developed laboratory based ELISAs for the detection of peanut and hazelnut. Lateral flow devices for rapid, quantitative assessment of peanut and hazelnut content were produced at CSL.

This thesis reports on the accomplishment and results of a collaborative trial that was coordinate by the project partner IFA-Tulln for the external validation of the 4 newly developed test kits. Eight laboratories from five different states within the European Union (Austria, Germany, Italy, Netherlands, U.K.) participated in the trial. Nine, in part complex food matrices, were spiked with various amounts of milled peanut or hazelnut powder. Samples were extracted, measured in duplicate and results evaluated statistically at IFA-Tulln. The aim of this study was to estimate the performance characteristics of the new test systems such as applicability to complex food matrices, within-laboratory and among-laboratories precision, recovery, sensitivity, limit of determination.

Additionally a food survey was performed with the 4 different test kits. Approximately 40 pre-packaged food samples from 11 different countries were analysed regarding their content of peanut and hazelnut respectively. The objectives to be investigated were on the one hand the performance of the newly developed test kits on commercial food products and on the other hand the real content of peanut and hazelnut in the various products compared to the labelling on the package.

Another topic of this thesis was the development of dipstick assays and microarrays for the detection of peanut and hazelnut in food. Monoclonal and egg yolk antibodies were used for the construction of the assays in a sandwich format.
On the one hand the two different assay formats were compared regarding the performance characteristics like sensitivity and recovery when applied on peanut / hazelnut extracts and spiked food matrices. Dipstick assays are already applied very commonly in food analysis due to their rapid and user friendly implementation. Contrary microarrays are a relatively new field of research and they are not established for the quantitative analysis of complex protein solutions by now.
A second aspect of this work was the investigation of the different features of monoclonal and egg yolk antibodies when applied in the two immunoassay formats.
Although egg yolk antibodies are an inexpensive, convenient and animal friendly alternative to antibodies derived from mammals, monoclonal antibodies are still employed in the majority of immunoassays due to their high specificity and reproducibility of homogeneous material.

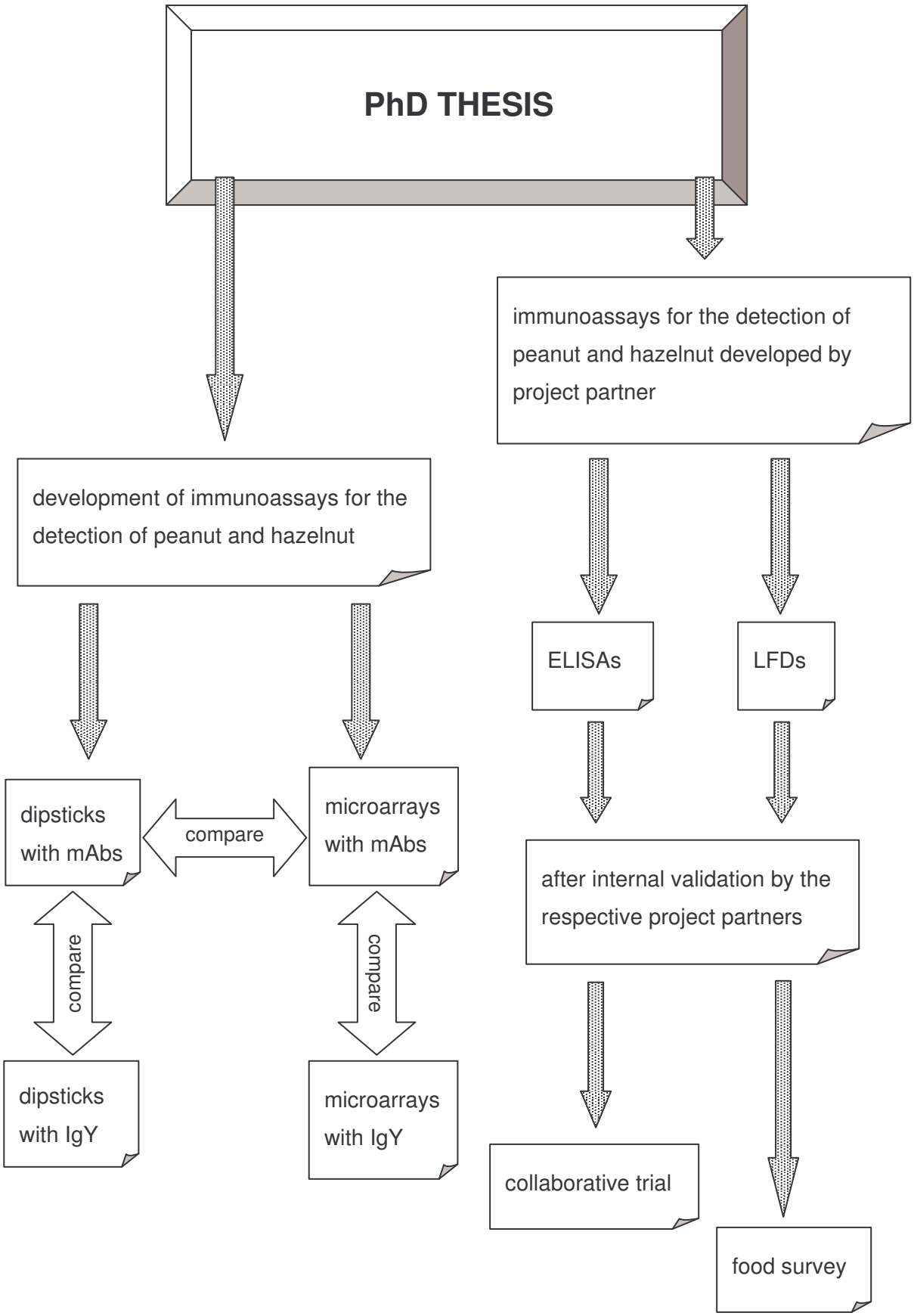


Figure I.1: Overview of the objectives of the PhD thesis

2 ALLERGY

The term "allergy" was originally introduced by the Austrian pediatrician Clemens von Pirquet in 1906, meaning "changed reactivity". Allergy is an immune malfunction whereby a person's body is hypersensitised to react immunologically to typically nonimmunogenic substances such as pollen, dust, mould, dander or certain foods [Wikipedia encyclopedia, 2005]. According to Gell and Coombs there are four classes of hypersensitivities [Elgert, 1996]:

Type I hypersensitivity:
Immediate (anaphylactic) hypersensitivity involves cell-bound IgE antibodies
Is exemplified by allergic asthma, allergic rhinitis (hay fever), atopic dermatitis (eczema), and acute urticaria (hives), it is manifested within minutes after a second exposure to the offending allergen (antigen).

Type II hypersensitivity:
Antibody–dependent cytotoxic hypersensitivity involves antibody responses against antigens on cells
Is also an immediate reaction that generally involves harmful immune responses to surface antigens of red blood cells, platelets, or granulocytes.

Type III hypersensitivity:
Immune complex–mediated hypersensitivity involves formation of IgG antibody-antigen aggregates
Is typified by the hives of serum sickness and other manifestations due to soluble immune complexes that may deposit themselves anywhere in the body.

Type IV hypersensitivity:
Cell-mediated (delayed type) hypersensitivity involves T cells and activated macrophages, not antibodies
Differs from the preceding three types in two important ways: (1) the hypersensitivity is mediated by T cell activated macrophages rather than by antibody, and (2) the reaction starts after a latent period of several hours and peaks at 48 to 72 h.

Allergy is the term commonly used for reactions caused by Type I Hypersensitivity, characterised by classical IgE mediation of effects [Roitt, 1991].

Mechanism of an allergic reaction [Elgert, 1996]
An allergen is a substance (an antigen) that elicits an allergic response rather than an immune response. Allergens are always proteins or are bound to proteins as haptens, allergenic molecules have a molecular weight between 15 and 40 kD.

Another characteristic that predisposes a molecule to be an allergen is the presence and spacing of two or more antigenic determinants (epitopes).

This antigenic determinant arrangement allows the cross-linking of adjacent mast cell- or basophil-bound IgE molecules.

When initially exposed to an allergen (sensitization) the individual does not exhibit any symptoms, but large amounts of IgE antibodies are produced that bind to the surface of mast cells or basophils. Clinical manifestations of immediate hypersensitivity appear after re-exposure to the same allergen. The allergen binds and cross-links two adjacent mast cell-bound IgE molecules, leading to mast cell degranulation and the release of mediator molecules (e.g. histamine, serotonin, heparin) that cause the symptoms of immediate hypersensitivity.

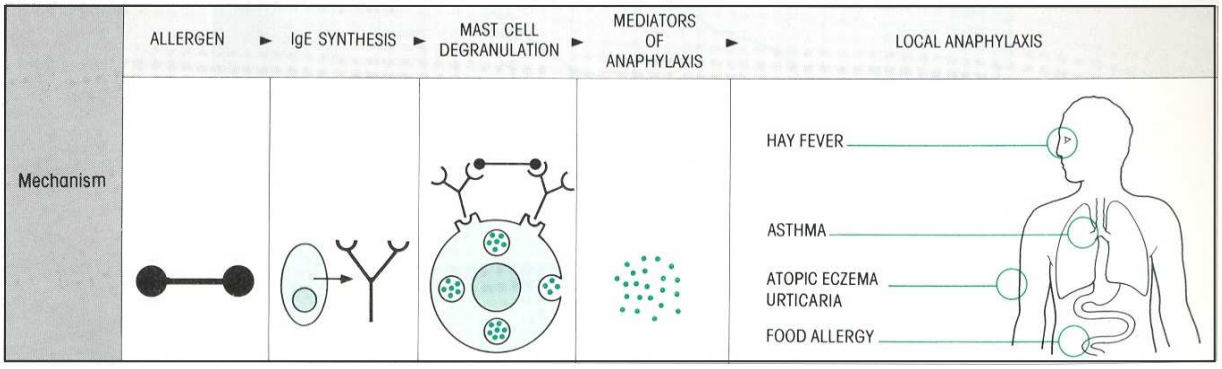


Figure I.2: Development of an allergic reaction

Diagnosis of allergy

In vivo tests for the diagnosis of allergy are skin prick test and patch test, where allergens are applied to the skin, bronchial, nasal or conjunctival provocation tests and food challenge [Durham, 1998].

The majority of commonly used in vitro tests detect allergen specific IgE in the patients serum like radio-immunoassay (RIA), radio-allergosorbant test (RAST) and enzyme linked immunosorbent assay (ELISA).

Treatment of allergy

There are limited mainstream medical treatments for allergies, probably the most important factor in rehabilitation is the removal of sources of allergens from the home environment, and avoiding situations in which contact with allergens is likely [Durham, 1998].

First line treatment for anaphylaxis is medication of adrenaline [Durham, 1998].

One approach of immunotheraphy is the hyposensitization, where the patient is gradually vaccinated against progressively larger doses of the allergen in question which can either reduce the severity or eliminate hypersensitivity altogether [Herxheimer, 1950].

2.1 Food allergy

Hippocrates first described adverse reactions to food over 2000 years ago. Other Greek scholars recorded adverse reactions to cow´s milk in the first and second centuries [Cohen and Saavedra-Delgado, 1989]. The first anaphylactic reaction to egg was recorded by Marcello Donati in the sixteenth century and to fish by Philipp Sachs in the seventh century [Harper, 1980]. In the early part of the twentieth century, physicians began reporting series of children with eczematous rashes exacerbated by food allergies [Blackfan, 1920]. However, it was not until 1950 that Loveless first used blinded, placebo-controlled food challenges to establish the diagnosis of food allergy and demonstrate the unreliability of patient history [Loveless, 1950].

Today food allergy is an important public health problem especially in industrial countries [Besler, 2001]. The prevalence of food allergy can be estimated up to 8% in children younger than 3 years of age and approximately 2% in adults [Sampson, 1999]. A limited number of foods are responsible for the vast majority of food-induced allergic reactions: milk, egg, peanut, fish, and tree nuts in children and peanuts, tree nuts, fish, and shellfish in adults [Sampson, 1999].

There is a great public interest in the topic of food allergies. However the public perceives food allergy differently from doctors – especially in relation to its symptoms and prevalence, the percentage of people perceiving their illness as being food dependent is much higher than the actual prevalence of food allergy [Durham, 1998]. Up to 30% of the general population believe they have a food allergy [Sloan et al, 1986; Woods et al, 2002] and up to 30% of parents believe that their children have a food allergy. It is important to distinguish between true food allergy and other adverse reactions to food, because the basic mechanisms differ strictly.

Adverse reactions to foods may be classified as due to either true food allergy or non-allergic food intolerance [Durham, 1998]:

- Food allergy due to IgE mediated mechanism (hypersensitivity type I)
  Either one or a limited number of specific foods provoke symptoms, usually within minutes:
  oral allergy syndrome - itching and swelling in the mouth and oropharynx
  gastrointestinal hypersensitivity - nausea, abdominal pain, colic, vomiting, diarrhea
  life threatening - exacerbation of asthma, laryngeal oedema, anaphylaxis
- Food allergy not involving IgE, in which other immunological mechanisms are implicated (for example, hypersensitivity type IV)
  Delayed reactions for example, eczema in children may be exacerbated by milk ingestion, and a small proportion of adults with severe contact dermatitis due to nickel may react to nickel in their diet.

- Non-allergic food intolerance
  Reactions to food can be pharmacological (anaphylactoid reactions, flushing, hypotension and urticaria upon ingestion of food with high histamine content), metabolic (abdominal symptoms and chronic diarrhoea after ingestion of milk by children with lactase deficiency), or toxic (contamination of food by chemicals or bacterial toxins)
- Food aversion (symptoms are often non-specific and unconfirmed by blinded food challenge)

2.1.1 "Hidden" allergens and food labelling

As mentioned before one of the main aspects in treatment of food allergy is the avoidance of the causal allergen. Indeed prevention of accidental ingestion of trace amounts of allergenic protein can be rather difficult due to the occurrence of so-called "hidden" allergens.
Hidden allergens are defined as potential allergenic material that should normally not be present in that food [Brett, 1998].
Contamination of putative safe food occurs for several reasons [Steinman, 1996]:

- manufacturers use previously manufactured products for manufacture of a secondary product and do not declare the ingredient on the new product
- in manufacturing plants the same equipment is used to make different products without adequate cleaning of the equipment
- the same serving utensils are used for different foods (e.g. in salad bars, ice cream parlors)
- misleading labelling

To improve consumer information and protection a new EU directive on food labelling has been enacted. Directive 2003/89/EC [European Commission, 2003] abolishes the 25% rule for compound ingredients, which stated that for some products it is not obligatory to label the components of compound ingredients that make up less than 25% of the final food product, thereby underlining the principle that all ingredients should be labelled, regardless of the quantity contained in the finished food. More specifically, Annex IIIa of Directive 2003/89/EC lists 12 ingredients which may induce food allergies or intolerances and for which any derogations to the obligatory declaration of food ingredients are not applicable. This requirement pertains to: cereals containing gluten, crustaceans, eggs, fish, peanuts, soybeans, milk and dairy products including lactose, nuts, sesame seeds, celery, mustard, and products thereof and sulphites. To meet the mentioned demands, powerful analytical methods are required to survey potentially contaminated food products on the market.

2.1.2 Peanut allergy

Peanut

Peanut (Arachis hypogea) is a species that belongs to the family Fabaceae (or Leguminosae) closely related to peas and beans [Loza and Brostoff, 1995]. The bushy annual plant is native to South America. After pollination of the colourful yellow flowers, the fruit develops into a legume containing 2 to 3 (rarely 1 or 4) seeds, which forces its way underground to mature.

Peanuts are grown throughout the tropical and warm temperate regions of the world, where there are no freezing temperatures, they do not tolerate frost [Putnam et al,2005], the pods ripen 120 to 150 days after the seeds are planted. Thousands of peanut cultivars are grown, with four major Cultivar Groups being the most popular: Spanish, Runner, Virginia, and Valencia. The major producers/exporters of peanuts are the United States, Argentina, Sudan, Senegal, and Brazil, these five countries account for 71 % of total world exports [Wikipedia, 2005]. Although India and China are the world's largest producers of peanuts, they account for a small part of international trade because most of their production is consumed domestically as peanut oil. Exports of peanuts from India and China are equivalent to less than 4 % of world trade [Wikipedia, 2005].

Peanuts are consumed chiefly as roasted seeds or peanut butter in the United States compared to use as oil elsewhere in the world. Approximately two-thirds of all U.S. peanuts are used for food products of which most are made into peanut butter, salted and shelled peanuts, confectionary products, and roasted-in-shell peanuts are the next most common uses for peanuts produced in this country [Putnam et al, 2005]. The remaining one-third of annual production is used for seed, feed, production of oil, or exported as food or oil. Also nonfood products such as soaps, medicines, cosmetics, and lubricants can be made from peanuts.

The peanut seed contains approximately 45-50% oil, 25-32% protein (average of 25% digestible protein), 8-12% carbohydrate, 5% water, 3% fibre and 2.5% ash [Arthur JC, 1953].

Peanut proteins were originally classified as albumins (water soluble) or globulins (saline soluble); the globulins were in turn subdivided into arachin and conarachin fractions (the major storage proteins) [Johns and Jones, 1916].

Peanut allergy

Seven different proteins were identified as peanut allergens, see Table I.1. The three major peanut allergens Ara h 1, Ara h 2, and Ara h 3 are recognised by more than 50% of peanut allergic individuals [Bannon et al, 2000]. Ara h 2 is the major allergen in Europe [Warner, 1999] while Ara h 1 is the major allergen in studies in the US [Stanley and Bannon, 1999].

Table I.1: List of peanut allergens

peanut proteins / glycoproteins	references
63 kDa (Ara h 1)	[Burks et al, 1991]
17 kDa (Ara h 2)	[Burks et al, 1992]
60 kDa (Ara h 3)	[Eigenmann et al, 1996]
37 kDa (Ara h 4)	[Kleber-Janke et al, 1999]
15 kDa (Ara h 5)	[Kleber-Janke et al, 1999]
15 kDa (Ara h 6)	[Kleber-Janke et al, 1999]
15 kDa (Ara h 7)	[Kleber-Janke et al, 1999]

Among food allergy, peanut allergy is common and severe, it is characterized by a high frequency of life-threatening anaphylactic reactions and typically lifelong persistence [Sampson et al, 1992]. Epidemiologic studies of the general population estimate a prevalence rate of 0.5% and peanut allergy accounts for 10-47% of food-induced anaphylactic reactions [Bannon et al 2000]. In a double-blind placebo-controlled food challenge with peanut, mild allergic symptoms were observed after ingestion of doses as low as 100 µg [Hourihane et al, 1997]. Since such small amounts of peanut protein can elicit an allergic response, and the food is ubiquitous in most food supplies, accidental ingestion with reaction is common, hidden peanut proteins have been reported as causes of adverse reactions to confectionary products, pastry and Asian food [Bannon et al, 2000].

2.1.3 Hazelnut allergy

Hazelnut [Wikipedia, 2005]

The Common Hazel (Corylus avellana) is a shrub that belongs to the Betulaceae or birch familiy and is native to Europe and Asia. The flowers are produced very early in spring before the leaves, and are monoecious, with single-sex catkins, the male pale yellow and 5-12 cm long, the female very small and largely concealed in the buds, with only the bright red 1-3 mm long styles visible. The fruit is a nut, produced in clusters of one to five together, each nut held in a short leafy involucre ('husk') which encloses about three quarters of the nut. The nuts fall out of the involucre when ripe, about 7-8 months after pollination. The preferred climate is characterised by a mild summer and cool winter, long periods of chilling are required to ensure fruitfulness. Common Hazel is cultivated for its nuts in commercial orchards in Europe, China, Australia and Turkey.

Hazelnuts are widely used in the food industry mainly for confectionary products owing to their nutritive value and taste.

The amount of hazelnut present in a recipe is usually considered as a mark of quality. Hazelnut oil, pressed from hazelnuts, is strongly flavoured and used as a cooking oil. The flesh of a typical nut includes 16% protein and 62% unsaturated oil. In addition it contains significant levels of Thiamine and Niacin and high levels of Calcium, Phosphorus and Potassium.

Hazelnut allergy

A number of hazelnut proteins have been classified as hazelnut allergens, Table I.2 shows a list of published data on the hazelnut allergens.

Table I.2: List of hazelnut allergens [Besler et al, 2001]

hazelnut proteins / glycoproteins	**references**
18 kDa	[Hirschwehr et al, 1992]
14, 18, 37, 40, 46, and 69 kDa	[Hirschwehr et al, 1992]
2 allergens <16 kDa, 17 kDa, and 42 kDa	[Caballero et al, 1997]
7, 9, 38, 42, and 50 kDa	[Schocker et al, 1999]

Due to consumption habits hazelnut allergy is of high relevance in Europe, with an estimated prevalence rate of 0.1-0.5% [Koppelman et al, 1999; de Groot et al, 1996]. The lowest dose observed to induce objective symptoms in an individual determined by double-blind, placebo-controlled food challenge was 1 mg hazelnut protein [Wensing et al, 2002]. Tree nuts including hazelnut are among the most common elicitors of food induced anaphylaxis [Sampson 1998 and 2003].

Beside food allergy to hazelnut also hazel pollen can induce allergic symptoms. The hazel pollen allergy is more frequent than the food related hazelnut allergy [Besler et al, 2001]. Hazelnut allergy is part of the phenomenon of pollen-associated food allergies, which are the most common food-induced allergic reactions in Europe [Ortolani et al, 1993]. About 70% of tree pollen allergic patients show IgE-mediated adverse reactions to hazelnut [Besler et al, 2001]. Cross-reactivities often occur among the major allergens of birch pollen and the proteins of, for instance, hazelnut, apple, carrot, potato and kiwi [Wigotzki et al, 2001].

For example the pathogenesis-related major birch pollen allergen Bet v 1 is cross-reactive to the major hazelnut allergen Cor a 1, of which four isoforms have been identified in hazel pollen and hazelnuts [Ebner et al, 1993]. A 14-kDa hazelnut allergen showed cross-reactivity to birch profiling (Bet v 2) [Diez-Gomez, 1999].

2.1.4 State of the art in the analysis of allergenic proteins in food

As mentioned before highly specific and sensitive analytical methods are required for the detection of allergens in foods and foodstuffs since even traces of allergens, in the µg/kg range, can elicit adverse reactions. Currently there are several technical possibilities for the detection of potential allergens in food products. The methods employed are either targeting the allergen (protein) itself or a marker that indicates the presence of the offending food. The ideal marker would the offending allergenic protein, however at present detecting the allergen per se is not always feasible, as the chemical properties may not be well characterize. Additionally, many allergenic foods contain multiple allergenic proteins that can vary in abundance. As markers for the presence of potentially allergenic food products or ingredients, specific proteins or DNA fragments are targeted [Poms et al, 2004]. Immunoassays, are presently the method of choice for detection and identification of a wide range of food components including food allergens [Besler, 2001]. Examples for protein-based methods involving immunochemical detection based on antibody-antigen interaction are radio-allergosorbent test (RAST), enzyme-allergosorbent test (EAST), rocket immuno-electrophoresis (RIE), immunoblotting, and enzyme linked immunosorbent assay (ELISA). Whereas RIE and immunoblotting render only qualitative or semiquantitative results, RAST, EAST and ELISA are quantitative methods. Presently only the ELISA technique is used in routine food analysis due to its high precision, simple handling and good potential for standardization [Poms et al, 2004]. Although specific IgE is required for allergen characterization it is not suitable for reliable allergen determination in food products, since the specificity of IgE from sensitized individuals differs considerably and the amount of serum is usually limited [Besler, 2001]. In order to overcome the disadvantages associated with the use of human serum IgE ELISA technique unlike RAST, EAST, RIE and immunoblotting is usually based on antisera specially raised in animals such as sheep, rabbits, mice or chicken see chapters 3.3, 3.4 and 3.5.

Methods operating on the DNA level are based on the polymerase chain reaction (PCR), with real-time PCR highly accurate quantitative results can be obtained [Poms et al, 2004]. However the employment of DNA analysis in allergen detection is discussed controversially, since proteins are the allergenic component and processing may differentially affect nucleic acids and proteins.

Currently, the ELISA technique is the most commonly method used to detect and quantify hidden allergens in food [Taylor and Nordlee, 1996; Poms et al, 2004]. Several ELISAs for the detection of peanut and hazelnut in food have been developed [Hefle et al, 1994; Yeung and Collins, 1996; Holzhauser and Vieths, 1999a, 1999b; Drs et al, 2004], also there is a number of commercially available ELISA kits, see Table I.3.

Table I.3: Commercial available ELISA test kits for the detection of peanut and hazelnut

test kit	supplier
RIDASCREEN® FAST Peanut ELISA	R-Biopharm AG, G
Prolisa Peanut PAK	Pro-Lab Diagnostics Inc., UK
Veratox Peanut Protein Test Kit	Neogen, USA
Peanut Residue ELISA	Elisa Systems (http://www.elisas.com.au/)
BioKits Peanut ELISA	Tepnel (http://www.tepnel.com/default.asp)
Peanut DiagnoKit™	ABKEM (http://www.abkemiberia.com/)
RIDASCREEN® FAST Hazelnut ELISA	R-Biopharm
Hazelnut Residue ELISA	Elisa Systems (http://www.elisas.com.au/)
Hazelnut DiagnoKit™	ABKEM (http://www.abkemiberia.com/)

However the ELISA technique requires laboratory equipment and skilled personnel, the minimum reaction time reported for an ELISA is 30 minutes [R-Biopharm, 2003 and 2005].
There is an increasing demand for rapid and easy to use assays particularly in food industry, for on site quality control, safety assurance and allergen monitoring. Testing has to be performed to determine the quality of bulk raw materials and at various steps during the production line. Often laboratory equipment and skilled personnel is not available but results are requested immediately. Therefore alternatives to the ELISA format have earned increasing importance in recent years: dipstick assays and lateral flow devices (LFD), see also chapter 5.2 and chapter 5.3. These assay formats are very inexpensive, rapid and portable, they do not require instrumentation and are extremely simple to perform. Currently, these test are only qualitative. A rather small number of such fast assay formats for the detection of peanut and hazelnut have been developed by now [Mills et al, 1997; Stephan et al, 2002; Blais et al, 2003]. The only commercial available rapid test for the detection of peanut in food is the Peanut Rapid Test Kit from Tepnel (http://www.tepnel.com/), there are so far no commercial rapid tests for the detection of hazelnut.
Another assay format that enables targeting of either DNA or protein, gained importance only very recently, the microarray (see also chapter 5.4), and could theoretically also be employed for the detection of food allergens. So far there are no reports on microarrays specific for peanut or hazelnut though application of antibody microarrays for the quantitative analysis of complex protein solutions still requires modifications and careful optimisation to overcome the limitations with regard to sensitivity and cross-reactivity [Angenendt, 2005].

3 ANTIBODIES

3.1 History

The term antibody was coined by Emil von Behring in 1890 to describe the antitoxin proteins, discovered by himself and Shibasaburo Kitasato, which appeared in the bloodstream of guinea pigs after bacterial infections [von Behring and Kitasato, 1890]. A German bacteriologist showed in 1894 that cholera bacteria were destroyed by antibodies (a process called bacteriolysis) [Pfeiffer and Isayev, 1894]. In 1898 a young Belgian bacteriologist, Jules Bordet, found that when cholera serum was heated to 56° C, it retained its antibodies but lost its ability to destroy bacteria [Elgert, 1996]. He concluded that the heated serum lost a bactericidal substance, originally called alexine but which became known as complement. This explained an important element of immunity: an antibody combines with an antigen, and only after the antibody reacts with complement is the antigen made harmless. Bordet also explained that antigens can be detected by their reaction to specific antibodies, and by the fact that antibody-antigen complexes precipitate out of a solution when they react to complement. The specific nature of the antigen-antibody reaction was used by Karl Landsteiner in 1900 to make his very important discovery of the human blood groups [Landsteiner, 1900]. Landsteiner also showed that individual antibodies react to the specific chemical structure of individual antigens. German medical scientist Paul Ehrlich developed the "side-chain" theory of immunity explaining that antibodies and antigens fit together in very specific molecular ways, like a key in a lock [Ehrlich, 1901]. In 1938 Tiselius identified antibodies as proteins of the gamma globulin portion of plasma [Tiselius, 1938], therefore they are also called immunoglobulines (Ig). In 1948 Astrid Fagraeus showed that antibodies are produced by plasma cells in the bone marrow and lymph nodes [Fragraeus, 1948]. The exact molecular nature of antibodies was difficult to discern, since the body produces about one million different antibodies, and they are all large molecules. A pioneer in this field was Linus Pauling, who published his first paper on antibody structure in 1940 [Pauling, 1940]. Investigations of protein sequence in the 1960s revealed that antibodies consist of an extensively constant and an extremely variable component [Grawunder and Haasner, 1992]. The reason for the vast diversity of the antibody structure was not fully understood until Dreyer and Bennett in 1965 hypothesised that 2 different genes could code for one antibody chain [Dreyer and Bennet, 1965].

3.2 Structure and function of antibodies

Antibodies are recognition proteins, unique to the immune system of vertebrates [Elgert, 1996]. They are produced by B cells that, upon stimulation, undergo repeated cell divisions, enlargement and differentiation to form a clone of antibody secreting plasma cells. The biological function of antibodies in the body is to bind pathogens and facilitate their elimination [Janeway and Travers, 1995].

Steps to B cell activation [Elgert, 1996] **:**

When a "foreign" substance or antigen enters the body it is recognized by the immune system. Normally antigens are proteins or polysaccharides derived from pathogens (e.g. bacterium, fungus, parasite, virus). The cells of the immune system recognize discrete and distinctive sites on the antigen, called epitopes. Antigen presenting cells (e.g. macrophages) incorporate and process the antigen and display the fragments via molecules called major histocompatibility complex (MHC) on the cell surface. Another type of cells of the immune system, the T cells recognize the antigen fragments presented by the macrophages and activate the B cells specific for the antigen to mature to plasma cells capable of antibody secretion. An additional stimulus for the B cell is the binding of extracellular antigen to the antibody molecules on its cell surface.

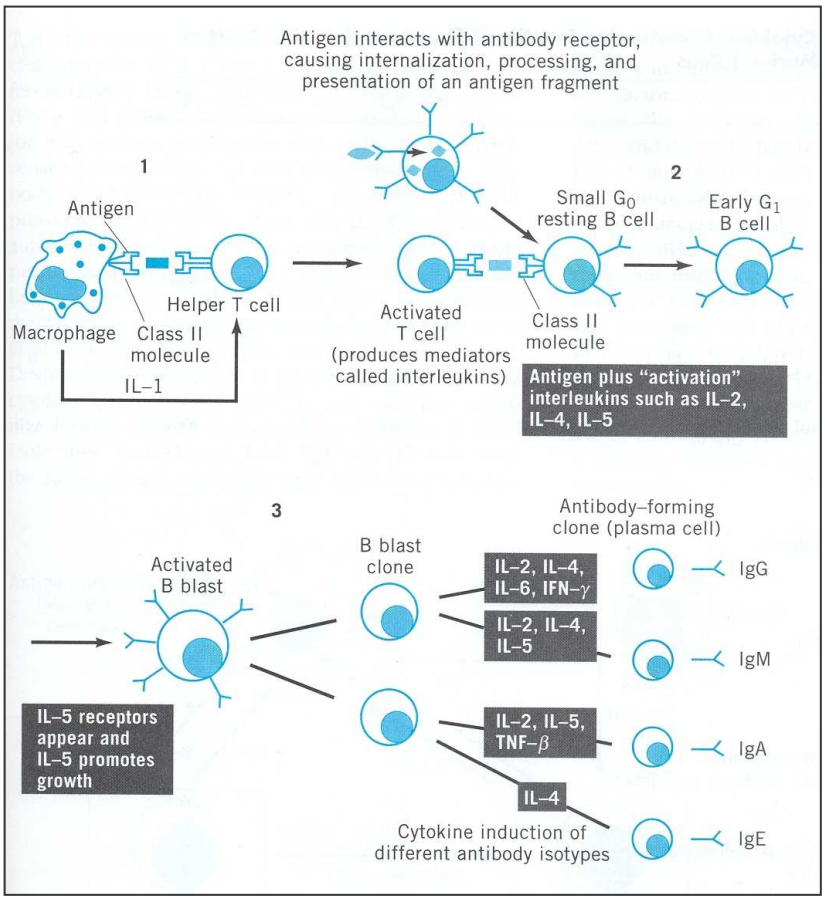


Figure I.3: Schema of B cell activation [Elgert, 1996]

Depending on the kind of cytokine induction the plasma cell can produce different antibody isotypes [Roitt, 1991]:

IgG most abundant Ig of internal body fluids particularly extravascular where it combats microorganisms and their toxins

IgM very effective agglutinator, produced early in immune response, effective first-line defence versus bacteraemia

IgA major Ig in sero-mucous secretions where it defends external body surfaces

IgE protection of external body surfaces, recruits anti-microbial agents raised in parasitic infections, responsible for symptoms of atopic allergy

Structure of antibodies [Elgert, 1996]**:**

Antibodies are large proteins shaped to form a Y. The antibody molecule is made up of two identical heavy and two identical light chains held together by interchain disulphide bonds [Roitt, 1991]. Each of the heavy and light chains consists of a constant (C) region at the carboxyl terminal end and a variable (V) region at the amino terminal end. While the respective amino acid sequences of the constant regions are almost identical the amino acid sequences of the variable regions vary greatly from one antibody to another. These variable sections, that make up the tips of the Y´s arms create a pocket uniquely shaped to enfold a specific antigen epitope. The unique sequence of amino acid residues for each V region leads to the large diversity of structure which accounts for antibody specificity. The region where the arms meet the stem of the Y is called hinge region and shows flexibility to facilitate antigen binding, only IgG, IgA and IgD (one of the main receptor on mature B cells) antibody molecules have hinge regions.

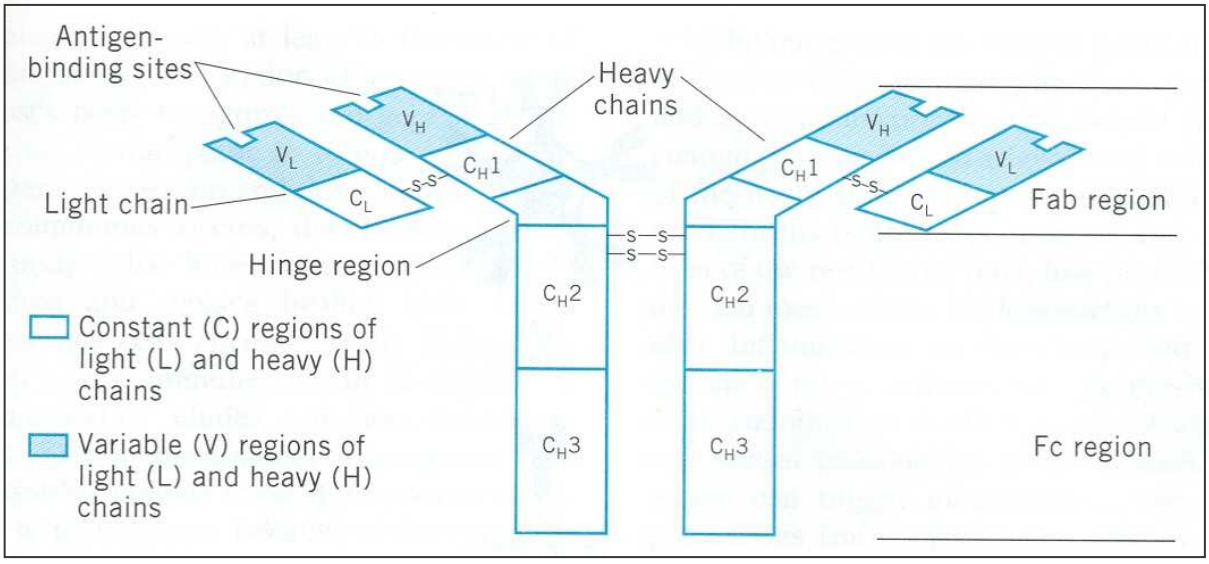


Figure I.4: Antibody structure [Elgert, 1996]

3.3 Polyclonal antibodies

Polyclonal antibodies are raised by repeated immunization of a suitable animal, e.g., rabbit, goat, donkey, or sheep, with a suspension of the appropriate antigen. This induces the B-lymphocytes to produce IgG immunoglobulins specific for the desired antigen. The IgG circulates in the blood stream of the animal and can be purified from the mammal's serum.
One characteristic of polyclonal antibodies is that they are derived from different antibody-producing B clones in the immunized animal [Chemicon, 2005]. This mixture of resulting antibodies may then recognize a variety of epitopes on the antigen, which can be an especially useful feature in some experimental procedures. Because these polyclonal mixtures of antibodies react with multiple epitopes on the surface of the antigen, they will be more tolerant of minor changes in the antigen, e.g., polymorphism, heterogeneity of glycosylation, or slight denaturation, than will monoclonal (homogenous) antibodies [Chemicon, 2005]. A major disadvantage of polyclonal antibodies is, that the amount of material derived from one animal is limited and when immunizing multiple animals, you will get a slightly different mixture of antibodies from each animal.

3.4 Monoclonal antibodies

Monoclonal antibodies (mAb) are antibodies that are identical because they were produced by one type of B-cells, all clones of a single parent cell [Wikipedia encyclopedia, 2005]. The technique for the production of monoclonal antibodies was developed in 1975 by Kohler and Milstein [Kohler and Milstein, 1975]. To produce monoclonal antibodies, B-cells are removed from the spleen of a mouse that has been immunized with the desired antigen. These B-cells are then fused with myeloma tumor cells that can grow indefinitely in culture (myeloma is a B-cell cancer). The fused hybrid cells (called hybridomas) will multiply rapidly and indefinitely (since they are cancer cells) and will produce large amounts of antibodies (IgG). The hybridomas are sufficiently diluted and grown, thus obtaining a number of different colonies, each producing only one type of antibody. The antibodies from the different colonies are then tested for their ability to bind to the antigen (for example with a test such as ELISA), and the most effective one is picked out. Monoclonal antibodies can be produced in cell culture or in animals. When the hybridoma cells are injected in mice (in the peritoneal cavity, the gut), they produce tumors containing an antibody-rich fluid called ascites fluid. For an overview of the steps in preparation of monoclonal antibody see Figure I.5.

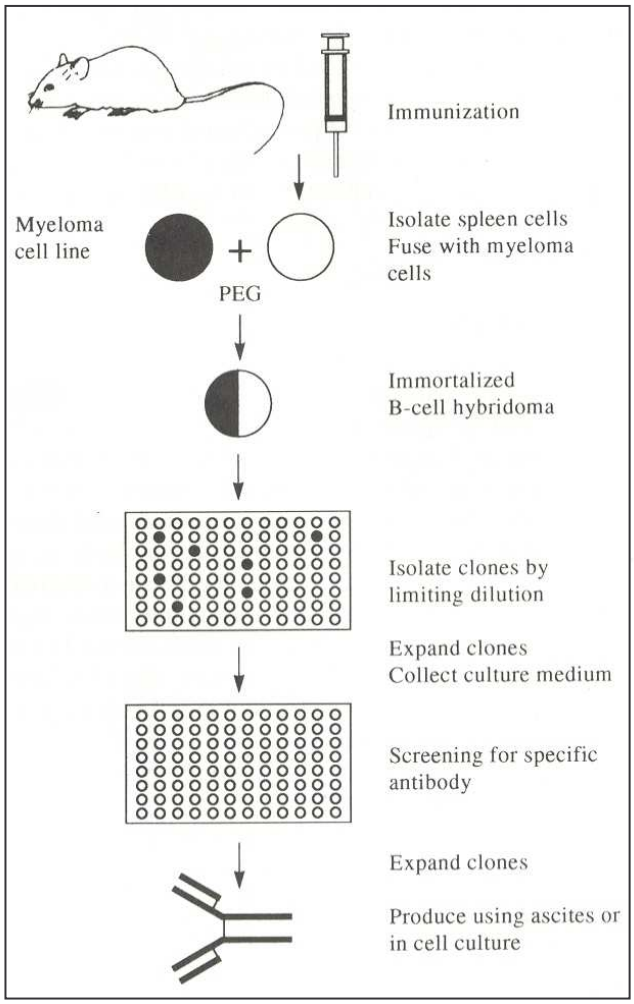


Figure I.5: Preparation of monoclonal antibodies [Wild, 1994]

Monoclonal antibodies can be produced in unlimited quantities and after further processing high- purity antibody can be obtained. Cell lines can be preserved at –80°C and reactivated to any later date thus providing reproducibility of identical material. Monoclonal antibodies are homogeneous and recognize all the same epitope, therefore they are highly specific. The disadvantage of a monoclonal lies in the high cost of production and, also the affinity varies strongly for the individual antibodies and might therefore be lower compared with their corresponding polyclonal counterparts [Wild, 1994].

3.5 Egg yolk antibodies

Egg yolk antibodies are polyclonal antibodies that are derived from the egg yolk of immunised chickens. Like mammals the birds provide their offspring with antibodies, while the egg is still in the ovary, hens transfer their serum immunoglobulin into the yolk [Klemperer, 1893].

The term IgY instead of IgG for hen´s main serum-immunoglobulin has been proposed by Leslie and Clem in 1969 [Leslie and Clem, 1969].
In their experiments they proved that IgY molecules are different from IgG. The molecular weight of IgY (180 kDa) is higher than the one of mammalian IgG (150 kDa) [Affiland, 2005]. Reason for that is the structural difference: the heavy chain of IgY has an additional constant domain instead of the hinge region of IgG, Figure I.6 [Schade, 2001].

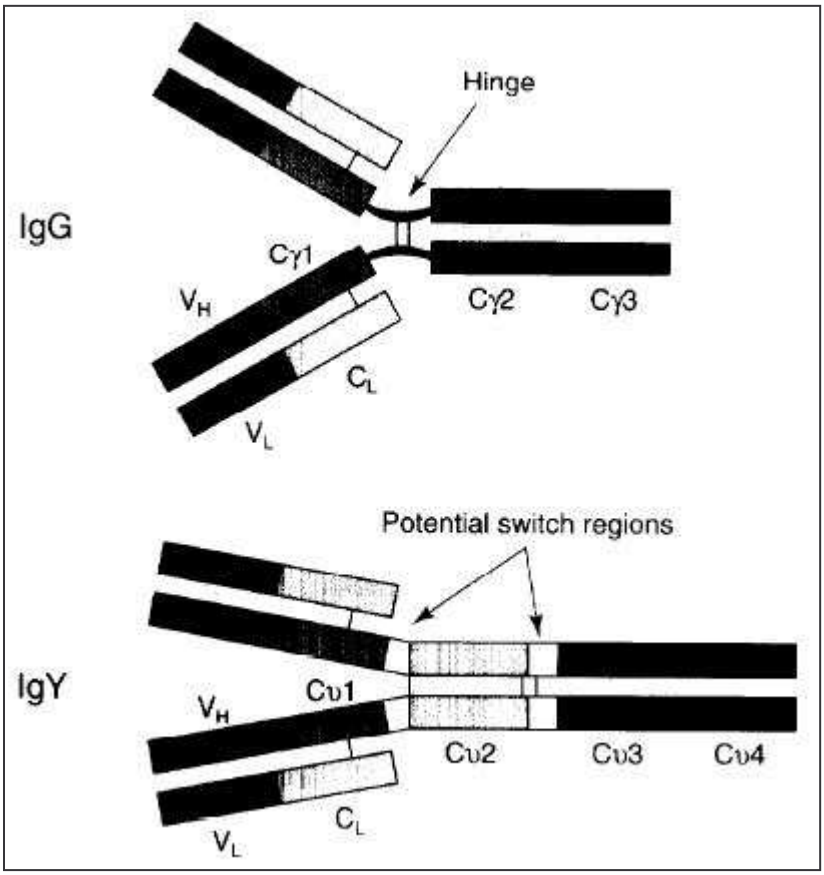


Figure I.6: Structural difference of IgG and IgY [Warr et al, 1995]

Further inflammatory response characteristics of IgY are that they do not bind to rheumatoid factor (an marker) in blood [Larson et al, 1998], they do not activate mammalian complement factors [Larson et al, 1992], they do not bind to cell surface Fc receptor [Schmidt et al, 1993], protein A [Kronvall et al, 1974] and protein G [Akerström et al, 1985]. Advantages of IgY antibodies are that their production is comparatively cheap, a single chicken can produce an enormous amount of antibody and animals do not have to be killed. However affinity of IgY to the antigen can be lower than for rabbit antibodies [AgriSera, 2005], also problems with high cross-reactivity of egg yolk antibodies are mentioned in literature [Drs et al, 2004]. Despite the fact that the use of chicken as immunization host brings many advantages to the production of polyclonal antibodies, the generation of egg yolk immunoglobulins is rarely chosen [Tini et al, 2002].

4 ANTIBODY LABELS

4.1 Colloidal gold

4.1.1 History

Colloidal gold has been used for various purposes during history, therefore a short overview is given in the following [Wikipedia encyclopedia, 2005].

Already in the Middle Ages colloidal gold was known and used for its health restorative properties. Since Ancient Roman times colloidal gold has been used to colour glass an intense red. In the 16th century, the alchemist Paracelsus claimed to have created a potion called Aurum Potabile (Latin: potable gold). In the 17th century the glass-colouring process was refined by Andreus Cassius and Johann Kunchel. In 1842, John Herschel invented a photographic process called chrysotype (from the Greek word for gold) that used colloidal gold to record images on paper. Colloidal Gold in a pure state was first prepared around the year 1857 by the distinguished English chemist, Michael Faraday.

However, the application of these inorganic suspensions to protein labelling did not occur until 1971, when Faulk and Taylor invented the immunological staining procedure [Faulk and Taylor, 1997]. Probes labelled with colloidal gold were originally used as electron dense markers for the visualization of cellular or tissue components in electron microscopy [Romano et al, 1974] and, in combination with the silver enhancement technique, as colour markers in light microscopy [Roth, 1982]. Since their introduction to microscopy, gold labels have also become recognized as very important tool for detection and quantitation of proteins, antigens and nucleic acids when used with other techniques such as blotting, flow cytometry, hybridisation, and DNA fingerprint identification [Brada and Roth, 1984], [Jackson et al, 1990]. More recently a very important use for gold conjugates has emerged in their incorporation into rapid test immunoassays [Shyu et al 2002], [Xiulan et al 2005]. In these techniques the unique red colour of the accumulated gold label provides a convenient and extremely sensitive method for visual one-step detection of sub nanogram quantities of proteins in solution.

4.1.2 Properties and use of colloidal gold conjugates

Colloidal gold, or more precisely gold nanoparticles are sub-micrometer sized particles of gold, usually found in the form of a suspension in water. The liquid appears to be either an intense red colour (for particles less than 100 nm), or a dirty yellowish colour (for larger particles) [Wessling, 1996].

Generally, gold nanoparticles are produced in a liquid by reduction of hydrogen tetrachloroaurate ($HAuCl_4$). After dissolving $HAuCl_4$, the solution is heated to boiling and rapidly stirred while a reducing agent is added.

This causes Au^{3+} ions to turn into plain gold atoms. As more and more of these atoms appear, the solution becomes supersaturated, and gradually gold starts to precipitate in the form of small sub-nanometer particles. The rest of the gold atoms that appear stick to the existing particles, and if the solution is stirred vigorously enough, the particles will be fairly monodisperse. Pioneered by Turkevich et al and refined by Frens, this recipe is the simplest one available [Turkevich et al, 1951], [Frens, 1973], it is used to produce modestly monodisperse spherical gold nanoparticles of around 10-20 nm in diameter.

Gold particles may be conjugated to a wide variety of molecules including proteins (e.g. antibodies), polypeptides, carbohydrates, polymers, polysaccharides, enzymes and nucleic acids. For the application in rapid test immunoassays conjugations of proteins (antibodies) to gold are of major importance. Preparing stable protein-gold complexes depends upon at least three physical phenomena [Hermanson, 1996]:

- **charge attraction** of the negative gold particle to positively charged protein
- **hydrophobic absorption** of the protein to the gold particle surface
- **dative binding** of the gold to sulphur where this may exist within the structure of the macromolecule

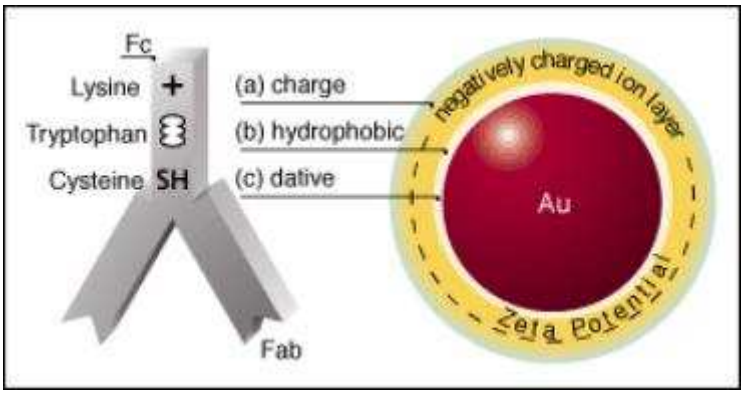


Figure I.7: Physical phenomena effectuating conjugation of proteins to colloidal gold [Chandler et al, 2000]

In the colloidal suspension, there exists a balance between the negative-charged repulsion and the attractive forces (Van der Waals attraction), that could cause coagulation. This balance can be breached by the addition of electrolytes to the solution that can mask the negative surface charge on each particle. At a certain concentration of electrolytes, the colloid will begin to collapse as the gold particles adsorb onto one another, forming large aggregates and ultimately falling out of suspension.

Electrolyte-mediated coagulation forms the basis for creating all gold conjugates with other molecules. If macromolecules such as proteins are present in the colloidal suspension as the electrolyte concentration is raised, then adsorption will occur with the protein molecules instead of with other gold particles. Thus, in place of aggregation and collapse of the suspension, labelling occurs.

4.2 Latex particles

Coloured latex particles represent another antibody label for one-step detection, very commonly used in rapid immunoassays, a short description is given in the following [Seradyn]:

The term “latex particles” actually refers to individual plastic spheres in the size range of 0.03 to 2 µm in diameter. A suspension of microparticles has the milky appearance of latex rubber. For this reason, microparticle suspensions have historically been referred to as latex.

The latex particles are usually polystyrene or carboxylate-modified particles prepared by emulsion polymerization methods. The microparticles incorporate dyes internally and are available in various colours. Proteins may be bound to polystyrene or carboxylate-modified particles by adsorption. Adsorption is mediated by hydrophobic and ionic interactions between the protein and the surface of the microparticles. Proteins may also be covalently attached to the surface of carboxylate-modified particles.

Polymeric microspheres have historically been the solid phase of choice for latex agglutination tests, particle enhanced turbidimetric assays and particle capture tests and assays [Bangs Laboratories Inc]. More recent applications include flow cytometric assays, colloidal arrays and biosensors.

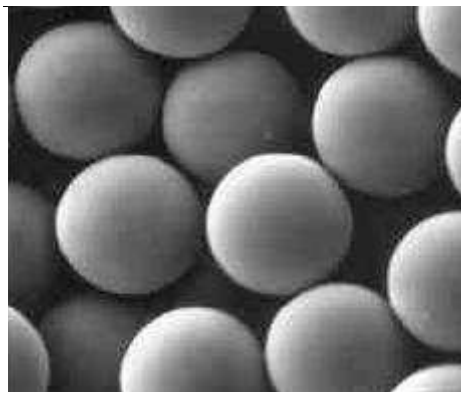


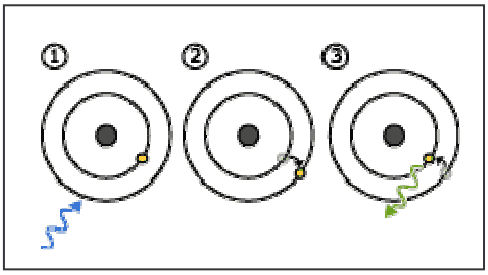
Figure I.8: Scanning Electron Microscopy image of polystyrene microspheres [Bangs Laboratories Inc]

4.3 Fluorescent dye

A fluorescent probe is a fluorophore designed to localize a specific region within a biological specimen or to respond to a specific stimulus [Invitrogen, 2005]. Fluorescent probes enable researchers to detect particular components of complex biomolecular assemblies, including live cells, with exquisite sensitivity and selectivity.

Fluorescent dye can be conjugated to oligonucleotides, proteins, antibodies and drugs, there is a wide choice of commercial products for fluorescence labelling. Therefore the technique has major applications in flow cytometry [Stewart and Stewart, 1994], DNA sequencing [Oefner et al, 1994], fluorescence in situ hybridization [Nederlof, 1990], fluorescence microscopy [Brelje et al, 1993] and recently also in DNA microarray [Schena et al 1995] and protein microarray [Luo and Diamandis, 2000].

Fluorescence is a luminescence which is mostly found as an optical phenomenon in cold bodies, in which a molecule absorbs a high-energy photon, and re-emits it as a lower-energy (longer-wavelength) photon. The energy difference between the absorbed and emitted photons ends up as molecular vibrations (heat) [Wikipedia encyclopedia, 2005].



1. energy is absorbed by the atom which becomes excited
2. the electron jumps to a higher energy level
3. soon, the electron drops back to the ground state, emitting a photon - the atom is fluorescing

Figure I.9: Principle of fluorescence

The fluorescence intensity can be measured with various fluorescence instruments depending on the respective type of application mentioned before (flow cytometer, spectrofluorometer, microplate reader, fluorescence microscope, fluorescence scanners).

Therefore fluorescent probes can be quantified unlike the antibody labels described before (colloidal gold, coloured latex particles).

4.4 Horseradish peroxidase

Hydrogen peroxide oxidoreductase (HRP) is the most widely used enzyme in the enzyme immunodiagnostic products, it belongs to the ferroprotoporphyrin group of peroxidases and is a hemoprotein isolated from horseradish roots (Armoracia rusticana) [Deshpande, 1996]. This enzyme catalyzes the oxidation of a number of organic or inorganic substrates by hydrogen peroxide, it has been found well suited for the preparation of enzyme conjugated antibodies, due in part to its ability to yield chromogenic products, and in part to its relatively good stability characteristics [Crowther, 2001].

A method for the conjugation of peroxidase to antibodies using sodium periodate was published by Nakanake and Kawaoi in 1974. Thereby, coupling is based on the fact that peroxidase is a glycoprotein containing 18% by weight sugars which can be oxidised by periodate to reactive aldehyde groups. These aldehydic groups are then able to react with the amino groups of an antibody in a subsequent step.

Peroxidase labelled immunoglobulins have been used successfully as immunohistological probes for the demonstration of tissue antigens [Nakane and Pierce, 1967], and in enzyme amplified immunoassay systems for the quantitative determination of soluble and insoluble antigens [Avrameas, 1969; Avrameas and Guilbert, 1972; van Weeman and Schuurs, 1974].

Especially for enzyme linked immunoassays (ELISA) peroxidase labelled immunoglobulins are used for detection very commonly. For the quantification of enzyme label 3, 3´,5, 5´-tetramethylbenzidine (TMB) is often the substrate of choice, because due to its excellent chromogenic properties in combination with its lack of toxicity problems it is regarded as one of the best HRP substrates [Aslam and Dent, 1998]. Nanomolar levels of HRP can be detected, and the formation of the oxidised blue product follows linear formation kinetics for up to three minutes; the absorption maximum of the blue-green product is 655 nm, but a stronger absorption is obtained at 450 nm in the presence of sulphuric acid [Aslam and Dent, 1998].

5 IMMUNOASSAYS

Modern immunoassays have arisen from the desire to detect and quantify complex biological molecules under conditions for which chemical and physical analytical techniques are either unsuitable or not available. The potential for high sensitivity and high specificity of the antibody-antigen reaction has attracted the attention of workers wishing to exploit these properties in the search for improved analytical techniques. Hence the specific binding of an antibody to the corresponding antigen has become the basic principle of immunoassays.
Today immunoassays are important analytical tools, not only for medical diagnostics but also for quality control of foods and feeds or for environmental research. They are inexpensive, fast and easy to perform, sensitive, and high throughput is possible, immunoassays for the detection of a plethora of analytes available.
Different types of immunoassays have been developed, some of them are introduced in the following.

5.1 ELISA

The term “ELISA” for enzyme linked immunoassay was first introduced in 1974 by Engvall and Perlmann as well as Van Weemen and Schuurs [Engvall and Perlmann, 1974; Van Weeman and Schuurs, 1974]. Principle of an ELISA is the detection and quantification of antigen with an enzyme labelled antibody and a subsequent substrate reaction, as mentioned above a frequently used enzyme is horseradish peroxidase.
ELISAs are traditionally carried out in 96 well plastic plates (microplates), thereby several samples can be analysed in parallel.
There are three basic ELISA principals [Crowther, 2001]:

- Non-competitive ELISA
- Competitive ELISA
- Sandwich ELISA

In addition several subtypes of this main methods exists, in the following only formats that have been applied in this work are described in detail. ELISAs provide the opportunity to analyse plenty of samples simultaneously, also they have the potential for a highly sensitive determination of the amount of analyte in the sample. However the total reaction time of an ELISA is normally longer compared with the LFD or dipstick immunoassay format. The minimum reaction time reported for an ELISA is 30 min [R-Biopharm, 2003]. Moreover certain laboratory equipment is necessary for the accomplishment of an ELISA.

5.1.1 Indirect non-competitive ELISA

The principle of a indirect non-competitive ELISA is illustrated in Figure I.10, the term “indirect” in this case indicates, that the specific antibody is not labelled itself, but detected with the help of an enzyme labelled second antibody. In the first step antigen = analyte (mainly protein in nature) is applied to the plastic solid phase of the microplate wells and attaches passively to the plastic during a period of incubation. After a washing step antibodies are added and those which are specific will bind to the antigen during incubation. Following a further washing step antibodies labelled with enzyme, directed against the particular species in which the original antibodies were produced (anti-species), are added. These bind to the first antibodies which are attached to the antigen. Excess conjugate is washed away after a period of incubation. Detection is completed through addition of substrate leading to a reduction of peroxide by the enzyme and resulting in a colour signal.

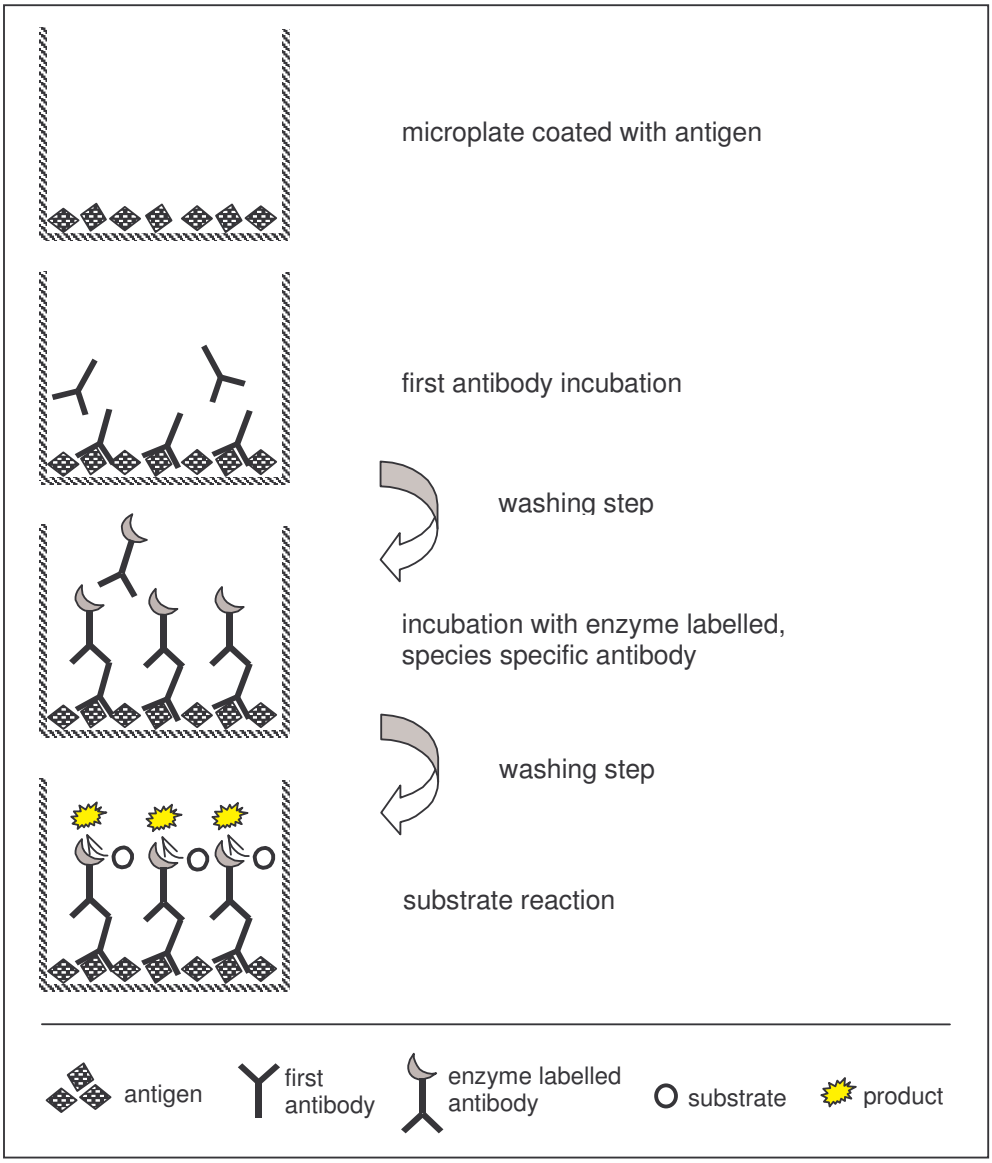


Figure I.10: Principle of an indirect non-competitive ELISA

5.1.2 Indirect competitive ELISA

The target analyte is adsorbed to the solid phase. In the first step of the assay the sample is incubated together with the first antibody enabling the competition between the coated antigen and the solubilised analyte for the specific antibody binding sites. After removing the unbound molecules in a washing step, a second antibody, which is species specific for the first antibody, and conjugated to an enzyme is added. After further washing and substrate addition, the bound first antibodies can be detected indirectly by the enzyme reaction of the second antibody-enzyme conjugate. The detected signal of the colour absorbance is conversely proportional to the concentration of the analyte.

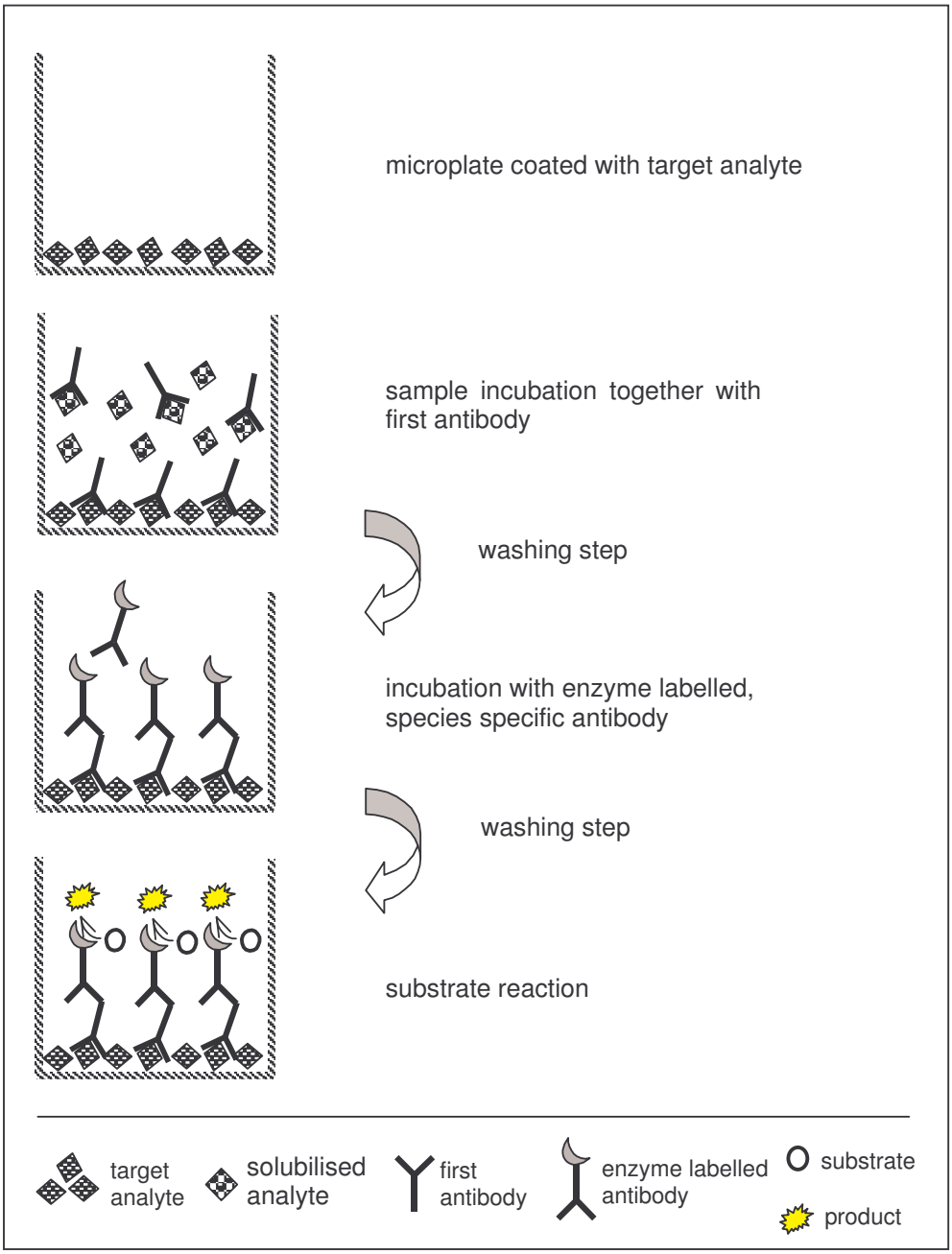


Figure I.11: Principle of an indirect competitive ELISA

5.1.3 Sandwich ELISA

Sandwich ELISAs belong to the most common types of immunoassays used in medical diagnostics and food control. In this assay format a specific antibody is adsorbed to the solid phase (e. g. the wells of a microplate). After incubation of the antigen solution (respective sample) the solid phase is washed to remove non-bound molecules. Subsequently the antibody-bound sample molecules are recognised by a second enzyme-linked antibody which binds to the antigen in the antigen-antibody complex. After that a further washing step is performed before the amount of analyte in the sample can be determined by a subsequent substrate conversion resulting in a colour reaction. This type of immunoassay is based on an excess of antibodies and the analyte must provide at least two epitopes (binding sites) requiring a certain molecular size. Hence, proteins are ideal analytes for this type of assays.

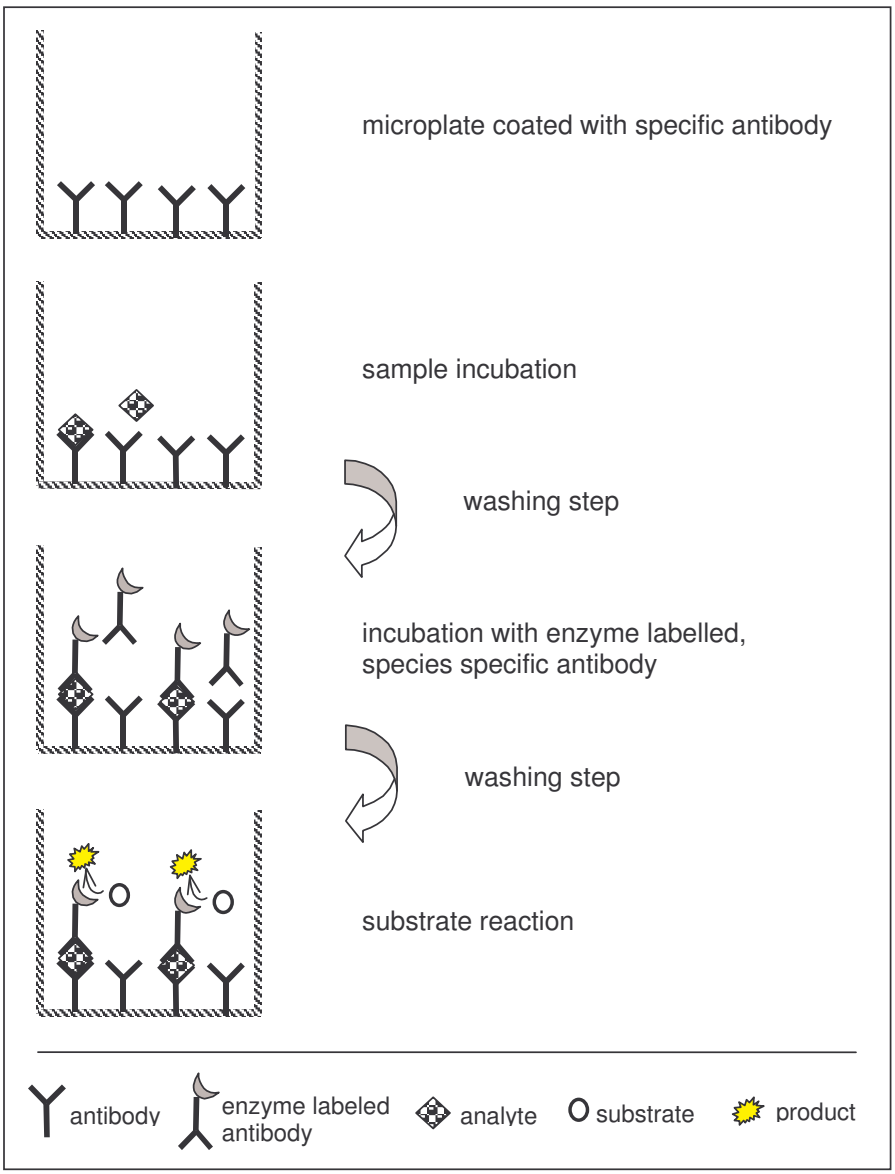


Figure I.12: Principle of a sandwich ELISA

5.2 Lateral Flow Device

Immunochromatographic assays, also called lateral flow tests or simply strip tests, are an extension of the technology used in latex agglutination tests, the first of which was developed by Singer and Plotz in 1956.

The basic principle behind the test is again the antibody-antigen reaction. Dyed microspheres or colloidal gold are bound directly to the specific antibody and therefore a one-step detection of the antigen is possible. Moreover the colour signal can be detected visually without any measuring instrument.

Further benefits of LFDs tests include:

- user-friendly format
- very short time to get test result
- long-term stability over a wide range of climates
- relatively inexpensive to make

These features make strip tests ideal for applications such as home testing, rapid point of care testing, and testing in the field for various environmental and agricultural analytes. In addition, they provide reliable testing that might not otherwise be available to third world countries. LFDs provide qualitatively, and in some cases semi-quantitatively results, the exact quantification of the analyte is impossible. However for many applications the information on presence or absence of the analyte is sufficient, examples therefore are some of the more common lateral flow tests currently on the market: tests for pregnancy, bacterial or viral infections. There are two different reaction schemes for lateral flow devices which basically agree in principle with the respective ELISA formats.

5.2.1 Sandwich LFD

Specific antibodies (detection antibodies) are labelled with dyed latex particles or colloidal gold and applied onto a release pad using an immersion procedure to produce a stable particle reservoir for release onto a nitrocellulose membrane. The specific capture antibodies are immobilized on the nitrocellulose strip to build the test line, additionally a control line consisting of anti-species antibodies directed against the detection antibodies is applied. The release pad and nitrocellulose membrane are assembled, together with an absorbent pad, into a plastic housing, for a schematic of the assay principle see Figure I.13. After the sample is added to the device the labelled detection antibodies are released from the release pad and begin to flow across the membrane together with the sample. If analyte is present in the sample, antibody binding occurs to produce a complex of antigen attached to labelled detection antibody.

As soon as these complexes reach the test line they bind to the immobilized capture antibodies via the antigen, which results in the formation of a visible band, indicating a positive result. Redundant labelled detection antibodies further transverse down the LF membrane and form a signal at the control band. The positive control line is a proof for the successful operation of the test and develops regardless of the presence of analyte in the sample. If the control line does not appear the measurement must be considered as invalid. Consequently only two bands, formed through accumulation of dyed detection antibody at both the test and control line indicates a positive result.
This format is used for testing of larger analytes with multiple antigenic sites, such as proteins.

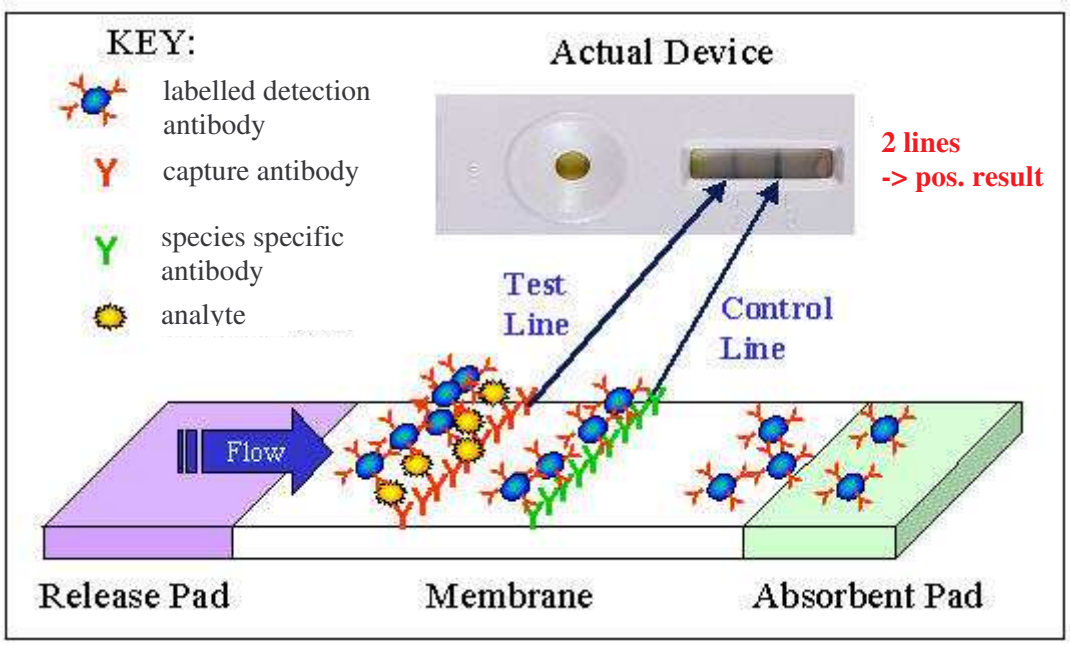


Figure I.13: Principle of a sandwich LFD (© by Chris Danks, CSL, U.K.)

5.2.2 Competitive LFD

The competitive LFD relies upon the competition for binding sites on the detection antibodies. Specific detection antibodies are labelled with dyed microspheres or colloidal gold and additional an internal control bead is produced by labelling such species antibodies that will be recognized by the species specific antibodies that build the control line. Both labelled antibodies are then applied onto a release pad, to produce a stable particle reservoir for release onto a nitro-cellulose-based membrane. Two lines of reagents are immobilised onto the membrane. The target reference or test line is comprised of target analyte.

The control line is a species specific antibody that recognizes the antibody used for internal control bead.

The release pad and membrane are assembled, together with an absorbent pad, into a plastic housing, for a schematic of the assay principle see Figure I.14.

When a sample is added to the device the both of the labelled antibodies are released from the release pad, and begin to flow across the membrane. If analyte is present in the sample, it will be bound by the labelled detection antibody. All the remaining detection antibodies that failed to bind to an antigen will attach to the immobilised target analyte test line as they traverse the membrane; thus producing a visible colour signal. The species specific antibodies on the control line capture the internal control beads to produce an internal control line. This is independent of analyte detection, and provides a visible confirmation of latex flow. Presence of target analyte in the sample, induces complete inhibition of binding of the labelled detection antibody to the test line, therefore a positive result is indicated as single line of dyed antibody binding at the control line only. Insufficient or no target analyte in the sample results in the accumulation of dyed antibodies at both the target and control line, presented as two lines.

This is used most often for small target molecules with single antigenic determinants, which cannot bind to two antibodies simultaneously.

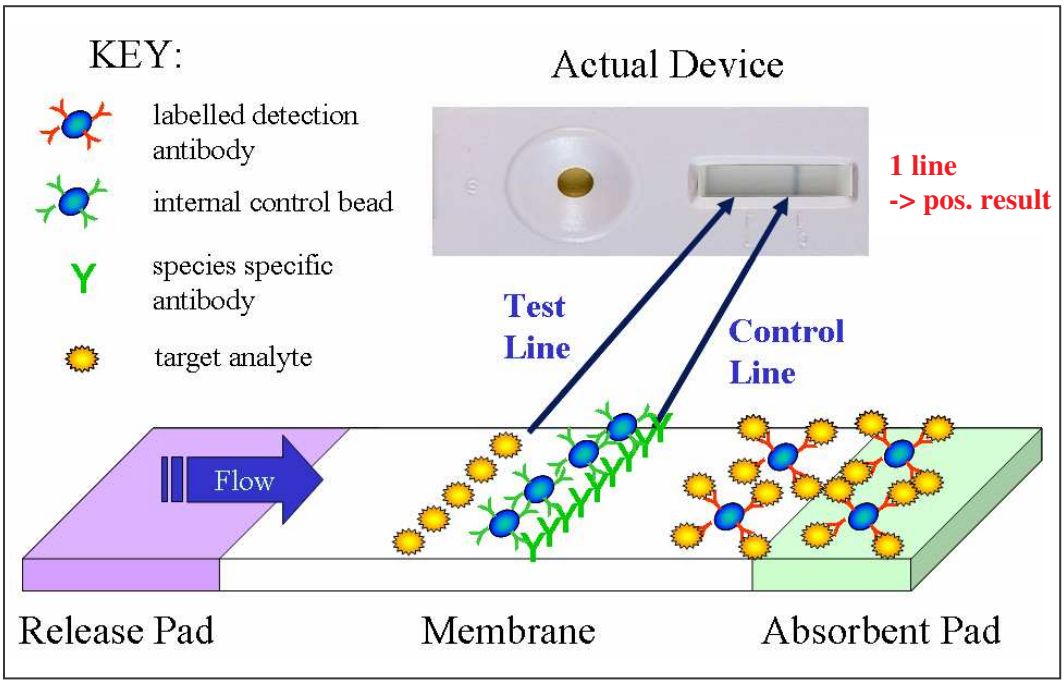


Figure I.14: Principle of a competitive LFD (© by Chris Danks, CSL, U.K.)

5.3 Dipstick

Another example for a qualitative immunoassay is the dipstick. The principle of this immunoassay format is similar to a sandwich ELISA, but carried out on a membrane. Capture antibodies are immobilized on nitrocellulose membrane or immunostick paddles, see Figure I.15. After sample incubation the analyte is usually detected visually in a one step reaction with the help of dyed detection antibodies (conjugated to dyed microspheres or colloidal gold). Hence there are also examples for dipsticks where enzyme conjugated detection antibodies are employed and a substrate reaction is necessary implementing a second step in detection [Mills et al, 1997; Stephan et al, 2002; Baumgartner et al, 2002]. Dipsticks are always built up in sandwich format and usually do not have an internal control unlike LFDs.

This assay format is intended for a very simple and rapid screening of samples by dipping the test device directly into the sample extract (-> therefore the name dipstick). Dipstick assays have found particularly use in medical diagnostic (e.g. urine samples) but also for quality control of foods and feeds or for environmental research.

Figure I.15: Example of an immunostick paddle [Canadawide Scientific]

5.4 Microarray

The basic concept of microarray technology was initiated by the ambient analyte model of Ekins and colleagues [Ekins et al, 1989; Ekins et al 1990], which states that “microspot” assays that rely on the immobilisation of interacting elements on a few square microns should, in principle, be capable of detecting analytes with a higher sensitivity than conventional macroscopic immunoassays. On the basis of such ideas, and boosted by the completion of whole-genome sequencing projects, DNA microarray technology rapidly became the first application of this model [Pease et al, 1994; Schena et al, 1995]. Shortly after a comparable technology for the analysis of proteins was developed [Anderson and Seilhamer, 1997; Ideker et al, 2001; Griffin et al, 2002]. To achieve this task, antibodies, being natural binders of proteins, were immobilised in an array on a solid support to create antibody microarrays. Microarrays usually consist of large numbers of molecules distributed in rows in a very small space with spot sizes <250 µm and therefore permits simultaneously characterization of complex analyte solutions with regard to many features [Angenendt, 2005]. Detection can be achieved in two ways: directly, by using labelled detection antibodies, or indirectly without any modification of the detection antibody involving species specific labelled secondary antibodies. Labelling is mainly performed either radioactively, using isotopes or fluorescently using Cyanine, Alexa or Oyster dyes [Angenendt, 2005].

Therefore laboratory equipment is necessary for the evaluation of microarrays, also total reaction time is usually longer then for the other immunoassays mentioned (ELISA, LFD, dipstick), however this format provides the potential to analyse a very high number of samples in parallel and furthermore a multitude of possible applications.

Although protein and antibody microarray technology are at an early stage of development, they are implemented in several areas such as autoantibody profiling [Robinson et al, 2002], characterisation of cytokine release [Lin et al, 2004], cancer research [Nam et al, 2003] or signal pathway characterisation [Grubb et al, 2003; Wulfkuhle et al, 2003].

The widespread application of antibody microarrays for the quantitative analysis of complex protein solutions still requires modifications and careful optimisation to overcome the limitations with regard to sensitivity and cross-reactivity [Angenendt, 2005].

6 VALIDATION

6.1 Definition

Validation is the process of demonstrating that an analytical procedure is suitable for its intended use. The definition used for “validation” in the EURACHEM Guide is “confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled” [EURACHEM, 1998].

In the validation process the following validation characteristics need to be evaluated:

[ISO VIM DGuide 99999, 2004]

- Accuracy

  The accuracy of an analytical measurement expresses the closeness of agreement between a quantity value obtained by measurement and the true value of the measurand.

- Precision

  The precision of an analytical measurement expresses the closeness of agreement between quantity values obtained by replicate measurements of a quantity, under specific conditions:

  a) Repeatability condition

     Condition of measurement in a set of conditions including the same measurement procedure, same operator, same measuring system, same operating conditions and same location, and replicated measurement over a short period of time.

  b) Reproducibility condition

     Condition of measurement in a set of conditions including different locations, operators, and measuring systems.

- Specificity

  Specificity is the capability of a measuring system, using a specified measurement procedure, to provide a measurement result for a quantity involving a specified component in a system undergoing measurement, without interference from other components in the same system.

- Detection limit [Eurachem, 1998]

  The lowest concentration of analyte in a sample that can be detected, but not necessarily quantitated under the stated conditions of the test.

- Quantification limit [Eurachem, 1998]

  The lowest concentration of analyte that can be determined with acceptable precision (repeatability) and accuracy under the stated conditions of the test.

- Linearity [Eurachem, 1998]
  Defines the ability of the method to obtain test results proportional to the concentration of analyte.
- Range [Eurachem, 1998]
  Set of values of measurands for which the error of a measuring instrument is intended to lie within specified limits.
- Robustness [Eurachem, 1998]
  The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

6.2 Interlaboratory study

Interlaboratory studies are organised to assess the method performance, to improve methods in a new sector of chemical analyses, to standardise methods, and to certify reference materials. Appropriate to the different objectives these studies can be classified into method performance studies, laboratory performance studies, and material certification studies according to Horwitz [Horwitz, 1995].

In a **method performance study** or so-called collaborative study each participant uses the same method on a set of identical samples to perform analyses by the same protocol. The obtained results are used to estimate the performance characteristics of the applied method. Usually these characteristics are within-laboratory and among-laboratories precision, and when necessary and possible, other pertinent characteristics such as systematic error, recovery, internal quality control parameters, sensitivity, limit of determination, and applicability.

Laboratory performance studies are conducted for the evaluation and/or improvement of laboratory performance. These studies consist of one or more analyses conducted by a group of participating laboratories on one or more homogeneous, stable test items by the method of their choice. The reported results are compared with those from other participants or with target or assigned values. Moreover, a laboratory performance study will be termed proficiency testing when the objective of the study is continuous assessment of laboratory or analyst performance.

A **material certification study** is an interlaboratory study that assigns a reference value (true value) to the analyte concentration in the test material with a stated uncertainty.

6.2.1 Reference material

Stable and homogeneous reference material is an essential requirement for method performance - and laboratory performance studies. It is crucial that the batch of test items provided to participants in each round be sufficiently homogeneous so that any results later identified as extreme are not attributed to any significant test item variability. Due to a lack of peanut and hazelnut containing matrix reference material, test material for interlaboratory studies is often prepared by the coordinator of the study. To comply the mentioned requirements homogeneity testing has to be carried out prior to the despatch of the test items to establish the homogeneity of the test material.

Homogeneity testing [ISO/DIS 13528, 2002]
Samples are prepared and packaged, a number g of the samples in their final packaged form is taken at random, where $g \geq 10$. Two test portions are prepared from each sample using techniques appropriate to the test material to minimise between-test-portion differences. The $2g$ test portions are measured in a random order, completing the whole series of measurements under repeatability conditions. General average, within-samples standard deviation and between-samples standard deviation is calculated.
An analysis of variances (ANOVA) [Sachs, 1992] is carried out. Therefore the F-values for target concentration levels of the individual samples are calculated. The F-value or variance ratio is a measurement of distance between individual distributions. The calculated F-values are compared with the upper critical values of F-distribution (evaluated from the degrees of freedom (total and inter) at 5% significance level). When the calculated F-value is below the critical value the sample can be regarded as being homogeneous.

II MATERIALS AND METHODS

1 ANTIBODIES

The first step in the development of immunoassays is the production of antibodies.
The monoclonal and polyclonal antibodies were prepared by different project partners.
The production of the egg yolk antibodies was part of the work conducted in this thesis.

1.1 Monoclonal and polyclonal antibodies

The monoclonal and polyclonal antibodies were kindly donated by RIKILT (Institute of Food Safety, Netherlands) and CSL (Central Science Laboratory, U.K.). Monoclonal antibodies were derived from immunized mice, polyclonal antibodies were generated in rabbits.
The immunogen for the peanut antibodies was produced by extracting a mix of 12 different peanut species each raw and roasted. The hazelnut immunogens were prepared accordingly from 12 different roasted hazelnut species. All antibodies were tested for cross-reactivities in a standard competitive ELISA protocol. None of the antibodies showed cross-reactivity against any of the following substances:
hazelnut / peanut, cashew nut, almond, walnut, pecan, coconut, chestnut, pistachio, brazil nut, pumpkin seed, sunflower seed, sesame, poppy seed, lupine, wheat, rye, corn, rolled oats, barley, rice, apple, mango, apricot, carob, raisins, soy bean, chickpea, pea, lentil, white bean, aranzini, lecithin, dried egg white, cocoa powder, cinnamon, cocoa butter, yeast, vanillin, potato starch, pectin, skim milk powder, cookies, birch pollen, hazelnut pollen, walnut pollen.

1.2 Egg Yolk Antibodies

1.2.1 Immunisation

Immunisation and animal housing were conducted by Ao. Univ. Prof. Dr. Marcela Hermann at the Department of Medical Biochemistry, Medical University of Vienna.
The immunogen for the peanut antibody was the high MW protein fraction (> 20 kD) of an TBS extract (procedure see chapter II.2.2.1) of roasted peanut. For the hazelnut immunogen a mix of 7 different roasted hazelnut species was extracted in the same way and used as a whole. For the first immunisation 250 µl immunogen solution were mixed with 250 µl Freund´s Complete Adjuvant and injected into the breast muscle of the chicken.

In week 6, 12 and 18 after the first immunisation booster injections were performed accordingly with a mix of immunogen / Freund´s Incomplete Adjuvant. Eggs were collected starting one week after the third immunisation until 3 months after the last booster injection and stored at 4 °C until further processing.

1.2.2 Isolation of egg yolk antibodies

Principle of the IgY isolation is the removal of lipids from the egg yolk and a precipitation of the proteins with polyethylene glycol (PEG) based on the method developed by Polson [Polson et al, 1985].

Buffers and Solutions

IgY preparation buffer, pH 7.5

Na_2HPO_4 90 mM
NaH_2PO_4 10 mM
NaCl 1 M
in dist. H_2O

For antibody isolation batches of five subsequently laid eggs were pooled. Without destroying the yolk-membrane the egg shell was broken, the yolk carefully separated from the white and washed with distilled water. Then the yolk surface was pricked and while keeping the membrane the content was collected. An equal volume of IgY preparation buffer was added and the mixture stirred for 15 min. After transferring the suspension into a centrifuge beaker 3.5% (w/v) of PEG were added and stirring was continued another 15 min, followed by 15 min of shaking to improve the precipitation. Subsequently, the mixture was centrifuged for 15 min at 14000 x g and 10 °C. The result of centrifugation consisted of two phases: a slimy precipitate and a clear liquid phase containing the antibodies. The clear, nearly colourless liquid was filtered through a gauze tissue covered funnel, the filtrate was collected and stored at –20 °C until further use.

1.2.3 Characterisation of egg yolk antibodies

Total protein concentration of the isolated egg yolk antibodies was determined using BCA Protein Assay Kit (procedure see chapter II.2.3). An indirect non-competitive ELISA protocol was performed to assess the sensitivity of the new antibodies. The specificity was checked in an indirect competitive ELISA format.

Thereby cross-reactivity of the peanut and hazelnut egg yolk antibody against the following substances was screened:
hazelnut / peanut, cashew nut, almond, walnut, pecan, coconut, chestnut, pistachio, brazil nut, pumpkin seed, sunflower seed, sesame, poppy seed, lupine, wheat, rye, corn, rolled oats, barley, rice, apple, mango, apricot, carob, raisins, soy bean, chickpea, pea, lentil, white bean, aranzini, lecithin, dried egg white, cocoa powder, cinnamon, cocoa butter, yeast, vanillin, potato starch, pectin, skim milk powder, cookies, birch pollen, hazelnut pollen, walnut pollen.

1.2.3.1 Indirect non-competitive ELISA

Buffers and solutions

Coating buffer, pH 9.6
Na_2CO_3 12 mM
$NaHCO_3$ 39 mM
NaN_3 0.01% (w/v)
in dist. H_2O

PBS, pH 7.5
Na_2HPO_4 180 mM
NaH_2PO_4 20 mM
NaCl 362 mM
in dist. H_2O

Blocking buffer, pH 7.5
PBS, pH 7.5 diluted with
dist. H_2O 1:4

Assay buffer, pH 7.5
PBS, pH 7.5 diluted with
dist. H_2O 1:4
Tween 20 0.1% (v/v)

Washing buffer, pH 7.5 (20x)
PBS, pH 7.5
Tween 20 0.08% (v/v)
in dist. H_2O, dilute 1:20 before use

Substrate buffer, pH 4

Citric acid 200 mM

Sorbic acid 0.7 mM

in dist. H_2O

adjust pH with conc. NaOH

TMB stock solution

Tetramethylbenzidin 6 mM

Dimethylsulfoxide 20% (v/v)

in Methanol

Substrate solution

(for one MTP, prepare just before use)

Substrate buffer 25 ml

30% H_2O_2 5 µl

TMB stock sol. 200 µl

Stop Solution

H_2SO_4 1 M

in dist. H_2O

For coating of the microplates TBS extracts (procedure see chapter II.2.2.1) from peanut or hazelnut respectively were diluted with coating buffer to give the following concentrations:
1 mg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml, 0.001 µg/ml.
Additionally there was a blank sample consisting of TBS buffer. 12x 200 µl of each concentration were added to a microplate with high binding property according to the schema displayed in Figure II.16:

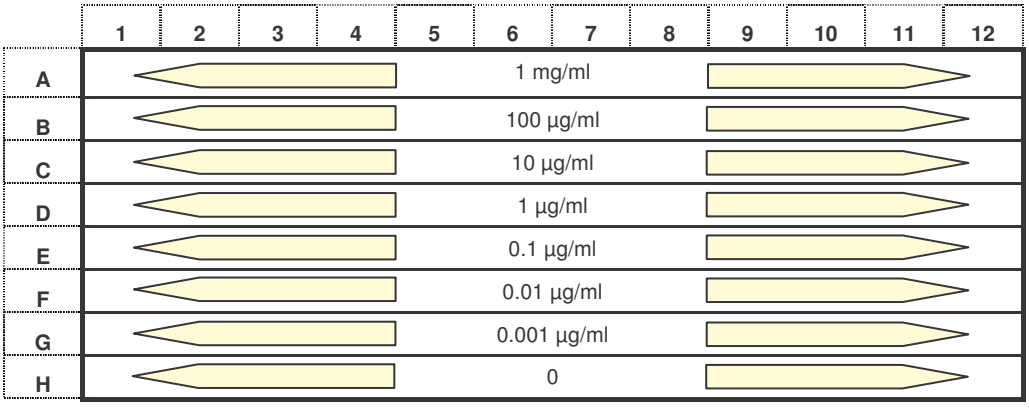


Figure II.16: Distribution of coating dilutions on the ELISA plate

The plate was covered and incubated over night at 4 °C.
Next day the coated plate was washed three times with Washing buffer. After that 300 µl Blocking buffer with 1% skimmed milk powder were added to each well and the plate was incubated for 1 h at 37 °C. Following a further washing step egg yolk antibody diluted with Assay buffer (1:8000, 1:2000, 1:500) was applied to the plate, 200 µl per well, according to the schema displayed in Figure II.17.

	1	2	3	4	5	6	7	8	9	10	11	12
A	IgY batch 1 - 1:8000	IgY batch 1 - 1:2000	IgY batch 1 - 1:500	IgY batch 2 - 1:8000	IgY batch 2 - 1:2000	IgY batch 2 - 1:500	IgY batch 3 - 1:8000	IgY batch 3 - 1:2000	IgY batch 3 - 1:500	IgY batch 4 - 1:8000	IgY batch 4 - 1:2000	IgY batch 4 - 1:500
B												
C												
D												
E												
F												
G												
H												

Figure II.17: Distribution of antibody dilutions on ELISA plate

The plate was shaken (fixed on a microplate shaker) at room temperature for 30 min and washed again three times with Washing buffer. For second antibody incubation a peroxidase labelled anti – IgY antibody was diluted 1:2000 with Assay buffer and 200 µl added to each well. After 30 min incubation shaking at room temperature and a further washing step 200 µl freshly prepared Substrate solution were added to the cavities. The plate was shaken at room temperature under light protection (substrate solution is light sensitive) for 15 min. Then the reaction was completed by adding 50 µl Stop solution to each cavity, and absorption at 450 nm was measured with a microplate reader. Calibration curves for all antibody dilutions were prepared by plotting the average blank-corrected measurement for each standard versus its concentration in µg/ml. Comparing the resulting calibration curves, the most sensitive IgY batches each were selected for further usage.

1.2.3.2 Indirect competitive ELISA

(Buffers and solutions see chapter II.1.2.3.1)

A microplate (high binding property) was coated with 10 µg / ml peanut or hazelnut TBS extract (procedure see chapter II.2.2.1) in coating buffer and incubated covered, overnight at 4 °C. Next day the plate was washed 3 times with Washing Buffer. 300 µl Blocking buffer with 1% BSA were added to each well and the plate was incubated for 1 h at 37 °C.

Meanwhile protein standards were prepared by diluting peanut or hazelnut extract with Assay buffer as follows: pure extract, 1:200, 1:600, 1:1800, 1:5400, 1:16200, 1:32000, blank. Cross-reactivity samples were produced by extracting the substances with TBS (procedure see chapter II.2.2.1). After the plate was washed again three times, 150 µl of the standards and cross-reactivity samples (diluted 1:200 in Assay buffer) were added in triplicates. Immediately after that 50 µl of the diluted IgY (1:20000 in Assay buffer) were pipetted into each well. The plate was incubated for 30 min shaking, at room temperature and washed three times with washing buffer. The peroxidase labelled second antibody (anti – IgY) was diluted 1:2000 with Assay buffer, 200 µl added to each cavity and the plate shaken for another 30 min at room temperature. After a further washing step 200 µl of freshly prepared substrate solution were added per well and the plate incubated covered 15 min shaking, at room temperature. 50 µl Stop solution were pipetted to each cavity, and absorption at 450 nm was measured with a microplate reader. The three OD values of each cross-reactivity sample were averaged and subtracted from the mean blank value. The 1:200 diluted standard was designated 100% and the percentages of the cross-reactivity samples calculated accordingly.

1.2.4 Purification of egg yolk antibodies

For isolation of the IgY antibodies from the egg yolk PEG precipitate, the samples were applied onto a HiTrap™ IgY Purification HP, 5 ml column. This ready to use column consists of a thiophilic adsorption medium with 2-mercaptopyridine coupled to Sepharose™ High Performance. Via thiophilic adsorption [Porath et al, 1985], promoted by water-structuring salts, immunoglobulins were isolated and purificated from chicken egg yolks.

Buffers and solutions

Binding buffer, pH 7.5

NaH_2PO_4 20 mM

K_2SO_4 0.5 M

in dist. H_2O

Elution buffer, pH 7.5

NaH_2PO_4 20 mM

in dist. H_2O

Cleaning buffer, pH 7.5

NaH_2PO_4 20 mM

Isopropanol 30% (v/v)

in dist. H_2O

The column was connected to a FPLC system, with a peristaltic pump a flow rate of 5 ml/min was maintained throughout the whole run, proteins in the effluent were checked with a UV/Vis detector at 280 nm. First the column was washed with 25 ml of each buffer, and then equilibrated with 25 ml Binding buffer. 5 ml sample obtained from the PEG precipitation (chapter II.1.2.2) were applied via a 10 ml sample loop. To remove unbound proteins the column was washed with 100 ml of Binding buffer. After this no more unbound protein was detected in the effluent and the immunoglobulin fraction was eluted with 50 ml of Elution buffer. Fractions of 2.5 ml were collected with an automated fraction collector. The column was regenerated with 50 ml of cleaning buffer and stored at 4 °C for further use. Collected fractions were characterized by SDS-Page (chapter II.2.4) and with BCA Protein Assay Kit (chapter II.2.3).

1.3 Production of colloidal gold – labelled antibodies

1.3.1 Preparation of colloidal gold

Colloidal gold particles were prepared by controlled reduction of gold chloride with sodium citrate using the procedure described by Frens [Frens, 1973].

Buffers and solutions

$HAuCl_4$ 0.01% (w/v) in Milli-Q H_2O

$C_6H_5Na_3O_7$ 1% (w/v) in Milli-Q H_2O

NaN_3 10% (w/v) in Milli-Q H_2O

250 ml of 0.01% Tetrachlorauric[III] acid Trihydrate (Figure II.18/1) were heated to boiling under reflux conditions, then 2.5 ml of 1% tri-Sodium citrate dihydrate were added under constant stirring (Figure II.18/2). After about 25 sec the slightly yellow solution turned blue (Figure II.18/3) and then after approximately 70 sec the blue colour suddenly changed to brilliant red (Figure II.18/4-5), indicating the formation of particles. The solution was allowed to boil for another 5 min to complete the reduction of the gold chloride.

For quality control of the gold particles 1 ml of the gold solution was applied to a plastic cuvette and an UV-spectrum (350 nm – 800 nm) was measured. After addition of 1.25 ml of 10% NaN_3 the gold suspension could be stored at 4°C for approx. 4 weeks.

1 2 3 4 5 6

Figure II.18: Steps in the preparation of colloidal gold

1.3.2 Labelling antibodies with colloidal gold

The minimum amount of protein needed to stabilize the colloidal gold was determined by the procedure of Horisberger [Horisberger and Rosset, 1977].

Buffers and solutions

PEG ***1% (m/v)***

0.2 M K_2CO_3 ***in dist. H_2O***

0.02 M K_2CO_3 ***in dist. H_2O***

NaCl ***10% (m/v) in dist. H_2O***

BSA ***2% and 6% (m/v) in dist H_2O pH 8.5***

NaN_3 ***10% (m/v) in dist H_2O pH 8.5***

Therefore 1% (m/v) of a 1% PEG solution were added to the colloidal gold to protect the electrode when adjusting the pH, then the pH of the gold suspension was adjusted to pH 8.5 with 0.2 M K_2CO_3 and 0.02 M K_2CO_3. 1 ml of gold suspension each was mixed with 100 µl antibody solutions at different concentrations (1 µg/ml –15 µg/ml) and incubated for 2 h, shaking at room temperature. After that 200 µl of 10 % NaCl were added to each reaction and colour reactions observed. The minimum concentration of antibody that did prevent a change of colour from red to blue was determined and further employed in the coupling reaction.

For performance of the coupling reaction the protocol from Verheijen et al [Verheijen et al,1998], was slightly modified. The pH of the gold suspension was adjusted as described before.

The sufficient amount of antibody to give a final concentration of 8 µg per millilitre gold suspension was calculated (usually 50 ml gold suspension were used for one coupling reaction, the concentration of the antibodies was 1-2 mg/ml). For preliminary dilution the calculated amount of antibody was added to 1 ml dist H_2O pH 8.5. Subsequent the antibody dilution was mixed with the gold solutions in a plastic centrifugation tube and incubated for 90 min at room temperature gently swirled on a rock and roll shaking platform. After this time the remaining binding sites were blocked by addition of 2.5 ml 2% BSA and incubation of the mixture as described before for further 90 min. To separate unbound antibody the gold conjugate was centrifuged for 30 min at 8000 x g. The labelled gold particles gathered at the bottom forming a dark red phase, the colourless supernatant was removed and the phase with the gold particles washed with 30 ml dist H_2O pH 8.5. Centrifugation was repeated and supernatant removed again. 400 µl of 6% BSA and 12 µl of 10% NaN_3 were added to the remaining 2 ml of dark red, gold labelled antibody solution. Until further use the reagent was stored at 4 °C.

1.4 Labelling antibodies with fluorescent dye

For labelling of the antibodies two different amine-reactive fluorescent dyes were used, following the manufactures protocol [Invitrogen, 2005]:
Alexa Fluor® 555 carboxylic acid, succinimidyl ester
Alexa Fluor® 647 carboxylic acid, succinimidyl ester

Buffers and solutions

1 M Carbonate buffer, pH 8.3

Solution I: Na_2CO_3 *1 M*
NaCl 0.5 M
in dist H_2O (~pH 11.4)

Solution II: $NaHCO_3$ *1 M*
NaCl 0.5 M
in dist H_2O (~pH 7.9)

Sol. II is titrated with Sol I until pH 8.3 is reached

PBS (10x)

KCl *27 mM*

KH_2PO_4 *15 mM*

NaCl *1.4 M*

Na_2HPO_4 *121 mM*

in dist. H_2O, dilute 1:10 and adjust pH 7.4 before use

NaN_3 ***1% (m/v) in dist H_2O***

For the coupling reaction it is essential that protein solutions are free of any amine-containing substances, hence the antibody has been dialyzed against PBS and thereby brought to a concentration of approx. 3 mg/ml (procedure see chapter II.2.7). 50 µl of the antibody solution were adjusted to a pH between 7.5 – 8.5 by addition of 5 µl Carbonate buffer. 50 µg dye were weighed into a reaction vial, the antibody solution was added and the mixture incubated for 1 h at room temperature with continuous stirring. A SigmaSpin™ Post-Reaction Purification Column was utilized to separate uncoupled dye via size exclusion. Therefore the column was placed in a collection tube and centrifuged for 2 min at 750 x g, the eluate was discarded and the column placed in a new collection tube. The coupling preparation was applied to the center of the column and centrifuged for 4 min at 750 x g.

The eluate (the dyed antibodies) was collected and, after addition of 2.5 µl 1% NaN_3, stored at 4 °C until further use.

2 PROTEINCHEMICAL METHODS

2.1 Preparation of spiked food matrix samples

2.1.1 Preparation of peanut and hazelnut powder

Raw / roasted peanuts and hazelnuts were frozen with liquid nitrogen, covered with icy distilled water and ground one minute using an Ultraturrax mixer. Then the mixture was pressed through a 125 µm sieve, oversized material was discarded and the sieved fraction lyophilised. The resulting powder was stored at -20 °C until further use.

2.1.2 Preparation of CMC-spiking-solution

Because it was desired to produce samples with very low spiking levels (approx. 2 ppm – 40 ppm), it was impossible to weigh these small amounts of nut powder with an analytical balance. Hence, a homogeneous carboxymethylcellulose (CMC) carrier gel with an adequate concentration of nut powder was prepared for spiking [Trucksess et al, 2004].
A 2% CMC-suspension was produced dissolving 12 g carboxymethylcellulose (CMC) in 600 ml distilled water, stirring for approx. 2 h at room temperature. After addition of 300 mg NaN_3 the mixture was further stirred for 30 min. For fabrication of the CMC-spiking-solutions, 50 mg peanut or hazelnut powder were added to 70 g CMC-suspension (0.714 mg nut in 1 ml suspension), and stirred for 4 hours at room temperature. The CMC-spiking-solutions were stored at 4 °C until further use.

2.1.3 Spiking of food matrices

The CMC-spiking-solution was brought to room temperature and stirred again for 1 h before use. Food matrices were homogenized either by melting in a water bath at 40 °C, or by grinding in a kitchen blender, depending on their consistency. The homogenized matrix material was mixed well and aliquots of 1 g weighed into plastic centrifugation tubes. The spiking-solution was diluted 1:10 or 1:5 with PBS buffer (chapter II.1.2.3) and appropriate amounts added to the matrix aliquots with a pipette and mixed well by shaking. If not extracted immediately samples were stored at –20 °C.

2.2 Sample extraction

2.2.1 Standard extraction procedure

Buffers and solutions

TBS Extraction buffer, pH 8.2

Tris *100 mM*
NaCl *1 M*
Tween 20 *1% (v/v)*
in dist. H_2O

TBS Extraction buffer was heated to 60 °C in a water bath. 10 ml of preheated buffer were added to 1 g of homogenized sample (peanut / hazelnut powder, spiked matrix or food sample) in a plastic centrifugation tube. The preparation was mixed well and incubated for 15 min in the water bath at 60 °C. After incubation the tube was shaken once more and centrifuged for 10 min at 8000 x g. With a 10 ml syringe the clear supernatant was detached as complete as possible without dispersing the pellet. To remove possible remaining turbidity supernatant was optionally filtered through 5 µm and 0.45 µm syringe filters if necessary. For utilisation within 5 days extracts were kept at 4 °C, for long time storage they were frozen at –20 °C.

2.2.2 R-Biopharm extraction procedure

Buffers and solutions

RIDASCREEN® Allergen Extraction Buffer

RIDASCREEN® Allergen Extraction Buffer was heated to 60 °C in a water bath. 1 g of skimmed milk powder was added to 1 g of the homogenized sample. 20 ml of preheated buffer were added to the preparation, mixed well and incubated for 10 min in the water bath at 60 °C. After incubation the tube was shaken once more and centrifuged for 10 min at 8000 x g. With a 10 ml syringe the clear supernatant was detached as complete as possible without dispersing the pellet. To remove possible remaining turbidity supernatant was optionally filtered through 5 µm and 0.45 µm syringe filters if necessary. For utilisation within 5 days extracts were kept at 4 °C, for long time storage they were frozen at –20 °C.

2.3 Determination of protein concentration (BCA)

For detection and quantification of total protein the BCA Protein Assay Kit based on a colorimetric reaction with bicinchoninic acid (BCA) was employed. This method combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) with the colorimetric detection of the cuprous cation (Cu^{1+}) using a reagent containing bicinchoninic acid [Smith et al,1985]. A purple-coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/ml).

Buffers and Solutions

BCA Reagent A

BCA Reagent B

Albumin Standard, 2 mg/ml

NaCl 0.9% (m/v)

The Albumin Standard was diluted with 0.9% NaCl to give the following concentrations:
25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 1500 µg/ml, 2000 µg/ml.
Additionally there was a blank standard consisting of 0.9% NaCl. The protein content of the sample was estimated and sample diluted with 0.9% NaCl in an adequate manner to fit in the working range. To minimize the required sample volume and allow a larger number of samples to be analyzed, a microplate (non-protein-binding property) was used as reaction vessel. 20 µl of each standard and unknown sample were applied to the wells of the microplate in triplicates. The BCA working reagent was prepared by mixing 20 ml of BCA Reagent A with 0.4 ml of BCA Reagent B. Then 200 µl of the working reagent were added to each well and the plate was gently shaken for approx. 30 s. The covered plate was incubated at 37 °C for 30 min. After the incubation step absorbance at 562 nm was measured on a microplate reader.
A calibration curve was prepared by plotting the average blank-corrected measurement for each standard versus its concentration in µg/ml. This calibration curve was used to determine the protein concentration of each unknown sample.

2.4 SDS-PAGE

The principle of SDS polyacrylamide gel electrophoresis (SDS-PAGE) is the separation of proteins based on their molecular weight. Sodium dodecylsulfate (SDS) is an anionic detergent that binds to the polypeptide backbone, thus conferring a negative charge to the protein that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. After visualization through a protein-specific staining technique the size of a protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight.

Buffers and Solutions

MES running buffer (20x)

MES	*1 M*
Tris	*1 M*
SDS	*69 mM*
EDTA	*21 mM*

in dist. H_2O, dilute 1:20 before use

Sample buffer (2x)

dist. H_2O	*3.8 ml*
0.5 M Tris-HCl, pH 6.8	*1 ml*
Glycerol	*0.8 ml*
10% (w/v) SDS	*1.6 ml*
0.5% (w/v) bromophenol blue	*0.4 ml*

SeeBlue® Plus2 Pre-Stained Standard

SDS-PAGE was conducted with the NuPAGE® Gel System (Invitrogen).
Pre-Cast 12% NuPAGE® Bis-Tris Gels for small to mid-size molecular weight proteins together with a XCell SureLock™ Mini-Cell electrophoresis unit were used. Pre-Cast gel, buffer core and buffer dam were placed in the in the electrophoresis unit and fixed with the gel tension wedge (see Fig. II.4). Then the buffer chamber was filled with approx. 600 ml MES running buffer, until the gel slots were submersed. 5 µl of protein sample were mixed with 5 µl of Sample buffer, samples with very high protein contents were diluted 1:4 in dist. H_2O prior to that. 10 µl of each sample and of a SeeBlue® Plus2 Pre-Stained Standard were pipetted into the slots. When finished the cell lid was placed on the unit and a power supply was connected.

The electrophoretic run was performed with the parameters 200 V / 400 mA set at 4 °C for 50 min. After the run, the gel was removed from its plastic shell and subjected either to a dyeing (procedure chapter II. 2.5) or blotting procedure (procedure chapter II.2.6).

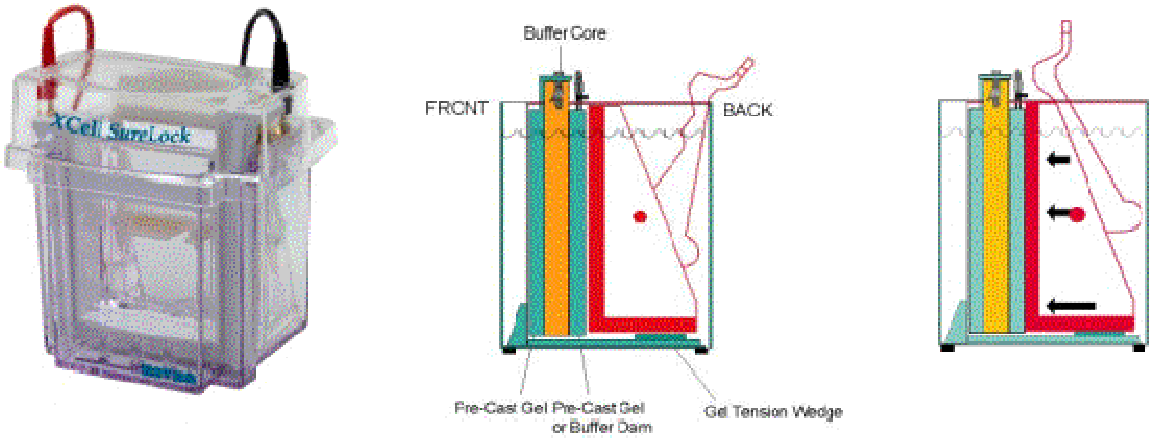


Figure II.19: Schema of the XCell SureLock™ Mini-Cell electrophoresis unit (© invitrogen)

2.5 Coomassie stain

Coomassie Brilliant Blue is a dye routinely use for visualizing protein bands on polyacrylamide gels [Neuhoff et al, 1985]. Coomassie blue binds non-specifically and stoichiometrically to virtually all proteins via physisoprtion. The used product SimplyBlue™ SafeStain constists of Coomassie® G-250, a very common type of stain.

Buffers and solutions

SimplyBlue™ SafeStain

Destain

NaCl *20% (w/v)*
in dist. H_2O

The gel was rinsed 3 times for 5 minutes with 100 ml distilled water to remove SDS and buffer salts, which interfere with binding of the dye to the protein. Then the gel was immersed in SimplyBlue™ SafeStain (~20 ml) and incubated for 1 hour at room temperature with gentle shaking. After that, the stain was discarded and the gel was rinsed 3 times for 5 minutes with 100 ml distilled water to remove redundant stain. To achieve maximum sensitivity and clear background the gel was immersed in Destain and incubated over night at room temperature with gentle shaking. Protein pattern was documented by scanning the gel with a flat bed scanner.

2.6 Western Blot

Western blotting is an analytical technology for the electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets [Towbin et al, 1979]. After SDS conferred a negative charge to proteins during the polyacrylamide electrophoresis procedure (chapter II.2.4), the proteins migrate from the gel via the nitrocellulose membrane to the anode when voltage is applied.
In the immunoblotting step the proteins immobilized on the membrane can then be probed with a primary antibodiy directed against a protein of interest. Detection with a secondary antibody can be conducted colorimetric, fluorescent or via radioactive enhancement.

Buffers and Solutions

Blotting buffer stock (10x)

Glycin	*1.9 M*
Tris	*143 mM*
in dist. H_2O	

Blotting buffer, pH 8.3

dist. H_2O	*720 ml*
Methanol	*200 ml*
Blotting buffer stock	*80 ml*

0.2 % Ponceau stain

Ponceau S	*2.6 mM*
Trichloracetic acid	*3% (v/v)*
in dist. H_2O	

The freshly-run SDS-PAGE gel, two pieces of filter paper and a nitrocellulose membrane were immersed in Blotting buffer. The nitrocellulose membrane was placed on a piece of filter paper, followed by the SDS-PAGE gel and one more filter paper on top of that. Air bubbles between the nitrocellulose and the gel were removed by rolling with a pasteur-pipette over the gel in all directions. The so formed blotting sandwich was placed between two foam sponges and put into a plastic cassette. The cassette was placed into a HOEFER Mighty small transfer electrophoresis unit filled with Blotting buffer in such an orientation, that the nitrocellulose was located between the SDS-PAGE gel and the anode so that proteins from the gel could migrate towards the membrane. The transfer tank was closed and connected to a power supply. Parameters for the transfer run were 400 V / 200 mA, for 90 min at 4 °C. Following the electrophoretic run the nitrocellulose membrane was removed and rinsed with dist. H_2O.

For visualization of the transferred protein pattern the membrane was immersed in 0.2% Ponceau stain and incubated for 30 min, gently shaking, at room temperature.
After the staining procedure the membrane was rinse shortly with dist. H_2O to remove redundant dye. Protein pattern was recorded using a flat bed scanner. Accordingly the membrane was cut into single strips with one protein lane each. Then the strips were placed in plastic incubation trays and completely destained by incubation in 1ml dist. H_2O each for 1 h shaking gently at room temperature.

Immunoblotting

(Buffers and solutions see chapter II.1.2.3.1)

The nitrocellulose strips were blocked in the incubation trays over night, shaking in 1ml Blocking buffer with 5% skimmed milk powder each at 4 °C. Next day strips were washed shaking 3 x 5 min in 1 ml Washing buffer. Primary antibodies (anti-peanut / hazelnut mAb or IgY) were initially diluted 1:500 in Assay buffer (in further experiments the dilution factor was adjusted individually for each antibody) and the strips incubated with 1 ml of the dilutions each for 30 min shaking at room temperature. After that, another washing step was performed as described before. The respective species-specific, alkaline phosphatase coupled, secondary antibodies were diluted 1:1000 in Assay buffer and strips incubated with 1 ml of the dilutions each, 30 min shaking at room temperature. A further washing step was performed. A substrate for the detection of alkaline phosphatase was prepared by dissolving SIGMA *FAST* BCIP/NBT Buffered Substrate Tablets in 20 ml dist. H_2O. Strips were incubated with 1 ml of substrate solution each for 15 min shaking, at room temperature. The reaction was terminated by washing the strips with dist. H_2O and protein bands documented by scanning the dried strips in a flat bed scanner.

2.7 Dialysis, concentration and ultrafiltration

Centricon Centrifugal Filter Devices YM-10 (10000 MW cut-off) and YM-50 (50000 MW cut-off) were used for dialysis, concentration and ultrafiltration.
Before use the devices where prerinsed, therefore 1 ml of dist. H_2O was applied to the sample reservoir and the device centrifuged for 10 min at 3000 x g, the filtrate was discarded. Then the sample was applied (2 ml maximum volume) and the device centrifuged at 3000 x g. To collect the retentate as complete as possible filtrate vial was removed, retentate vial was placed over sample reservoir and the whole was centrifuged inverted for 2 min at 300 x g.

For dialysis the described procedure was repeated three times, applying 1 ml of dialysis buffer to the sample reservoir after each centrifugation step.

For concentration of a sample centrifugation was performed (as described before) until the sample was reduced to the desired volume.

Performing ultrafiltration with Centricon Devices separation of substances according to the nominal molecular weight limits (10000 MW and 50000 MW) was conducted.

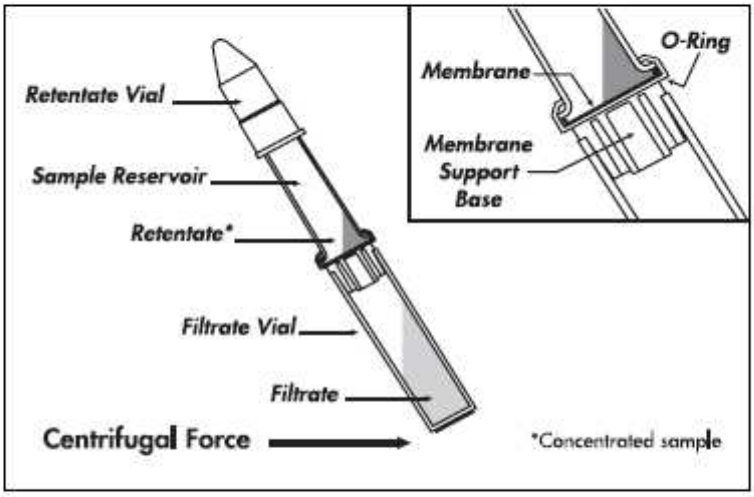


Figure II.20: Centricon Centrifugal Filter Device (© millipore)

3 IMMUNOASSAYS

3.1 Sandwich dipstick with colloidal gold labelled antibodies

Buffers and Solutions

TBS, pH 7.5

Tris *20 mM*

NaCl *1 M*

in dist. H_2O

Blocking buffer, pH 7.5

(see chapter II.1.2.3)

For easier handling and to minimize the reaction volume of the dipstick during the time of development, incubation was carried out in a 24 well polystyrene plate, therefore nitrocellulose membrane was cut into squares of 1 cm x 1 cm. The capture antibody was diluted with TBS to give a concentration of 1 mg/ml. 1 µl of the capture antibody dilution was applied to a nitrocellulose squares with a pipette. The membrane was allowed to dry for approx. 10 min at room temperature and subsequently placed in the 24 well plate. 500 µl Blocking buffer with 5% BSA were added and membrane incubated for 90 min, gently swirled on a rock and roll shaking platform at room temperature. After blocking the membrane was washed for 10 sec under running tap water. 300 µl of sample was added and membrane incubated for 15 min gently swirling on a rock and roll shaking platform at room temperature. Membrane was again washed for 10 sec under running tap water. The gold coupled detection antibody was diluted 1:10 with TBS, 300 µl of the antibody dilution was added to the membrane which was then incubated for 10 min as described before. Finally the membrane was shortly washed once more and a positive signal was indicated as visible red coloured dot. Results were documented by scanning the membrane with a flat bed scanner.

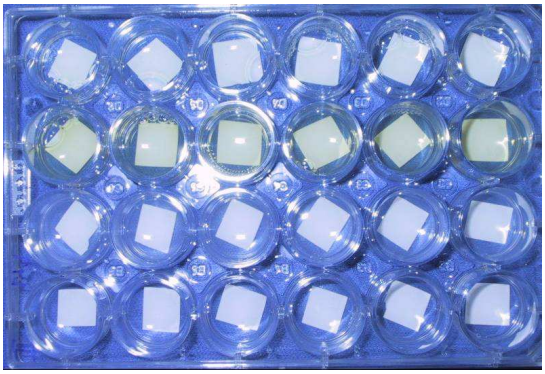


Figure II.21: Gold dipstick sample incubation

3.2 Microarray with fluorescence labelled antibodies

The practical work on microarrays was carried out by Dr. Markus Janotta, University of Applied Science Tulln.

Buffers and Solutions

Genetix spotting solution

Genetix blocking buffer

PBST, pH 7.4

PBS (see chapter II.1.4)
Tween 20 0.1% (w/v)

Microarrays were performed on Genetix aldehyde modified slides. To allow incubation with different samples at the same time, the slides were covered with a bonding sheet with 24 round clearances (∅ 4.4 mm) to generate individual reaction wells.

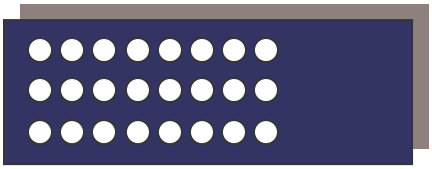


Figure II.22: Schema of a prepared slide

The capture antibody was diluted with Genetix spotting solution to give a concentration of 1 mg/ml. The capture antibody solution was spotted on the aldehyde slides at 60% relative humidity, with a QArrayMini microarrayer using aQu High Precision SPLIT Microarray Pins (Genetix). Up to 100 dots with a diameter of 180-220 µm were spotted into one reaction well. Slides were placed for 1 h in an 60% relative humidity environment and then incubated overnight at 4 °C with 10 µl Genetix Blocking Buffer / 1% skimmed milk powder per well. Next day blocking buffer was removed and the slides were incubated with 10 µl sample per well, for 90 min at room temperature. Washing was performed with PBST, therefore the wells were rinsed briefly first and then incubated with PBST shaking for approx. 10 sec, after that wells were rinsed once with PBS and once with dist H_2O. Fluorescence labelled antibody was diluted 1:100 in PBS. 10 µl of the detection antibody dilution were added per well and slide incubated for 90 min at room temperature. After a further washing procedure (as described above) the slides were dried with compressed air and fluorescence measured with a GenePix® Personal 4100A microarray scanner under following conditions:

Table II.4: Conditions for measurement with the GenePix® Personal 4100A microarray scanner

fluorophores	laser excitation	emission filter	PMT gain	pixel size
Alexa Fluor® 555	532 nm	550 – 600 nm	700	20 µm
Alexa Fluor® 647	635 nm	655 – 695 nm		

All data was analysed with GenePix Pro 5.1 software.

4 COLLABORATIVE TRIAL

The collaborative trial was carried out for external validation of the new ELISA and LFD test kits developed at R-Biopharm and CSL respectively.

4.1 Peanut ELISA

Buffers and solutions
Apart from the specific antisera buffers and solutions were the same as for the commercial RIDASCREEN FAST Peanut Enzyme immunoassay (R-Biopharm).

Design of the peanut ELISA:
For the peanut ELISA in sandwich format, polyclonal rabbit antibodies were used as capture and detection antibody. Detection antibodies were conjugated with horseradish peroxidase. The calibrants were prepared extracting (procedure see chapter II.2.2.2) a mix of 12 different peanut varieties, each raw and roasted.

Test procedure:
Samples were prepared as described in chapter II.2.2.2. 100 µl of samples and standards each were added to the coated antibody wells. After 10 minutes incubation at room temperature, wells were washed 3 times with 250 µl Washing buffer. Peroxidase conjugated antibody was added and plate incubated for further 10 minutes. Subsequent to a final washing step, Substrate solution was added and the wells incubated for 10 minutes, prior to halting the reaction by addition of Stop solution (1N H_2SO_4). The absorbance of individual wells was determined at 450 nm within 30 minutes using a microplate reader. A calibration curve was prepared by plotting the blank-corrected 450 nm measurement for each standard versus its concentration in mg/kg peanut extract. This calibration curve was used to determine the peanut concentration (mg/kg) of each unknown sample.

Internal validation:
The performance characteristics of the test were determined by the co-ordinating laboratory (R-Biopharm) as follows:
Serial dilutions of peanut containing extracts from dark chocolate, milk chocolate, cookie and cereal samples were measured with the laboratory-based peanut ELISA to define the internal assay validation data (LOD, LOQ, within assay variance, between assay variance).

4.2 Hazelnut ELISA

Buffers and solutions

Apart from the specific antisera buffers and solutions were the same as for the commercial RIDASCREEN FAST Hazelnut Enzyme immunoassay (R-Biopharm).

Design of the hazelnut ELISA:

For the hazelnut ELISA in sandwich format, polyclonal rabbit antibodies were used as capture and detection antibody. Detection antibodies were conjugated with horseradish peroxidase. The calibrants were prepared extracting (procedure see chapter II.2.2.2) a mix of 12 different roasted hazelnut species.

Test procedure:

The test procedure for the hazelnut ELISA was the same as for the peanut ELISA, described before. Only the washing steps were carried out slightly different: wells were washed 5 times (instead of 3 times) with 250 µl Washing buffer each.

Internal validation:

The performance characteristics of the test were determined by the co-ordinating laboratory (R-Biopharm) in the same way as for the peanut ELISA.

4.3 Lateral Flow Devices for peanut and hazelnut

Design of the lateral flow devices

For the Lateral Flow Devices in sandwich format monoclonal antibodies were used as capture and detection antibody. Detection antibodies were conjugated to blue dyed latex particles.

Test procedure:

Samples were prepared as described in chapter II.2.2.2. 80 µl of extracted sample was applied into the sample well of the Lateral Flow Device.

Sample flow across the viewing window was visible. Result was determined reading the control line and test line visually after 15 min as positive (+) or negative (-), for a schema of interpretation see Table II.5

Table II.5: Interpretation of LFD results

control line	test line	interpretation
-	-	invalid assay
-	+	invalid assay
+	-	negative result
+	+	positive result

Internal validation:

The performance characteristics of the tests were determined by the coordinating laboratory (CSL), as follows: Serial dilutions of peanut and hazelnut containing extracts and buffer blanks were measured with the LFDs and also with the developed ELISAs to obtain reference values. At each concentration level 10 independent replicates were measured with the LFDs. The lowest concentration where 10 out of 10 samples were determined positive was defined as limit of detection according to the guidelines for qualitative measurements described in EURACHEM Guide 1998.

4.4 Sample preparation

Food matrices were chosen according to the probability of possible contaminations with peanut / hazelnut in a real market situation. Confectionary products were included mainly because of their high frequency of contamination. Salami was also included in the test set up due to a hazelnut containing salami produced by a commercial supplier in Germany.

Because of the number and complexity of the selected food matrices it was decided to use commercial products as “blank” matrices. All “blank” food matrices were purchased in local food stores in Austria. These included: butter cookies, vanilla ice cream, salami, salad dressing containing herbs, instant soup, cornflakes and plain yogurt. The only matrix for which no “blank” material was available (conc. > 1.5 mg/kg) was dark chocolate. Therefore a batch of 1 kg blank dark chocolate was produced in-house and used as blank and subsequently for spiked samples. The “blank” samples were checked for the absence of peanut / hazelnut, therefore material was homogenized, extracted (procedure see chapter II.2.2.2) and 2 extracts each measured in duplicate, on commercially available peanut and hazelnut ELISA (RIDASCREEN® FAST Peanut ELISA, RIDASCREEN® FAST Hazelnut ELISA, R-Biopharm).

After homogenisation each sample aliquot (1 g of material) was spiked individually rather than to prepare self made peanut/ hazelnut containing products as it is usually conducted for the production of reference material. This procedure should ensure a higher accuracy of spiking value and better homogeneity of the hazelnut proteins in the sample.
Since only roasted peanuts / hazelnut are employed in food production, the material used for spiking was roasted peanut / hazelnut powder only (production described in chapter 2.1.1).
Spiking was carried out as described in chapter II.2.1. Thereby samples with the following spiking concentrations of whole milled peanut and hazelnut powder in mg/kg food matrix were obtained:
cookies: 5 mg/kg, salami: 8 mg/kg, instant soup: 15 mg/kg, cornflakes: 35 mg/kg,
yogurt: 6 mg/kg.

Some samples were spiked alternatively:
Ice cream containing 20 mg/kg peanut was prepared by weighing the required amount of spiking solution to 500 g of the blank vanilla ice cream, stirring for approx. 2 hours.
Ice cream containing 20 mg/kg hazelnut was prepared by adding appropriate amounts of a commercial hazelnut ice cream (purchased in a food store in Germany, labelled as containing 1% hazelnut) to 500 g of the blank vanilla ice cream and stirred for approx. 2 hours.

A true blank dark chocolate was prepared in house, stirring the following ingredients at 40 °C in a kitchen blender: 28.13% cocoa powder, 45% sugar, 26.88% cocoa butter, 0.5% lecithin.
Dark chocolate, containing 2.5 mg/kg hazelnut was prepared by mixing the melted dark chocolate blank with the required amount of a melted dark chocolate containing 74 mg/kg milled hazelnut powder, for 4 hours at 40 °C. Dark chocolate containing 10 mg/kg hazelnut was prepared following the same procedure.

Another set of samples was provided by one of the project partners (R-Biopharm):
milk chocolate with 10 mg/kg, milk chocolate with 40 mg/kg, dark chocolate with 10 mg/kg and dark chocolate with 40 mg/kg whole peanut (samples were produced in a food pilot plant spiking blank chocolate with a peanut paste).

A range of different peanut and hazelnut spiking values was chosen (2.5 – 35 mg/kg) to check the performance of the tests over the whole working range, additionally blank samples of dark chocolate, cookies and ice cream were included. In total there were 25 sets of different samples. For a complete list of samples see Table II.3.
All samples were stored at –20 °C until extraction by the participants in the collaborative trial (for approx. 4 weeks).

Table II.6: Complete list of samples for the collaborative trial

matrix	spiking value mg/kg	destination of material
milk chocolate	blank	R-Biopharm
milk chocolate	10 peanut	R-Biopharm
milk chocolate	40 peanut	R-Biopharm
dark chocolate	blank	R-Biopharm
dark chocolate	10 peanut	R-Biopharm
dark chocolate	40 peanut	R-Biopharm
dark chocolate	blank	self-made
dark chocolate	2.5 hazelnut	self-made
dark chocolate	10 hazelnut	self-made
cookie	blank	food store
cookie	5 peanut	self-made
cookie	5 hazelnut	self-made
ice cream	blank	food store
ice cream	20 peanut	self-made
ice cream	20 hazelnut	self-made
salami	8 peanut	self-made
salami	8 hazelnut	self-made
salad dressing	4 peanut	self-made
salad dressing	4 hazelnut	self-made
instant soup	15 peanut	self-made
instant soup	15 hazelnut	self-made
cornflakes	35 peanut	self-made
cornflakes	35 hazelnut	self-made
yogurt	6 peanut	self-made
yogurt	6 hazelnut	self-made

4.5 Homogeneity study

To control the spiking values of the in-house spiked sample sets and to assess the material homogeneity control measurements with the RIDASCREEN FAST peanut and hazelnut ELISA tests (R-Biopharm) were carried out. For the individually spiked samples, 5 aliquots were taken randomly from each sample set (consisting of 50 plastic centrifugation tubes, containing 1g of spiked material each). For the alternatively spiked samples ice cream and dark chocolate 5 aliquots of 1 g each were taken randomly from the spiked sample sets (consisting of 500 mg spiked material). Samples were extracted (procedure see chapter II.2.2.2) and diluted with RIDASCREEN® Allergen Extraction Buffer to fit into the working range, if necessary. Each extract was measured in duplicate on two different ELISA plates: peanut samples on peanut ELISA (procedure see chapter II.4.1), hazelnut samples on hazelnut ELISA (procedure see chapter II.4.2).

The obtained nut concentrations in the test samples in mg/kg were subjected to a statistical analysis of variances (ANOVA) [Sachs, 1992]. To determine if a set of sample is homogeneous the calculated F-value was compared with the critical tabular F_{max}-value, if the calculated F-value was below the critical F-value the sample set was regarded as being homogeneous. For calculation of the statistical values see chapter II.4.8.

4.6 Workflow of the collaborative trial

Eight laboratories from five EU-member states (Austria, Germany, Italy, Netherlands, U.K.) participated in the collaborative trial.

Every participant received a whole set of samples, sent at 4°C via air express with 24 h delivery from the coordinator of the collaborative trial. The required number of test kits were sent to the participants directly from the respective project partner (ELISA kits from r-Biopharm, LFD kits from CSL). Additionally, every participant obtained a detailed instruction protocol to make sure that the measurements were carried out in conformity, as well as report forms for ELISA and LFD results. Samples were supplied with numerical codes in a way that the composition of the individual samples was unknown to the participants.

Chocolate and ice cream samples were portioned 10 g each, participants were required to weigh out two aliquots of 1 g each. All other samples were ready to use for extraction in 50-ml centrifugation tubes, 1 g each, participants received 2 aliquots of each of these. Finally, each laboratory had to analyse 2 lots of 25 different samples. First all samples were extracted according to the procedure described in chapter II.2.2.2.

Then each sample was measured in duplicate using peanut and hazelnut ELISA, for some samples a dilution step with RIDASCREEN® Allergen Extraction Buffer was indicated to reach concentrations within the working range.

In addition to the calculated final results, the participants were requested to return the original absorbance readings of the micro-plate reader of all samples and standard solutions to the coordinator of the collaborative trial.

Finally the undiluted extracts of all the samples were measured in duplicate using peanut LFD and hazelnut LFD. The participants were asked to comment the visual readings as + (for a positive result) and – (for a negative result).

4.7 Evaluation of results

4.7.1 Evaluation of ELISA results

The mean of the duplicate measurements of each sample was calculated. Only values within the working range (2.5 mg/kg – 20 mg/kg) were incorporated in the further evaluation.

Elimination of outliers

Elimination of outliers was achieved by applying the Hampel outlier test (Huber´s method). This test is recommended for statistical evaluation of interlaboratory comparison tests by Davies [Davies, 1988], advantages of this method are the easy performance procedure and the applicability for a small number of values.

The test was carried out performing the following steps:

1. Calculation of the median xm of all results xi. xi ranging from x1 to xn (potential outliers included)
2. Calculation of absolute residuals |ri| of single values xi from median xm: |ri| = |xi-xm|
3. Calculation of the median of absolute residuals rm.
4. A value was considered to be an outlier if the absolute residuals |ri| of the suspected value was 1,483*(1+1,9/(total number of results-0,8)^1,2)*3 times larger than rm. This factor corresponds to calling those observations outliers which are 3 times larger than the standard deviation from the mean.

Statistical evaluation

The spiking concentration based on the added volume of the gravimetrically prepared spiking solution was defined as assigned target value for each sample [ISO/DIS 13528, 2002].

For the statistical evaluation following characteristics were worked out (for calculations see chapter II.4.8):

RSD_r intra laboratory relative standard deviation

RSD_R inter laboratory relative deviation

Recovery

4.7.2 Evaluation of LFD results

The number of positive and negative results per sample was evaluated.

Statistical analysis was carried out by a project partner (CSL).

The **sensitivity**, the percentage of samples that were correctly identified as positive and the **specificity**, the proportion of samples correctly identified as negative were determined.

Also two parameters that can be regarded as analogous quantities to repeatability and reproducibility for qualitative data, first described by Langton et al, were calculated [Langton et al, 2002]:

To assess within-laboratory repeatability, **accordance** measures were computed.

Accordance is the percentage chance that two samples sent to the same laboratory under standard repeatability conditions will both give the same result. It was calculated by averaging the probability that two samples will give the same result over all laboratories.

Inter-laboratory reproducibility was measured by **concordance**. This is the percentage chance that two samples sent to different laboratories will both give the same result.

Confidence intervals have been created using standard methods for binomial data

4.8 Statistical calculations and formulas

Key:

X	value
N	total number of values
k	number of groups
n	number of values in a group
T	sum of values in a group
m	arithmetic mean
df	degree of freedom
SS	sum of squares
MS	mean square (variance)
F	variance ratio
RSD_r	intra laboratory relative standard deviation
RSD_R	inter laboratory relative standard deviation

Formulas:

$m = \text{Sum } X / N$

$df\ (\text{total}) = N - 1$

$df\ (\text{inter}) = k - 1$

$df\ (\text{intra}) = df\ (\text{total}) - df\ (\text{inter})$

$SS\ (\text{total}) = \text{Sum } X^2 - (\text{Sum } X)^2 / N$

$SS\ (\text{inter}) = \text{Sum } T^2 / n - (\text{Sum } X)^2 / N \qquad (\text{Sum } T^2 = T_1^2 + T_2^2 + T_n^2)$

$SS\ (\text{intra}) = SS\ (\text{total}) - SS\ (\text{inter})$

$MS\ (\text{inter}) = SS\ (\text{inter}) / df\ (\text{inter})$

MS (intra) = SS (intra) / df (intra)

F = MS (inter) / MS (intra)

$RSD_r = \sqrt{}$ MS (intra)

$RSD_R = \sqrt{}$ MS (inter)

Recovery (%) = added mg/kg nut / measured mg/kg nut * 100

5 FOOD SURVEY

The consumer agencies of the 11 participating countries composed sets of approx. 40 food products, consisting of the 6 different categories (milk chocolate, dark chocolate, cookies, cereals, ice cream, yogurt).

Each participating laboratory was responsible for the samples of 1 or 2 countries. The consumer agencies disposed the samples directly to the corresponding laboratory via air express at 4 °C.

Table II.7: Distribution of samples in the food survey

laboratory	country	no of samples	kind of samples
IFA	Austria	40	milk chocolate, cookies, cereals, yoghurt
	Norway	40	milk chocolate, cookies, cereals, ice cream
RIKILT	Belgium	40	milk chocolate, dark chocolate, cookies, cereals
	France	41	milk chocolate, dark chocolate, cookies, cereals, ice cream, yogurt
CLS	Portugal	40	milk chocolate, dark chocolate, cookies, cereals
	Spain	40	cookies, cereals, ice cream, yogurt
TUM	Greece	35	milk chocolate, dark chocolate, cookies, cereals
	Slovenia	43	milk chocolate, dark chocolate, cookies, cereals, ice cream, yogurt
R-Bio	Czech Republic	40	milk chocolate, cookies, cereals
UNIMI	Italy	40	milk chocolate, dark chocolate, cookies, cereals, ice cream
CMMC	U.K.	40	milk chocolate, cookies, yogurt

Participants homogenized each food product as a whole, according to its consistency, either with a kitchen blender, or in a water bath at 40 °C. Material was mixed well, an aliquot of 1 g was taken and extracted as described in chapter II.2.2.2. The extracts were measured undiluted in duplicates using peanut and hazelnut ELISA (procedure see chapter II.4.1 and chapter II.4.2). The raw data (OD values) for all samples and standard solutions were sent to the coordinator of the Food Survey. Concentrations of peanut and hazelnut in the food products (mg/kg) were read from calibration curves calculated with RIDAWIN® Software.

Additionally all extracts were measured undiluted in duplicates using peanut and hazelnut LFD (procedure see chapter II.4.3). Results were determined after 10 min by visual interpretation and sent to the coordinator of the Food Survey.

6 EQUIPMENT AND REAGENTS

6.1 Laboratory equipment

name	type	supplier
Aldehyde modified slide		Genetix
Centricon; 10 kDa, 2 mL	YM-10	Millipore
Centricon; 50 kDa, 2 mL	YM-50	Millipore
Centrifuge	Avanti™ 30	Beckman
Centrifuge	J2-MI	Beckman
Centrifuge beaker	250 mL	Beckman
Electrophoresis Power Supply	600	amersham pharmacia
Electrophoresis unit	XCell SureLock™ Mini-Cell	Invitrogen
Filter paper	Gel-Blotting-Paper GB003	Schleicher & Schuell
FPLC controller	LCC-501 Plus	amersham pharmacia
FPLC fraction collector	Frac-100	amersham pharmacia
FPLC pump	LKB P-500	amersham pharmacia
FPLC software	OS/2 Warp	amersham pharmacia
Heating Mantle	ISOPAD Typ GSB 250 ml	Heraeus
HiTrap™ IgY Purification HP	Column 5 ml	amersham pharmacia
Incubator	Kelvitron t	Heraeus Instruments
Kitchen blender	Bosch universal	Bosch
Magnetic stirrer	IKAMAG RET-G	IKA
Magnetic stirring bar	15 x 4.5 mm	VWR
Magnetic stirring bar	40 x 8 mm.	VWR
Magnetic stirring bar	60 x 9 mm	VWR
Microarray scanner	GenePix® Personal 4100A	Molecular Devices
Microarray Split Pins	aQu High Precision 150 µm	Genetix
Microplate reader (photometer)	Spectra II	SLT Labinstruments
Microplate reader (photometer)	Sunrise Remote Control	TECAN
Microplate shaker	MTS 4	IKA
Microplate washer	Columbus	SLT Labinstruments
Microplates (high binding property)	ELISA-Plate, Microlon, 96 W	greiner bio-one
Microplates (non binding property)	ELISA-Plate, Microlon, 96 W	greiner bio-one
MilliQ H_2O generation system	MilliQ Plus PF	Millipore
Nitrocellulose membrane, 0.45 µm	Protran BA 85	Schleicher & Schuell
NuPAGE 12% Bis-Tris-Gel		Invitrogen
pH electrode	SenTix® 21	WTW
pH meter	pH 537	WTW
Pipette 0.5-10 µl		Eppendorf
Pipette 100-1000 µl	Eppendorf research	Eppendorf
Pipette 10-100 µl	Eppendorf research	Eppendorf
Pipette 25 - 250 µL	8-fold	Micronic
Pipette 5-5000 µl	Acura® 831	Socorex
Pipette Tips 25-250 µL	Precision Tip	Micronic
Pipette Tips 10 µl	white	greiner bio-one
Pipette Tips 100 µl	yellow	greiner bio-one
Pipette Tips 1000 µl	blue	greiner bio-one
Pipette Tips 5000 µl	Proline	Biohit
Plastic centrifugation tube	15 mL	Sarstedt
Plastic centrifugation tube	50 mL	Sarstedt
Plastic cuvette	1.5 ml semi-micro PS	Plastibrand
Post Reation Clean-Up Columns	SigmaSpin S-5059	Sigma Aldrich
PS Multiwell Plate	24 well, hydrophobic surface	greiner bio-one
QArrayMini microarrayer		Genetix
Reaction vial 1 ml	Microcentrifugation tube	greiner bio-one
Reaction vial 2 ml	Mµlti Dolphin	Roth

name	type	supplier
Ridasoft Win	V 1.22	R-Biopharm
Rock and roll shaking platform	Red Rotor	Hoefer Scientific Instruments
Software	BIOLISE V 2.0 Rev 15	BIOLISE
Software	Magellan V 3.0	TECAN
Software	GenePix Pro 5.1	Molecular Devices
Syringe filter; 0.45 µm	30/0,45 RC-GF51	Wagner Munz
Syringe filter; 5 µm	FP30/5,0 CN	Wagner Munz
Transphor Electrophoresis Unit	TE-22	Hoefer Scientific Instruments
Ultraturrax	T50	IKA
UV detector	LKB Control unit UV 1	amersham pharmacia
UV/Vis Spectrometer	Lambda 16	Perkin Elmer
Vortex	MS 1 Minishaker	IKA
Water bath		GFL

6.2 Reagents

substance	supplier
Alexa Fluor® 555 carboxylic acid, succinimidyl ester	Invitrogen
Alexa Fluor® 647 carboxylic acid, succinimidyl ester	Invitrogen
Anti Chicken IgY from rabbit AP conjugated	Jackson Immuno Research
Anti Mouse IgG from sheep, AP conjugated	Sigma-Aldrich
BCA Albumin Standard Ampules	Pierce
BCA REAGENT A, 1000 mL	Pierce
BCA REAGENT A, 250 mL	Pierce
BCA REAGENT B	Pierce
Blocking buffer	Genetix
Bromophenol Blue	Fluka
BSA (Bovine serum albumin)	Sigma-Aldrich
Carboxymethylcellulose Sodium Salt	Sigma
Citric acid monohydrate	Merck
Dimethyl sulfoxide	Merck
Di-sodium hydrogen phosphate dihydrate	Merck
EDTA (Ethylendiaminetetraacetic acid)	Sigma
Ethanol	Baker
Ethylenglycol	Merck
Extra Feine Kartoffel Steinpilz Suppe	Maggi
FAST BCIP/NBT Buffered Substrate Tablets	Sigma-Aldrich
Glycerol	Merck
Glycin	Merck
Isopropanol	Baker
MES (2-[N-Morpholino]ethanesulfonic acid)	Sigma
Methanol	Baker
Perhydrol® 30% H_2O_2	Merck
Polyethylene glycol MW 8.000	Sigma-Aldrich
Ponceau S	Merck
Potassium carbonate anhydrous	Fluka
Potassium chloride	Merck
Potassium dihydrogen phoshpate anhydrous	Serva
Potassium sulfate	Merck
Rabbit anti-Chicken-IgG peroxidase conjugate	VWR
RIDASCREEN® Allergen Extraction Buffer	R-Biopharm
RIDASCREEN® Allergen Stop Solution	R-Biopharm
RIDASCREEN® Allergen Substrate Solution	R-Biopharm
RIDASCREEN® Allergen Washing Buffer	R-Biopharm
SDS (Sodium dodecylsulphate)	Carl Roth GmbH & Co
See Blue Pre-Stained Standard	Invitrogen
Simply Blue™ SafeStain	Invitrogen
Skim milk powder	Merck
Sodium azide	Merck
Sodium carbonate anhydrous	Merck
Sodium chloride	Baker
Sodium dihydrogen phosphate monohydrate	Merck
Sodium hydrogen carbonate	Baker
Sorbic acid (Potassium salt)	Sigma
Spotting Solution	Genetix
Sulfuric acid 95-97%	Merck
TEMED (3,3´,5,5´-Tetramethyl benzidine)	Boehringer Mannheim
Tetrachlorauric[III] acid Trihydrate	Sigma-Aldrich
Trichloracetic acid solution 100%	Sigma Diagnostics
Tris (Tris(Hydroxymethyl)aminomethan)	Merck
tri-Sodium citrate dihydrate	Merck
Tween 20 (Polyoxyethylene sorbitan monolaureate)	Sigma-Aldrich

6.3 Food material

matrix	product	supplier
Raw hazelnuts		Masterfood
Raw peanuts		Masterfood
Cookies	Leibniz Butterkeks	Bahlsen
Cookies	Leibniz Vollkornkeks	Bahlsen
Cornflakes	Clever Cornflakes	Delikatessa GmbH
Hazelnut ice cream	Vanille-Haselnuß-ÖKO-Eiscreme	Rewe-Handelsgruppe
Ice cream	Vanillepflanzenfetteis	Eskimo-IGLO
Instant soup	Extra Feine Kartoffel Steinpilz Suppe	Maggi
Instant soup	Rindessa Feine Rindsbouillon	Knorr
Salad dressing	Dressing Kräuter	Mautner Markhof
Salami	Kantwurst	Stastnik
Yogurt	Joghurt 1% cremig gerührt	NÖM

III RESULTS AND DISCUSSION

1 ANTIBODIES

1.1 Monoclonal and polyclonal antibodies

In the first stage of the EU project large numbers of monoclonal and polyclonal antibodies directed against peanut and hazelnut were produced at RIKILT (Netherlands) and CSL (U.K.). All antibodies were characterized thoroughly by the respective project partner, employing different ELISA techniques:

Indirect non-competitive ELISA:

- assessment of the necessary dilution factors for the individual antibodies
- first information about sensitivity of the antibodies

Indirect competitive ELISA

- providing more detailed information about sensitivities
- appreciation of detection limits
- investigation of cross-reactivities

On base of the ELISA results a range of suitable antibodies was selected according to following selection criteria:

- final dilution — high dilution factor; good producing cells; highly sensitive antibodies
- working range — low limit of detection; high sensitivity
- cross-reactivity — no cross-reactivity; high selectivity

Sets of the selected antibodies were provided to the other project partners for assay development.

1.2 Egg yolk antibodies

The production of the egg yolk antibodies was part of the work of this thesis.

Immunisation and animal housing were conducted by Ao. Univ. Prof. Dr. Marcela Hermann at the Institute for Medical Biochemistry, Medical University Vienna.

1.2.1 Characterisation and selection of egg yolk antibodies

For the production of peanut and hazelnut specific egg yolk antibodies two chickens were immunized. From each chicken approx. 50 eggs were collected. For extraction of the antibodies from egg yolk batches of 5 eggs each were combined.

To be able to characterize the different batches and gain first information on sensitivity of the antibody, indirect non-competitive ELISAs were carried out for each batch. A typical calibration curve is shown in Figure III.23 (for plate layout see chapter II.1.2.3.1).

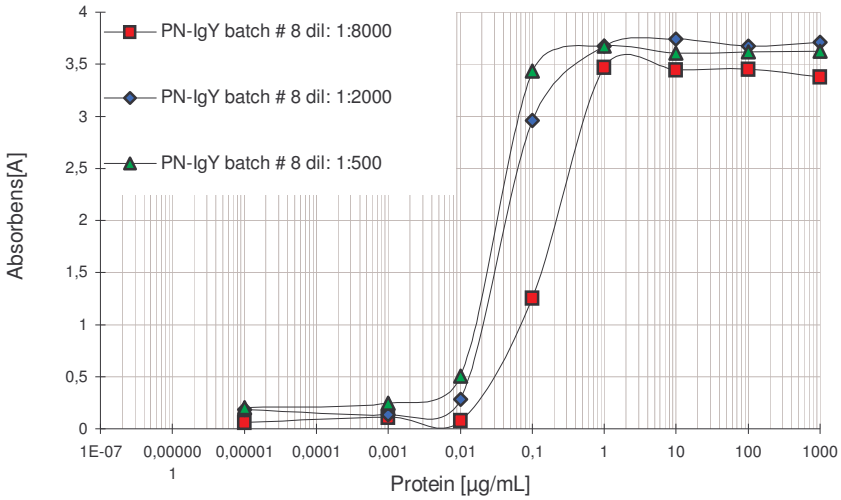


Figure III.23: Indirect non-competitive ELISA calibration curve

The most sensitive antibody batches (the ones with highest reactivity at high dilution factors) were further characterized in an indirect competitive ELISA format to gain more detailed information about sensitivity and cross-reactivity.

All batches of both antibodies showed cross-reactivities with various substances. For each antibody the batch with the greatest sensitivity was chosen for further applications, characteristics of these two antibody batches are displayed in Table III.8.

Table III.8: Characteristics of chosen egg yolk antibody batches

egg yolk antibody	protein content [mg/ml]	cross-reactivity
anti-peanut	22.13	soy bean, pectin, almond, vanillin, pecan, bean, hazelnut, lupine
anti-hazelnut	22.19	pumpkin seed, sunflower seed, poppy seed, soy bean, pecan

Because of the high cross-reactivity of the egg yolk antibodies they would not be suitable for application in the development of commercial immunoassays, nevertheless they were further employed in this work to study the behaviour of IgY in comparison to monoclonal antibodies when incorporated in sandwich immunoassays.

1.2.2 Purification of egg yolk antibodies

The egg yolk extracts contained beside the desired antibodies large amounts of other proteins like non specific antibodies and egg yolk proteins. This condition is of minor relevance in ELISA techniques where a second antibody is employed for detection. For immunoassay formats with a one step detection, where the detection antibody is directly labelled these large amount of additional proteins represent a drawback. In the coupling reaction the antibody labelling efficiency is reduced because the coupling agent also attaches to the unspecific protein. For the assay performance this means a decrease of specificity and sensitivity and background problems.
Therefore the selected antibody batches were further purified by thiophilic interaction chromatography separating the IgY antibodies from the other proteins.
Figure III.24 shows a typical chromatogram of such a purification procedure.

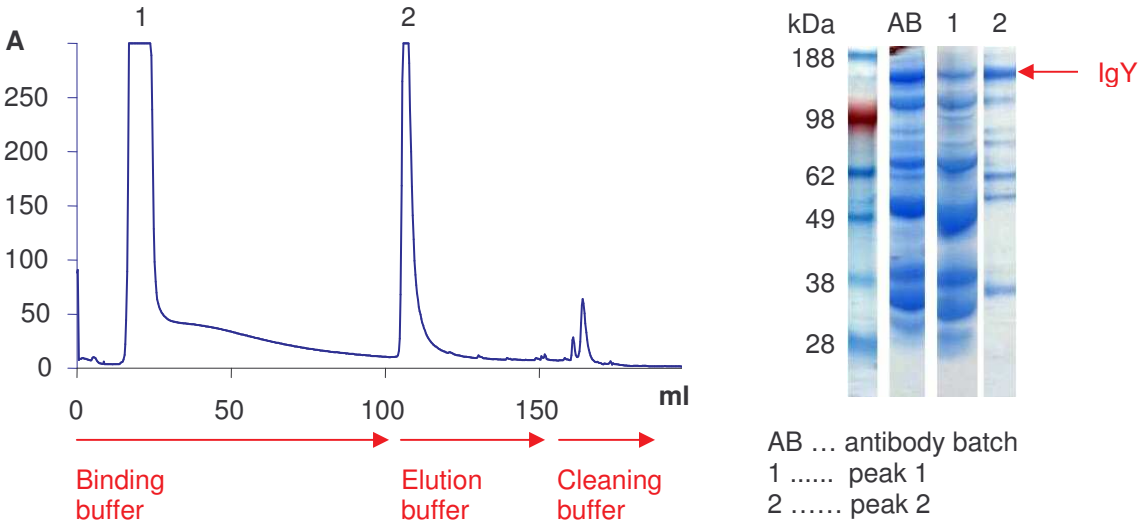


Figure III.24: Thiophilic interaction chromatography

The chromatogram monitored the eluation of proteins from the column. Two prominent peaks were detected, peak 1, eluated with Binding buffer contained the proteins that did not bind to the column, peak 2 represented the IgY that was bound to the column and eluated with Elution buffer.

For verification the fractions of the two peaks and a sample of untreated antibody batch were separated on SDS-PAGE (see Figure III.24). The untreated antibody batch showed a prominent protein band at 180 kDa (the size of IgY) beside a great amount of other proteins over the whole MW range. Peak 1 showed a minor band at 180 kDa and also broad range of other proteins.

The eluate of peak 2 separated on SDS–PAGE displayed a 180 kDa band for IgY, with a slightly reduced intensity compared with the corresponding band for the eluate of peak 1, and a few minor bands for other proteins.

Protein determination evidenced that 100 mg protein applied to the column resulted in approximately 10 mg IgY preparation.

Hence these results confirmed that via thiophilic interaction chromatography the IgY antibodies were isolated from the egg yolk extract and the major part of the undesired other proteins was eliminated. However there was also a minor decrease in antibody concentration but this was accepted in favour of the improved coupling properties of the isolate.

1.3 Selection of antibodies for immunoassay development

To find the best matching pairs of antibodies for the development of fast sandwich enzyme immunoassays all disposable antibodies were tested in a sandwich dipstick format with colloidal gold. Thereto monoclonal and polyclonal were employed in various combinations in the position of capture and detection antibody, incubating with different concentrations of nut extract. Selection criteria were a short reaction time (15 min sample incubation, 10 min detection antibody incubation), a high assay sensitivity (low limit of detection) and a strong signal intensity.

The following monoclonal antibody pairs were selected for further assay development:

<u>peanut</u>

capture antibody CSL-PN-mAb-68

detection antibody CSL-PN-mAb-68

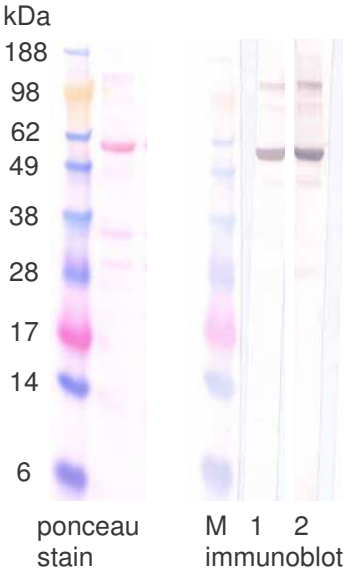
<u>hazelnut</u>

capture antibody RIKILT-HN-mAb-6E1

detection antibody RIKILT-HN-mAb-4F2

1.4 Western Blot of the selected antibodies

To check the binding property of the selected monoclonal antibodies and the egg yolk antibodies TBS extracts of peanut and hazelnut were separated by SDS-PAGE and blotted to nitrocellulose. A ponceau stain was performed to visualize the protein pattern on the membrane and subsequently immunoblots with the respective antibodies were carried out.

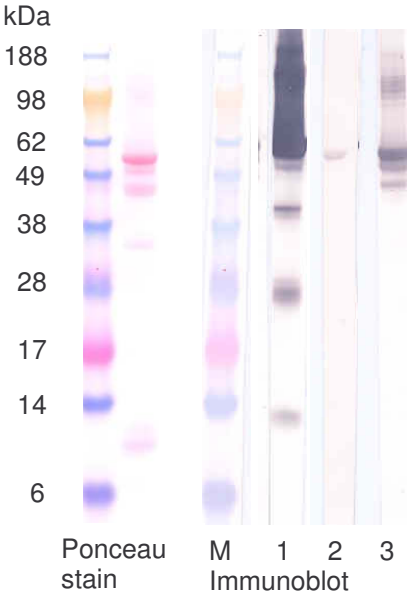


M	Marker
1	CSL-PN-mAb-68 (1:100)
2	PN-IgY (1:400)

Figure III.25: Western Blot with peanut antibodies

On the Ponceau stain for the peanut extract (see Figure III.25) bands between 25 and 130 kDa were visible. The most prominent band located near the 62 kDa marker was also the one mainly recognized by the two antibodies in the immunoblot. Both antibodies additionally detected some minor bands at 49 kDa, and 98 kDa.

Ara h 1 the major peanut allergen was found to be a 63 kDa glycoprotein and represented 12-16% of the total protein content of a peanut TBS extract established under similar conditions as the extracts used in this experiment [Koppelman et al, 2001]. Considering the high percentage of Ara h 1 in total protein it might be possible that immunization lead to antibodies directed against the major peanut allergen. For a confident verification of this thread additional experiments would have been necessary. However this approach has not been further investigated because specific allergen detection is in fact not so relevant for a commercial peanut immunoassay. For the allergic consumer only the information on presence or absence of peanut in a food is important. Moreover not every patient is sensitized to the same peanut allergen therefore a potential allergen test would have to recognize all possible peanut allergens to grant safety for all allergic consumers, which is impossible because there might be allergens not discovered yet.



M	Marker
1	RIKILT-HN-mAb-6E1 (1:3000)
2	RIKILT-HN-mAb-4F2 (1:200)
3	HN-IgY (1:100)

Figure III.26: Western Blot with hazelnut antibodies

Figure III.26 shows the Ponceau stain and immunoblot of hazelnut TBS extract. On the Ponceau stain bands between 10 kDa and 98 kDa were visible. The most prominent band was located between the 49 kDa marker and the 62 kDa marker, this band was also detected by all three antibodies in the immunoblot. RIKILT-HN-mAb-6E1 (lane1 of immunoblot) was highly reactive and recognized the whole area between 49 kDa and 200 kDa, as well as bands at ~12 kDa, ~27 kDa and ~40 kDa. The high reactivity of this antibody might be the reason why it worked well as capture antibody. RIKILT-HN-mAb-4F2 (lane 2 of immunoblot) only recognized the band between 49 kDa and 98 kDa. The hazelnut egg yolk antibody bound to all the three bands around 49 kDa in about the same intensity as they were displayed in the Ponceau stain.

In conclusion it was found out, that all the three employed hazelnut antibodies are mainly directed against a protein of about 55 kDa. None of the proteins described as hazelnut allergens has a correlating molecular weight [Hirschwehr et al, 1992]. However, as mentioned above for a hazelnut immunoassay employed on food it is not crucial to detect allergen, but presence or absence of hazelnut.

2 COLLOIDAL GOLD

2.1 Quality of the prepared colloidal gold

The quality of each freshly prepared gold suspension was controlled measuring a UV-spectrum (350 nm – 800 nm). Figure III.27 shows a typical UV-spectrum recorded against a background solution of dist. H_2O.

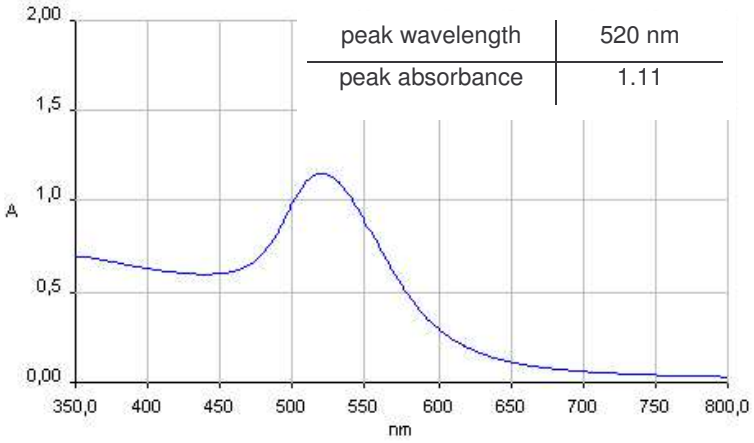


Figure III.27: UV-spectrum of colloidal gold

In literature [Sugunan et al 2005; Mayer et al 2002] and by commercial supplier (spi Supplies) colloidal gold particles with sizes from 15 nm – 50 nm are described to have an optical density of 1.1 +/- 0.1 at 520 nm. A more detailed determination of the particle size is usually performed using transmission electron microscope (TEM). As this technique was not available there is no information on uniformity and size of the gold particles. Nevertheless the quality of the produced colloidal suspension was considered satisfying due to the correlation of the data from the UV-spectrum with those in literature.

2.2 Stability of the colloidal gold

In the colloidal suspension exists a balance between the negative-charged repulsion and the attractive forces that could cause coagulation. As particles approach each other, an energy barrier must be traversed to overcome the repulsive character and enter the region on Van der Waals attraction. This barrier can be breached by the addition of electrolytes to the solution that can mask the negative surface charge on each particle.

At a certain concentration of electrolytes, the colloid will begin to collapse as the gold particles adsorb onto one another, forming large aggregates and ultimately falling out of suspension accompanied by a change of colour from red to blue. If macromolecules such as proteins are present in the colloidal suspension as the electrolyte concentration is raised to surpass the negative repulsion effects, then adsorption will occur with the protein molecules instead of with other gold particles. Thus, in place of aggregation and collapse of the suspension, labelling occurs and the red colour does not change.

The minimum amount of protein needed to stabilize the colloidal gold was discovered probing aliquots of gold (labelled with different concentrations of antibody) with NaCl and observing the colour of the solution visually [Horisberger and Rosset, 1977]. The lowest concentration to prevent aggregation (and change of colour) was found to be 8 µg/ml.

Further a proportion of 8 µg antibody / ml gold was used for the coupling reactions.

A: gold / 0 µg antibody
B: gold / 1 µg antibody
C: gold / 2 µg antibody
D: gold / 4 µg antibody
E: gold / 6 µg antibody
F: gold / 8 µg antibody
G: untreated gold

Figure III.28: Probing of different labelled gold aliquots

3 DEVELOPMENT OF IMMUNOASSAYS

Dipsticks and microarray sandwich-immunoassays for peanut and hazelnut were developed employing the selected best matching pairs of monoclonal antibodies described in chapter III.1.3. The aims were a short assay time, a low detection limit and the possibility to measure a preferably broad range of food matrices. Parameters like concentration of capture antibody, blocking - agent and - concentration were varied until optimal conditions were found. During the development stage of the test systems dilutions of nut TBS-extracts were employed as samples. Furthermore different food matrices, spiked with certain amounts of nut were analyzed. (Spiking concentrations of these samples had been verified in ELISA measurements before).

Subsequent concentrations for all samples are specified in µg nut / 10 ml extract because a dilution factor of 1:10 must be considered through extraction. This means the actual limit of detection is lower about one order of magnitude ($\mu g 10^{-1}$/ml) than the indicated values.

Table III.9: Concentration values of the different kinds of samples

kind of sample	**extraction**	**measured nut concentration**	**actual nut concentration**
milled nut powder	1 g/10 ml	µg /10 ml	$\mu g 10^{-1}$/ml
spiked matrix	1 g/10 ml	µg/g (10 ml) = ppm	$\mu g 10^{-1}$/ml

3.1 Sandwich Dipstick with colloidal gold labelled antibodies

As dipstick assays are qualitative screening methods the results have the character of binary responses and can therefore only be expressed as positive or negative which indicates whether the analyte is above or below a specified limit. In case of detection with colloidal gold coupled antibodies results are gained by visual estimation of a colour signal. The development of a visible red signal was judged as positive result, absence of colour was assessed as negative result.

3.1.1 Detection limits

Dilutions of nut TBS-extracts at a range of concentration levels as well as blank samples were analyzed in order to determine the detection limits of the dipstick tests. At each concentration level 10 independent replicates were measured. The lowest concentration at which 10 out of 10 samples were determined positive was defined as limit of detection. This method for finding the limit of detection in qualitative measurements was described in EURACHEM Guide 1998.
Reaction with the blank samples could be ruled out for peanut and hazelnut dipstick.
For the peanut dipstick the limit of detection for nut extract was determined 30 µg/10ml nut (see Figure III.29).

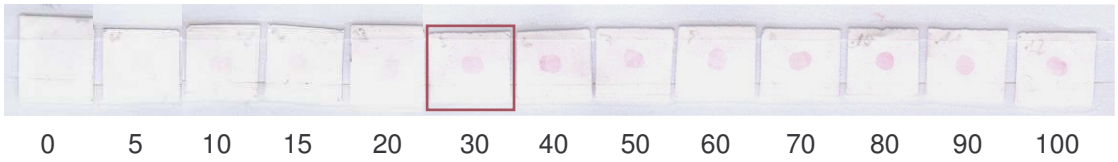


Figure III.29: Dipstick results detecting various peanut concentrations in µg nut/10ml

For the hazelnut dipstick the limit of detection for nut extract was determined 10 µg / 10 ml nut (see Figure III.30).

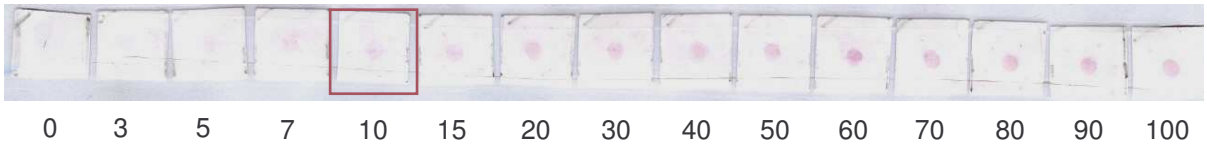


Figure III.30: Dipstick results detecting various hazelnut concentrations in µg nut/10ml

The sensitivity of the dipstick tests when measuring nut extract was satisfying, considering the actual lower limit of detection (about a factor 10) as mentioned above. This would mean the tests are able to detect 3 µg/ml peanut and 1 µg/ml hazelnut respectively.
However in practical application the tests will be applied to food samples, which again might reduce sensitivity due to matrix effects.

3.1.2 Measuring matrix samples

Matrix samples were prepared spiking dark chocolate, milk chocolate, cookies, cornflakes, instant soup, ice cream and yogurt with peanut and hazelnut CMC-spiking-solution at a concentration of 100 µg nut / g matrix (ppm). TBS extracts of the spiked and blank matrices were produced. The peanut and hazelnut containing extracts were diluted with the blank extracts to reach the desired nut concentrations and additionally blank extracts were analyzed. Preliminary tests have shown that matrix samples, measured with the dipstick assays resulted in a stronger background signal compared with the measurements of nut extracts. Analysis of blank matrices did not result in any positive signals. 6 out of the 7 matrices were successfully analyzed with peanut and hazelnut dipstick and detection limits for these matrices were determined as described before (results see Table III.10). Analysis of spiked samples of the dark chocolate matrix was not possible with the dipstick assays. Dipstick results for a sequence of nut concentration in the different matrices are displayed in Figure III.31 and Figure III.32.

As expected the detection limits for the individual food matrices where higher than for the nut extracts only. The performance of the hazelnut dipstick regarding stability and sensitivity was better than for the peanut dipstick, this phenomenon was also reported by the other project partners developing immunoassays. Highest limit of detection in the peanut dipstick was observed for the milk chocolate, maybe due to the rather complex matrix structure. Also the other matrices had clearly higher detection limits in the peanut dipstick than in the hazelnut dipstick. An option for improvement would be to reduce the extract volume and thus the dilution factor.

Table III.10: Detection limits for matrix samples on peanut and hazelnut dipstick

matrix	LOD for peanut	LOD for hazelnut
milk chocolate	100 ppm	30 ppm
cookies	60 ppm	30 ppm
cornflakes	50 ppm	30 ppm
instant soup	70 ppm	30 ppm
ice cream	60 ppm	30 ppm
yogurt	40 ppm	30 ppm

Figure III.31: Matrix samples on peanut dipstick

Figure III.32: Matrix samples on hazelnut dipstick

3.1.3 Measuring peanut and hazelnut simultaneously

One attempt was a dipstick assay for the detection of peanut and hazelnut simultaneously. Therefore both peanut and hazelnut capture antibodies were applied onto one piece of membrane (CSL-PN-mAb-68 in the upper left corner, RIKILT-HN-mAb-6E1 in the lower right corner). Sample incubation performed with peanut and / or hazelnut extract and detection was accomplished either with peanut or hazelnut detection antibody, as well as with both of them. Figure III.33 shows the results for the tested combinations.

	PN HN	**sample**	**detection antibody**
1)		100 µg/10 ml peanut	CSL-PN-Mab-68
2)		100 µg/10 ml hazelnut	RIKILT-HN-Mab-4F2
3)		100 µg/10 ml peanut	CSL-PN-Mab-68 RIKILT-HN-Mab-4F2
4)		100 µg/10 ml hazelnut	CSL-PN-Mab-68 RIKILT-HN-Mab-4F2
5)		100 µg/10 ml peanut 100 µg/10 ml hazelnut	CSL-PN-Mab-68 RIKILT-HN-Mab-4F2

Figure III.33: Dipstick for the detection of peanut and hazelnut simultaneously

Incubation with peanut and peanut–mAb (1) lead to a clear positive signal for peanut and some weak reaction at the position of the hazelnut capture antibody, probably due to a weak peanut affinity of the hazelnut capture antibody.

Analysis of hazelnut and incubation of hazelnut-mAb (2) resulted in a clear positive signal at the hazelnut position without any interference.

In the dipstick format where peanut- and hazelnut-mAbs were employed as detection antibodies simultaneously for the analysis of a peanut sample (3) a clear signal for peanut and some interference for hazelnut was observed. Hazelnut sample again lead to a clear sample for hazelnut and some interference for peanut (4). Reasons for the observed interferences could be a weak cross-reactivity of the antibodies or simply the higher concentration of gold-coupled antibodies in the detection reagent.

The attempt to analyse peanut and hazelnut simultaneously lead to weak signals for peanut and hazelnut (5). This first effort showed that it is generally possible to detect peanut and hazelnut simultaneously in one dipstick assay, although optimization work especially on cross-reactivity and sensitivity is necessary and the capability to detect peanut and hazelnut simultaneously also in food matrix has to be verified.

3.1.4 Egg yolk versus monoclonal antibodies

To gain information on the potential use of egg yolk antibodies in fast immunoassays, the egg yolk antibodies were incorporated in the sandwich assay and compared with the selected monoclonal antibody pairs. Therefore, the egg yolk antibodies were employed as capture as well as detection antibody, additionally different combinations of egg yolk and monoclonal antibodies were tested. Sample incubation was performed with diluted nut extracts at concentrations of 10 µg/10 ml, 50 µg/10 ml, 100 µg/10 ml, 1000 µg/10 ml peanut and hazelnut respectively, as well as mere buffer for blank samples.
Results for measurements of peanut samples are displayed in Figure III.34, results for measurements of hazelnut samples are displayed in Figure III.35.
These experiments showed the same results for peanut and hazelnut.
Dipsticks with egg yolk antibodies exclusively did not yield a detectable signal (results not shown), also combinations of monoclonal antibody as capture antibody and IgY as detection antibody did not result in any positive results. The IgY for capture and a monoclonal antibody for detection was the only combination with egg yolk antibody that enabled detection of peanut / hazelnut. It was possible to detect approximately 50 µg/ml peanut extract and 100 µg/ml hazelnut extract. Still the combinations with egg yolk antibodies led to weaker signals and a less sensitive detection of nut than the combinations of monoclonal antibodies exclusively with an limit of detection of 30 µg/ml for peanut and 10 µg/ml for hazelnut respectively.

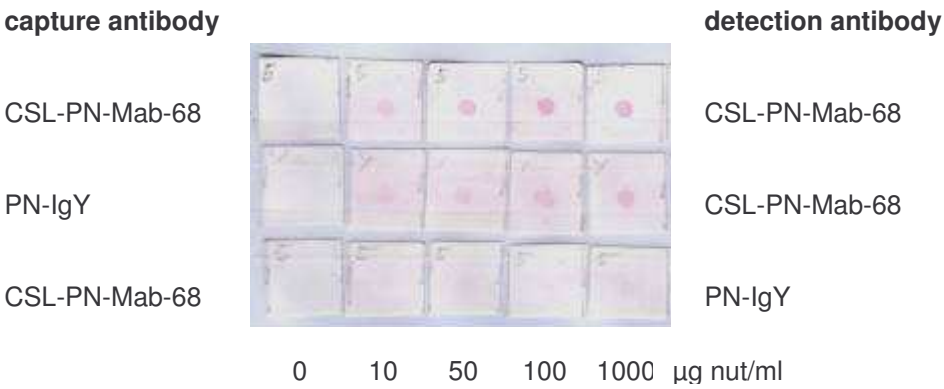


Figure III.34: Results for peanut samples on dipsticks with egg yolk antibodies

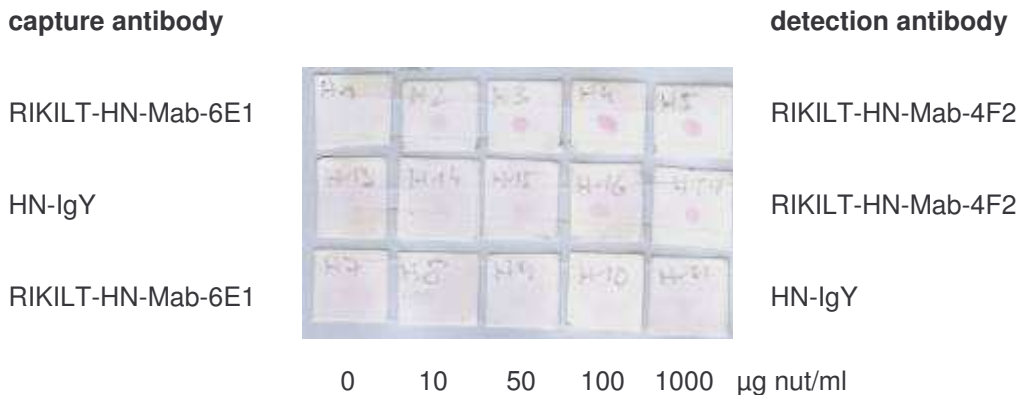


Figure III.35: Results for hazelnut samples on dipsticks with egg yolk antibodies

3.2 Microarray

The practical work on microarrays was performed by Dr. Markus Janotta, Univ. of Applied Science, Tulln. In some preliminary experiments the best matching pairs of monoclonal antibodies were also applied in a microarray format. Thereto the capture antibody was spotted 9-12 times into the individual reaction well of an aldehyde coated glass slide and an assay in sandwich format was performed. The detection antibodies were labelled with a fluorescent dye and results gained by measuring the fluorescence intensity. This detection method enables results to be expressed numerically and therefore also to be quantified. During the development of the microarrays background problems and weak antibody binding occurred. In order to overcome this difficulties parameters like blocking agent and washing steps were changed, unfortunately the problems could not be eliminated completely and results were not always reproducible. Nevertheless by means of the conducted experiments first information was gained on the performance of the microarray format as immunoassay for the detection of peanut and hazelnut in comparison with the dipstick format.

3.2.1 Detection limits

Ranges of lowest possible detection rather than defined detection limits were acquired due to the weak repeatability of the results. Therefore, dilutions of nut TBS-extracts at different concentration levels as well as buffer blanks were analysed. The lowest nut concentration that could be clearly differentiated from buffer blank was estimated as limit of detection. Fluorescence signals were expressed numerically and the signal with a value approximately double than that of the buffer blank was defined as limit of detection (see Figure III.36.B and Figure III.37.B). The peanut microarray with a detection range between 0.6 µg/10 ml and 0.8 µg/10 ml was found to be more sensitive than the hazelnut microarray with a detection range around 2 µg/10 ml. The inferior sensitivity of the hazelnut assay was caused by an increased occurrence of the mentioned problems with reproducibility in the performance of the hazelnut microarray conferred to the peanut microarray, which gets apparent comparing the scans of the observed fluorescence signals for the peanut and the hazelnut assay (Figure III.36.A and Figure III.37.A). Signals in the hazelnut assay got blurred after the washing step due to weak binding of second antibody. A strong background can be observed and also buffer blank exhibits fluorescence, probably due to insufficient blocking. For the peanut assay, the signals are clear, background low and buffer blank does not exhibit fluorescence. Fluorescence intensities were also expressed quantitatively in diagrams with a logarithmic scale (Figure III.36.C and Figure III.37.C). When evaluating the peanut assay a continuous rise of fluorescence intensity correlating with nut concentration can be seen, for hazelnut the slope is less constant with some outliers.

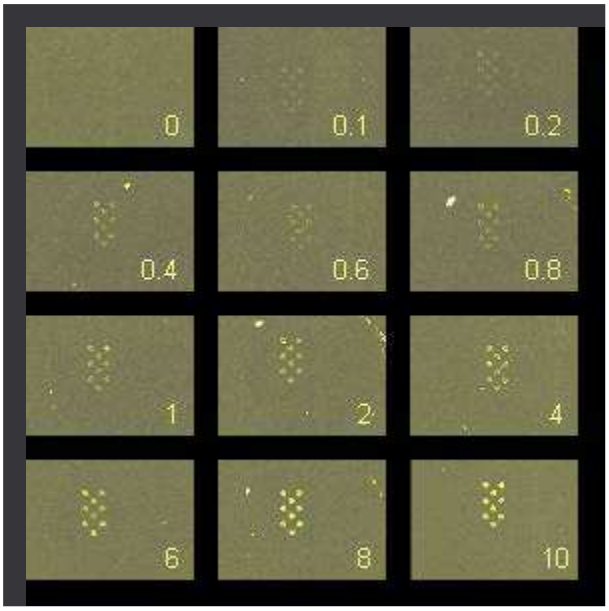


Figure III.36.A: Scan of observed fluorescence signals for peanut samples [µg peanut/ 10 ml]

peanut (µg/10 ml)	fluorescence (au)
0	509.1
0.1	706.4
0.2	779.2
0.4	1097.9
0.6	932.4
0.8	1416.8
1	1560.5
2	2377.5
4	2475
6	2969.4
8	4919.3
10	6372.6

Figure III.36.B: Fluorescence signals for peanut samples expressed numerically

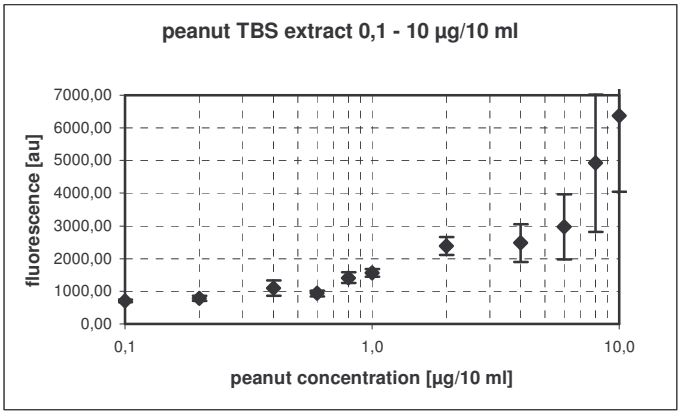


Figure III.36.C: Logarithmic scale of fluorescence signals for peanut samples

Figure III.36: Results for peanut microarray

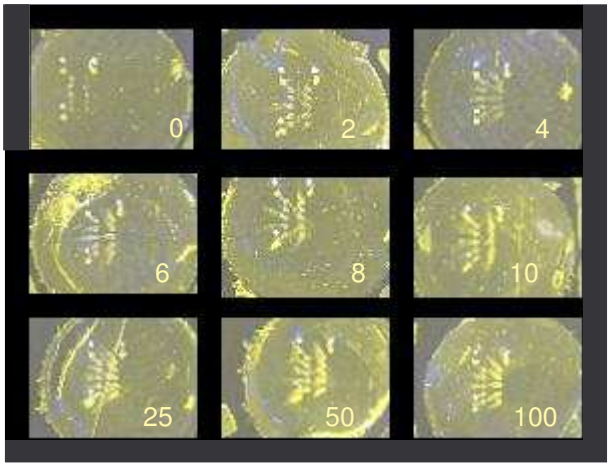


Figure III.37.A: Scan of observed fluorescence signals for hazelnut samples [µg hazelnut / 10 ml]

hazelnut (µg/10 ml)	Fluorescence (au)
0	2458.2
2	4816.3
4	5943.9
6	5629.6
8	3352.4
10	7694
25	7948.5
50	9331
100	9432.6

Figure III.37.B: Fluorescence signals for hazelnut samples expressed numerically

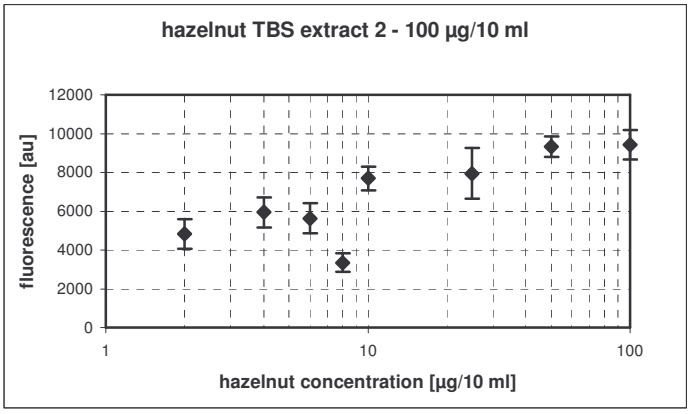


Figure III.37.C: Logarithmic scale of fluorescence signals for hazelnut samples

Figure III.37: Results for hazelnut microarray

3.2.2 Measuring matrix samples

Because of the mentioned problems with the hazelnut microarray, measurements of matrix samples were conducted only with peanut microarray. According to the measurements with the peanut dipstick assay TBS extracts of the following peanut spiked matrices were applied: dark chocolate, milk chocolate, cookies, cornflakes, instant soup, ice cream and yogurt. The peanut containing extracts were diluted with blank extracts of the correlating matrices to reach the desired peanut concentrations.

A preliminary detection limit between 1 ppm – 2.5 ppm was evaluated for most matrices, only for dark chocolate the preliminary limit of detection was higher with approximately 10 ppm.

Measurements of all matrices with peanut concentrations of 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm and blank matrix respectively were performed, results are displayed in Figure III.38.

Fluorescence intensity of the results ranged between 5000 and 60000 [au] for all spiked matrices but dark chocolate. For the dark chocolate matrix fluorescence intensity of the signals was lower, correlating with the higher limit of detection limit for this matrix. In most cases fluorescence intensity increased correlating with nut concentration. Exceptions were dark chocolate cookies and instant soup, here the fluorescence intensity decreased for the 50 ppm samples. This effect could be due to a “upper limit of detection”, the impossibility to measure very high peanut concentrations above a certain limit, as it is known from other immunoassay formats (e.g. “hook effect” for LFD).

These first experiments verified that it is possible to detect peanut in complex food matrices with a microarray immunoassay format.

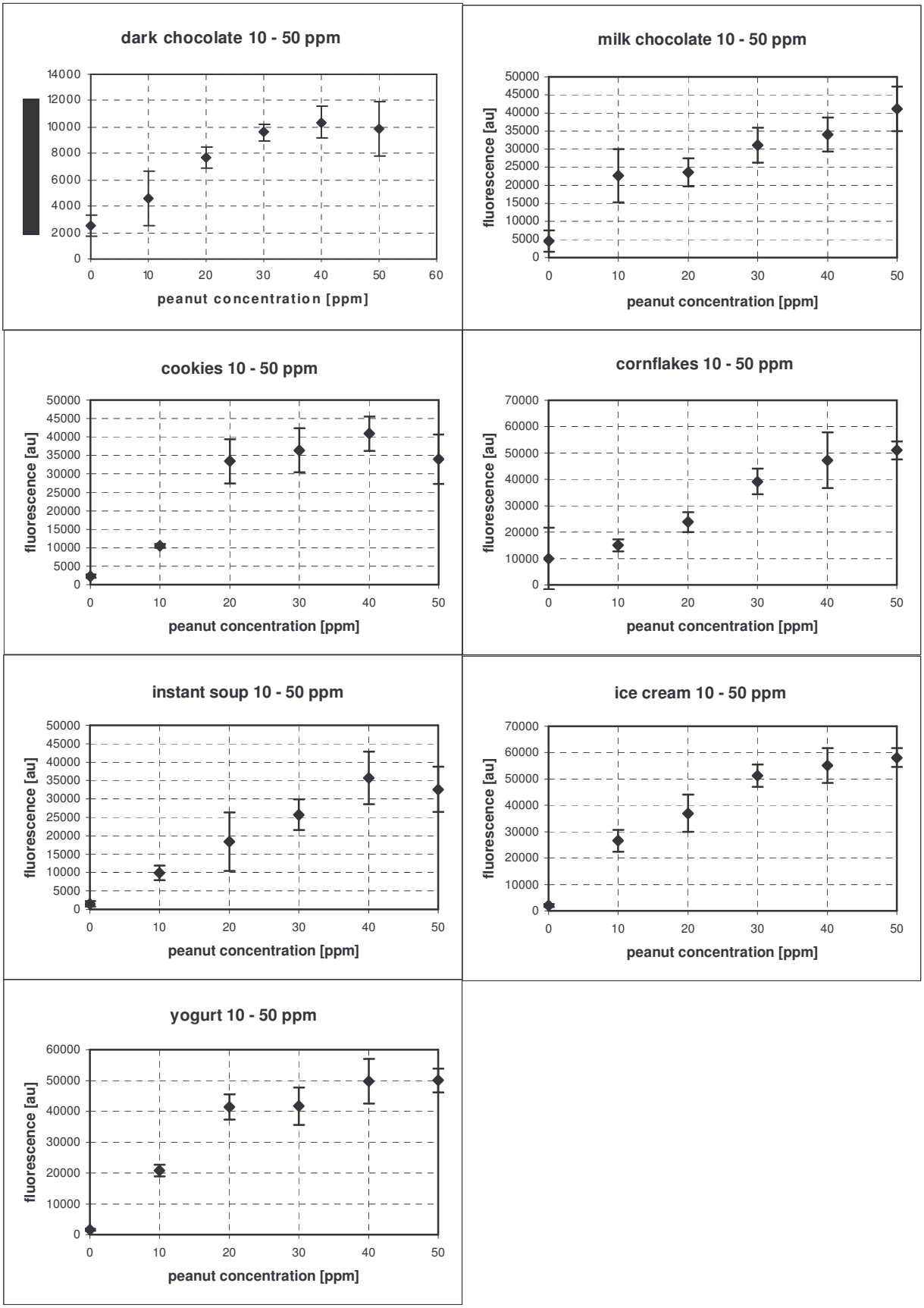


Figure III.38: Results for matrix samples measured with peanut microarray

3.2.3 Measuring peanut and hazelnut simultaneously

With the microarray format like with the dipstick it was also possible to measure peanut and hazelnut simultaneously. The capture antibodies of the two best matching pairs of monoclonal antibodies were immobilized in one reaction well of an aldehyde coated glass slide, peanut and / or hazelnut TBS extract was incubated and for detection either one or both monoclonal antibodies coupled to fluorescence dye were employed. No interference or cross-reactivities were observed and nut concentrations of 10 µg/10 ml could be easily detected (see Table III.11).

Table III.11: Results for microarray measurements of peanut and hazelnut simultaneously

capture antibody	peanut	hazelnut	peanut + hazelnut	detection antibody
CSL-PN-mAb-68 + RIKILT-HN-mAb-6E1	+	-	+	CSL-PN-mAb-68
CSL-PN-mAb-68 + RIKILT-HN-mAb-6E1	-	+	+	RIKILT-HN-mAb-4F2
CSL-PN-mAb-68 + RIKILT-HN-mAb-6E1	+	+	+	CSL-PN-mAb-68 RIKILT-HN-mAb-4F2

3.2.4 Egg yolk versus monoclonal antibodies

According to the dipstick experiments also egg yolk antibodies were incorporated into the microarray format and compared with the selected monoclonal antibody pairs. Therefore, the egg yolk antibodies were employed as capture as well as detection antibody, additionally different combinations of egg yolk and monoclonal antibodies were tested. Results for measurements of peanut samples are displayed in Table III.12, results for measurements of hazelnut samples are displayed in Table III.13. These experiments showed the same results for peanut and hazelnut. Combinations with egg yolk antibodies exclusively did not yield a detectable signal, also combinations of IgY as capture antibody and a monoclonal antibody as detection antibody did not result in any positive results. The only combination including egg yolk antibody that enabled detection of peanut / hazelnut was a monoclonal antibody for capture and IgY as detection antibody. It was possible to detect nut concentrations down to 10 µg/10 ml nut. However this combinations led to weaker signals and a less sensitive detection of nut than the combinations of monoclonal antibodies exclusively (limit of detection between 0.6-0.8 µg/10 ml for peanut extract and 2 µg/10 ml for hazelnut .extract respectively).

Table III.12: Results for peanut samples measured on microarray with egg yolk antibodies

capture antibody	detection antibody	signal intensity
CSL-PN-mAb-68	CSL-PN-mAb-68	++
PN-IgY	CSL-PN-mAb-68	-
PN-IgY	PN-IgY	-
CSL-PN-mAb-68	PN-IgY	+

Table III.13: Results for hazelnut samples measured on microarray with egg yolk antibodies

capture antibody	detection antibody	signal intensity
RIKILT-HN-mAb-6E1	RIKILT-HN-mAb-4F2	++
HN-IgY	RIKILT-HN-mAb-4F2	-
HN-IgY	HN-IgY	-
RIKILT-HN-mAb-6E1	HN-IgY	+

3.3 Comparison of the methods and conclusion

The fundamental difference between the two methods is, that the dipstick format is a classic immunochemical assay, intended to provide a rapid and easy-to-use tool for on site control of presence / absence of an analyte of interest. In contrast protein microarrays were initially developed as a high through-put characterisation method for a large number of proteins in parallel, requiring very small sample volumes for applications like screening of proteoms and analysis of patient sera.

This study was the first attempt to investigate the performance of a sandwich microarray format for the analysis of peanut in complex food matrices in comparison with a sandwich dipstick format employing the same antibodies. Table III.14 provides an overview of the major differences in performance of the methods that were observed.

Although the present longer reaction time of the microarray compared with the dipstick could probably still be reduced the dipstick format rather meets the requirements of a rapid and easy-to-use immunoassay, also because of the independence of laboratory equipment.

However due to the instrumental evaluation the microarray has the potential to reach lower detection limits compared with the dipstick, especially when measuring food matrices. Nevertheless, regarding recommended detection limits for food products between 1 and 100 ppm [Poms et al, 2004], the obtained results for both methods are located in an acceptable range. The detection of peanut and hazelnut simultaneously was possible with both assay formats, a feature that would represent a valuable enhancement for a commercial immunoassay.

An interesting observation was the difference in performance of the identical pairs of antibodies employed in both formats. In the dipstick format the hazelnut monoclonal antibodies were even more sensitive than the pair of monoclonal peanut antibodies, in the microarray format their performance was worse due to the weak binding of second antibody. Also the experiments with the egg yolk antibodies lead to different results for the two formats. Application of egg yolk antibodies exclusively was ineffective in both cases. The combination egg yolk antibody for capture and monoclonal antibody for detection lead to weak signals for 50-100 µg/ 10 ml nut in case of the dipstick. Combinations with monoclonal antibody for capture and IgY as detection antibody enabled detection down to 10 µg/ 10ml nut in case of the microarray. The conclusion from these observations is that there must be some differences in the antibody binding mechanism between the two methods although a sandwich format with identical antibodies is employed in both cases.

Comparing monoclonal - and egg yolk antibodies the results attested, that egg yolk antibodies are presently not able to meet the performance of monoclonal antibodies in immunoassays, especially regarding sensitivity and selectivity, as previous studies have already indicated [Drs, 2004].

Comparing the results of the two immunoassays it is impossible to judge one method superior to the other, the decision which method is more convenient will mainly depend on the specific requirements of each individual situation. The dipstick provides an easy-to-use format with qualitative results for on-site testing yielding a quick read-out. On the other hand if a fast response time is not so much a demand and laboratory equipment is available, the microarray format offers a more sensitive method for quantitative measurements.

Table III.14: Comparison of dipstick and microarray format

sandwich dipstick	**sandwich microarray**
25 min total reaction time	180 min total reaction time
no laboratory equipment necessary	fluorescence detector necessary
1 sample each	at least 24 samples each
better performance with hazelnut mAbs	better performance with peanut mAbs
sensitivity in two-digit ppm range	sensitivity in one-digit ppm range
matrix effect	weak matrix effect
analysis of dark chocolate matrix impossible	analysis of dark chocolate matrix possible
IgY works only in position of capture antibody	IgY works only in position of detection antibody
simultaneous detection of peanut and hazelnut possible	simultaneous detection of peanut and hazelnut possible

4 COLLABORATIVE TRIAL

The collaborative trial was carried out for external validation of the new ELISA and LFD test kits developed at R-Biopharm and CSL respectively. The aim of the study was to estimate the performance characteristics of the new test systems such as applicability to complex food matrices, within-laboratory and among-laboratories precision, recovery, sensitivity, limit of determination.

4.1 Internal validation data for the peanut ELISA

Within assay variance for samples 5-10 mg/kg (n=10): 5.9%
Between assay variance for samples 5-10 mg/kg (n=6): 5.3%
Limit of detection: 1.5 mg/kg
Limit of quantification: 2.5 mg/kg
Working range: 2.5 mg/kg – 20 mg/kg
(defined as the concentration range covered by the calibrants)

4.2 Internal validation data for the hazelnut ELISA

Within assay variance for samples 5-10 mg/kg (n=10): 8.6%
Between assay variance for samples 5-10 mg/kg (n=6): 5%
Limit of detection: 1.5 mg/kg
Limit of quantification: 2.5 mg/kg
Working range: 2.5 mg/kg – 20 mg/kg
(defined as the concentration range covered by the calibrants)

4.3 Internal validation data for the LFDs

The limit of detection for both lateral flow devices was determined 2 mg/ml.
Further it was observed that measurement of samples with very high content of peanut / hazelnut resulted in a reduced intensity of test line. This phenomenon was called “hook effect” and seemed to be caused by cross-linking of latex particle and significant accumulation at the release pad/membrane interface.

4.4 Homogeneity study

To control the spiking values of the in-house spiked sample sets and to assess the material homogeneity control measurements with the peanut and hazelnut ELISA tests were carried out. The obtained peanut and hazelnut concentrations in the test samples in mg/kg were subjected to a statistical analysis of variances (ANOVA).

Additionally the recoveries of nut material for each concentration and food matrix was calculated and described as a ratio in percent (%) of added and determined nut content. Results are displayed in Table III.15.

Table III.15: Results homogeneity study

matrix	spiking value [mg/kg]	result mean [mg/kg]	recovery [%]	RSD [%]	F-value	F_{max}-value
dark chocolate	2.5 hazelnut	3.5	140.8	5	0.39	5.19
dark chocolate	10 hazelnut	9.8	97.8	6	3.52	5.19
cookie	5 peanut	4.0	80.2	17	6.36	5.19
cookie	5 hazelnut	5.9	118.4	5	11.34	6.38
ice cream	20 peanut	16.4	81.8	6	0.88	5.19
ice cream	20 hazelnut	20.1	100.5	4	0.65	5.19
salami	8 peanut	7.2	90,0	4	0.81	5.19
salami	8 hazelnut	8.3	103.3	2	1.4	6.38
salad dressing	4 peanut	3.4	84.5	15	3.71	5.19
salad dressing	4 hazelnut	4.4	110.8	6	0.65	6.59
instant soup	15 peanut	10.3	68.8	10	0.89	5.19
instant soup	15 hazelnut	14.9	99.6	4	1.79	6.39
cornflakes	35 peanut	24.4	69.9	4	1.12	6.59
cornflakes	35 hazelnut	36.1	103.2	9	4.16	6.39
yogurt	6 peanut	5.3	88.2	10	2.04	5.19
yogurt	6 hazelnut	7.1	118.0	4	0.66	5.19

The measured recoveries were between 68.8 and 140.8% and therefore in an acceptable range. Statistical analysis of variance revealed that for all the samples apart from samples with the cookie matrix, the calculated F-values were below the critical tabular F_{max}-values, P=0.05. It was therefore concluded that the difference between the two mean squares was not significant, the between-samples measurement variation was zero, and that all samples excluding the cookie samples could be regarded homogenous.

Reason for the deviating results obtained for the cookie samples was the effect of cluster formation during spiking matrices with a powdery consistency, leading to uneven extraction and thus deviating protein content in the extract.

Relative standard deviations for the cookies matrices were 17% for the peanut sample and 5% for the hazelnut sample respectively. In a similar study, between-sample errors of up to 20% were considered acceptable [Poms et al, 2005], therefore also the cookie samples were included in the collaborative trial.

4.5 Results from the interlaboratory ELISA measurements

All of the 50 samples (25 different samples / 2 lots each) were measured in duplicate on peanut – and hazelnut ELISA. Every participant returned the results to the coordinator of the collaborative trial who accomplished evaluation.

4.5.1 Blank samples

The blank samples measured on the peanut ELISA were all below the limit of quantification. Using the hazelnut ELISA, the blank milk chocolate and blank dark chocolate samples provided by R-Biopharm showed measurable values of hazelnut. It turned out that all the peanut chocolate samples produced in the food pilot plant contained hazelnut contaminations of approx. 4 mg/kg.

4.5.2 Cross-reactivity testing

4.5.2.1 Peanut samples measured on hazelnut ELISA

The mentioned hazelnut contamination of the chocolates supplied by the project partner was also observed when measuring the peanut spiked chocolate samples on hazelnut ELISA.
For the following peanut spiked samples: cookies (5 mg/kg), salami (8 mg/kg), salad dressing (4 mg/kg), and yogurt (6 mg/kg) the measurements with hazelnut ELISA lead to concentrations values below the limit of quantification.
For the peanut spiked samples: ice cream (20 mg/kg), instant soup (15 mg/kg) and cornflakes (35 mg/kg) up to three out of the 16 measurements were found positive for hazelnut, with concentrations between 3 mg/kg and 6 mg/kg.

4.5.2.2 Hazelnut samples measured on peanut ELISA

The hazelnut spiked dark chocolate samples measured on peanut ELISA showed values of peanut in the range of 4.2–11.8 mg/kg. It turned out that the reason for these cross-reactive results actually was a previously unidentified peanut contamination of the hazelnut chocolate used for spiking when preparing these samples.

Two positive peanut measurements out of 16, with a mean of 4 mg/kg, were found for cornflakes spiked at 35 mg/kg hazelnut. All other hazelnut samples were negative on peanut ELISA.

Although only a marginal number of false positive values with concentrations not considerably above the limit of quantification were found among peanut samples measured on hazelnut ELISA and hazelnut samples measured on peanut ELISA, further experiments were carried out to clarify this phenomenon.

For this purpose, additional aliquots of all the suspect samples were extracted and cross-reactivity measurements performed. Concentrations were calculated with optimised ELISA software tools from R-Biopharm, which were also employed during in-house validation of the prototype ELISA kits. This time, no cross-reactive results and no matrix effects were observed. Hence the individual software packages used by the participating laboratories might have led to false positive results due to a reduced accuracy in the lower concentration range. Considering these findings the cross-reactive results were interpreted as inaccuracy in data interpretation and not as cross-reactivity of the ELISA test systems or cross-contamination of the samples.

4.5.3 Results below the limit of quantification

Measurements performed using the peanut ELISA for the analysis of the peanut spiked cookie samples (5 mg/kg) showed that only 7 out of 16 results from the 8 laboratories were above the limit of quantification with a mean of 3.15 mg/kg. For salad dressing spiked with 4 mg/kg peanut none of the findings on the peanut ELISA were above the limit of quantification.

For hazelnut spiked samples, using hazelnut ELISA the cookies (5 mg/kg) were found positive (> LOQ) in only 2 out of 16 samples, with a mean of 3.5 mg/kg. For salad dressing spiked with 4 mg/kg hazelnut, only one positive result (2.4 mg/kg) out of 16 measurements was detected.

For further investigation of these false negative findings new samples were prepared by spiking 20 aliquots each of the matrices cookies, cornflakes and salad dressing with freshly prepared peanut and hazelnut spiking solutions at a concentration of 15 mg/kg respectively.

ELISA measurements of 10 aliquots per sample performed directly after spiking lead to recoveries of 98% - 106%. The remaining sample aliquots were stored for 4 weeks at -20°C. After that period of time again 10 aliquots per sample were extracted and measured on the respective ELISA tests. Again recoveries for the different spiked matrix materials were evaluated. The decrease in recovery ranged between 15% - 20% for the cookie and cornflakes samples, for the salad dressing samples a reduction in recovery of 70% after 4 weeks was observed.

The outcome of this experiments indicates a certain minor decrease in spiking concentration after a storage time of 4 weeks in general, as documented for the matrices cookie and cornflakes. For the spiked salad dressing matrix a remarkable severe decrease in recovery was observed indeed. A verification of the pH value revealed an acidic pH for the salad dressing matrix (pH 4). It was believed that protein structure and content were influenced in the respective sample under this conditions during the storage period of 4 weeks. Therefore matrix effects that lead to falsification of the measurable protein content were regarded as responsible for the severe decrease in recovery. Considering the originally low spiking levels of 4 mg/kg for salad dressing and 5 mg/kg for cookies it is coherent that these samples are affected even more severe by a decrease of spiking concentration potentially leading to results below the limit of quantification of the ELISA test.

4.5.4 Statistical evaluated results

Statistical analysis was carried out on samples with results within the working range. The mean of all measurements, the relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), and the recovery, (assuming the spiked peanut and hazelnut content as target value) were all calculated (see chapter 4.5.4.1 and chapter 4.5.4.2).

Additionally graphs were prepared, showing the results of the collaborative trial obtained for each peanut and hazelnut sample (see Figure III.39 and Figure III.40).

Interpretation of the graphs:

In some cases values for individual laboratories are missing due to elimination of outliers. Furthermore, results outside the working range were not included. The given datapoints represent the overall mean of each sample consisting of 2 subsamples (■). Datapoints representing a single value were indicated by a different symbol (♦). Laboratory 6 returned single values for some samples due to errors in sample preparation.

Comparing the graphs for the different samples (both peanut and hazelnut) results from laboratories 1 and 8 tended to show higher recoveries in most cases.

Investigations revealed that these trends were due to a slightly different incubation procedure carried out by the two laboratories compared to the other participants: The ELISA plates were shaken during incubation, which may facilitate the access of the antibodies on the binding sites, which lead to higher signals.

In contrast, results for the hazelnut samples from laboratory 3 showed low recoveries compared to the results of the other laboratories in most cases. Investigations revealed that absorbance readings for the hazelnut calibrants of this laboratory were suspiciously high which led to underestimation especially in the lower concentration range.

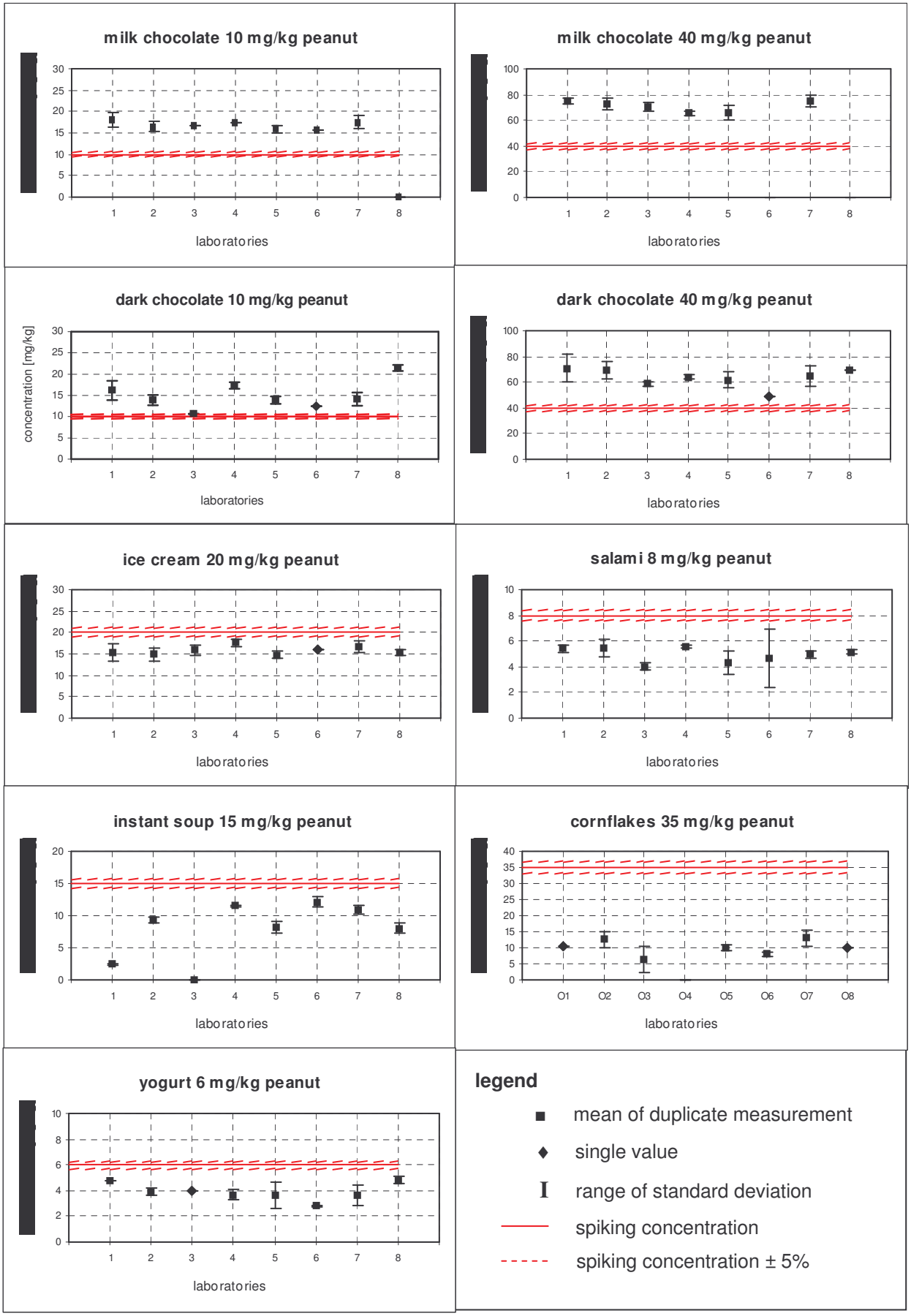


Figure III.39: Graphs for results of the peanut samples measured with peanut ELISA

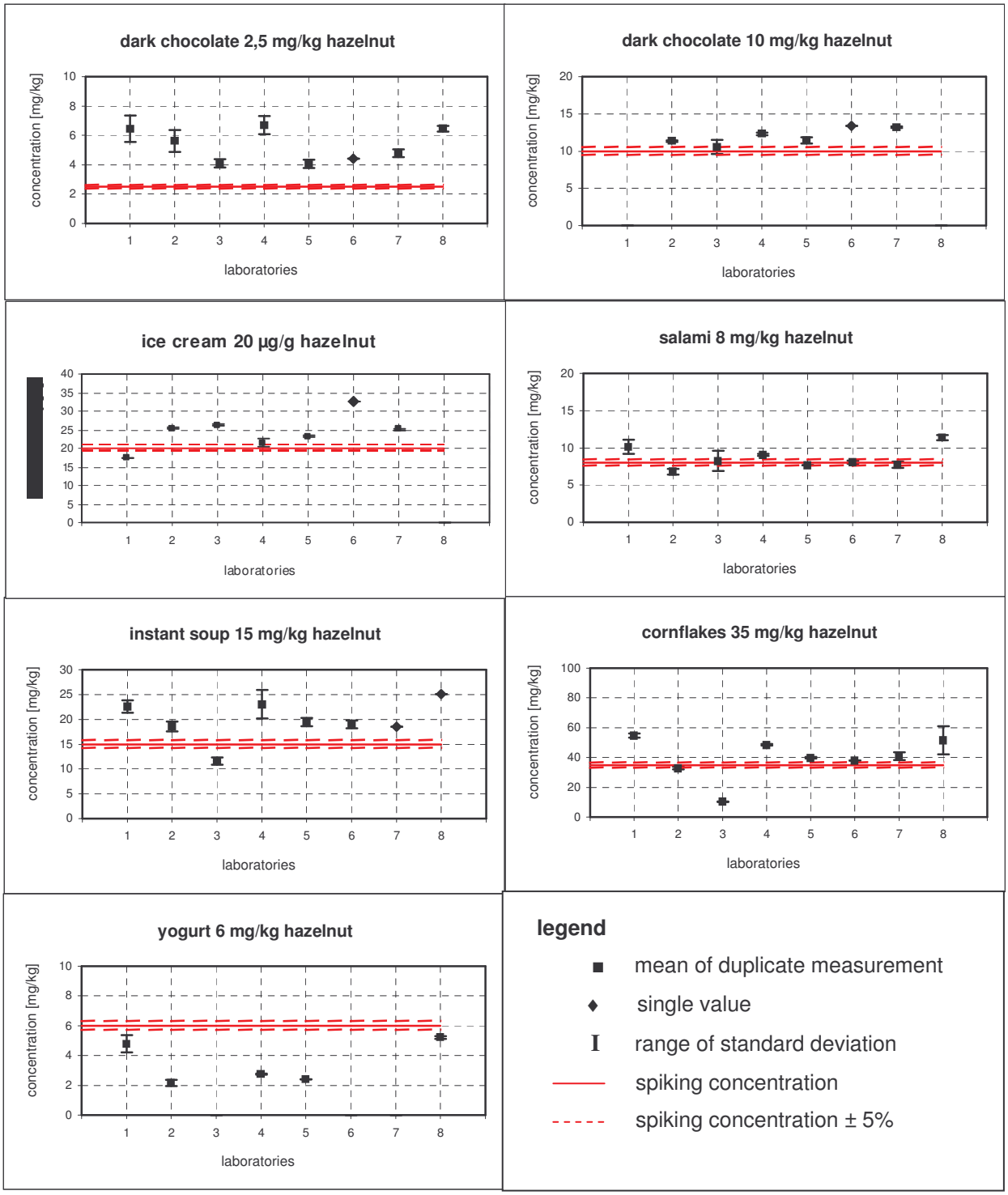


Figure III.40: Graphs for results of the hazelnut samples measured with hazelnut ELISA

4.5.4.1 Statistical evaluated results for peanut samples

Statistical data for peanut spiked samples measured on peanut ELISA are displayed in Table III.16.

Table III.16: Statistical data for peanut samples

measured values	matrix	spiking value [mg/kg]	results mean [mg/kg]	RSD_r [%]	RSD_R [%]	recovery [%]
13	milk chocolate	10	16.9	7	7	169
12	milk chocolate	40	70.8	5	9	177
15	dark chocolate	10	15.2	8	30	152
14	dark chocolate	40	64	10	13	160
15	ice cream	20	15.8	8	9	79
16	salami	8	4.9	19	16	62
14	instant soup	15	8.9	7	52	60
12	cornflakes	35	11.6	22	37	33
13	yogurt	6	3.9	14	24	65

The between-laboratory relative standard deviations (RSD_R) are < 50 % for the majority of samples. The highest RSD_R values in this study were found for samples with powdery consistency: 52% RSD_R for instant soup spiked with 15 mg/kg peanut and 37% RSD_R for cornflakes spiked with 35 mg/kg peanut. As mentioned before (see chapter 4.4) problems during the spiking procedure of matrices with a powdery consistency can lead to uneven extraction and thus deviating protein content in the extract. Hence the high RSD_R values can be explained due to complicacies during sample preparation rather then imprecision in the performance of the test system.

Comparing the results for recovery it was apparent, that the chocolate samples were continuously underestimated, while the in house spiked samples were underestimated. Further investigations revealed that the spiking values of chocolate samples provided by the project partner were evaluated falsely due to an error during control measurements and indeed contained higher values of peanut.

Also the underestimation of the recovery rates for the in house spiked samples was further investigated. Analysis of the peanut spiking solution used for spiking these samples after the collaborative trial revealed, that the peanut content of the solution was not stable over the time of the study. Indeed the recovery of the peanut spiking solution was only 40% after the end of the collaborative trial. Hence, underestimation of these peanut samples was clearly due to the instability of the peanut spiking solution.

4.5.4.2 Statistical evaluated results for hazelnut samples

Statistical data for hazelnut spiked samples measured on hazelnut ELISA are displayed in Table III.17.

Table III.17: Statistical data for hazelnut samples

measured values	matrix	spiking value [mg/kg]	results mean [mg/kg]	RSD_r [%]	RSD_R [%]	recovery [%]
15	dark chocolate	2.5	5.4	10	29	215
11	dark chocolate	10	11.9	4	12	119
14	ice cream	20	23.9	2	24	119
16	salami	8	8.6	7	24	108
14	instant soup	15	19.4	8	28	129
16	cornflakes	35	39.6	9	50	113
10	yogurt	6	3.46	8	58	58

Recoveries for the majority of samples ranged between 108% and 129% of the spiked value, outlying values for recovery were observed for dark chocolate, 2.5 mg/kg hazelnut (215 %) and yogurt, 6 mg/kg hazelnut (58%).

Considering the deviating result for the dark chocolate, 2.5 mg/kg hazelnut two complicating factors combined for this sample. First dark chocolate is known to be a rather challenging matrix for ELISA measurements, in fact this can not be the principal reason, because the recovery of the dark chocolate 10 mg/kg was thoroughly acceptable (119%). So for second the spiking value of 2.5 mg/kg coincides with the LOD of the assay, it is also known that the accuracy of an ELISA measurement decreases at the endpoints of the calibration curve. Hence the overestimation of the dark chocolate sample (2.5 mg/kg hazelnut) is evoked by the combination of these two complicating factors.

Also the low recovery (58%) for the yogurt sample (6 mg/kg hazelnut) was further investigated. Similarly to the salad dressing samples, verification of the pH value revealed a rather acidic pH for the yogurt matrix (pH 4.5). Thus it was again concluded, that matrix effects occurring at such low pH values lead to this underestimation.

Comparing the results for relative standard deviations between the laboratories (RSD_R) by neglecting the results for the yogurt matrix, the deviation was highest for the cornflakes sample (50% RSD_R). Reason for the deviation obtained for this matrix was again the spiking problem mentioned before (see chapter 4.4). Thus as for the peanut ELISA all discrepancies were on account of sample-related problems rather than impaired performance of the hazelnut ELISA.

4.5.5 Conclusion of the ELISA results

The application of the newly developed test kits on various food matrices in the collaborative trial revealed that the developed ELISA system cannot be regarded as suitable tool for the determination of peanut and hazelnut in matrices with an acidic pH value (< pH 4.5) like salad dressing or yogurt due to the protein derogating effects of such matrices during storage.

The hazelnut ELISA was successfully validated for the 5 food matrices dark chocolate, ice cream, salami, instant soup and cornflakes. Relative standard deviations for the validated matrices varied from 2%-10% (RSD_r) and 12%-50% (RSD_R) respectively, the recoveries ranged from 108%-215%.

Unfortunately validation of the peanut ELISA in the collaborative trial was not possible due to the problems with sample material.

Due to the numerous problematic samples also the performance of the assays in the low concentration range (< 5 mg/kg) could not be evaluated in the collaborative trial.

For both ELISAs the between-laboratory relative standard deviation (RSD_R) was < 50 % for almost all samples with exclusion of some samples with powdery consistency (due to problems during spiking procedure, as mentioned). The overall mean RSD_R for results obtained with the peanut ELISA was 22 % and the mean RSD_R for results obtained with the hazelnut ELISA was 28%. In a similar study it was stated, that due to the complexity of food allergen analysis, the ad-hoc group of CEN TC 275 WG 12 on method performance criteria discussed a value of 50% (and may be even more) for the between-laboratory relative standard deviations (RSD_R) to be acceptable for very low concentration levels (e.g. < 5 mg/kg) [Poms et al, 2005]. Though there were several samples with higher concentration levels (> 5 mg/kg), the overall mean RSD_R values for results were far below 50% for both assays (22 % for peanut ELISA and 28% for hazelnut ELISA). Therefore the comparability of quantitative results was regarded satisfying for an ELISA under the chosen conditions.

4.6 Results from the interlaboratory LFD measurements

All of the 50 samples (25 different samples / 2 lots each) were measured undiluted in duplicate on peanut – and hazelnut LFD, results were read visually. Every participant returned the results to the coordinator of the collaborative trial who accomplished evaluation.

Basically 32 measurements were performed per sample, reason for a reduced number of results were invalid lateral flow devices and errors in performance of measurement.

A result was regarded as positive when more than 50% of the measurements were positive.

4.6.1 Results peanut LFD

The results for measurements of all samples with peanut LFD are summarized in Figure III.41

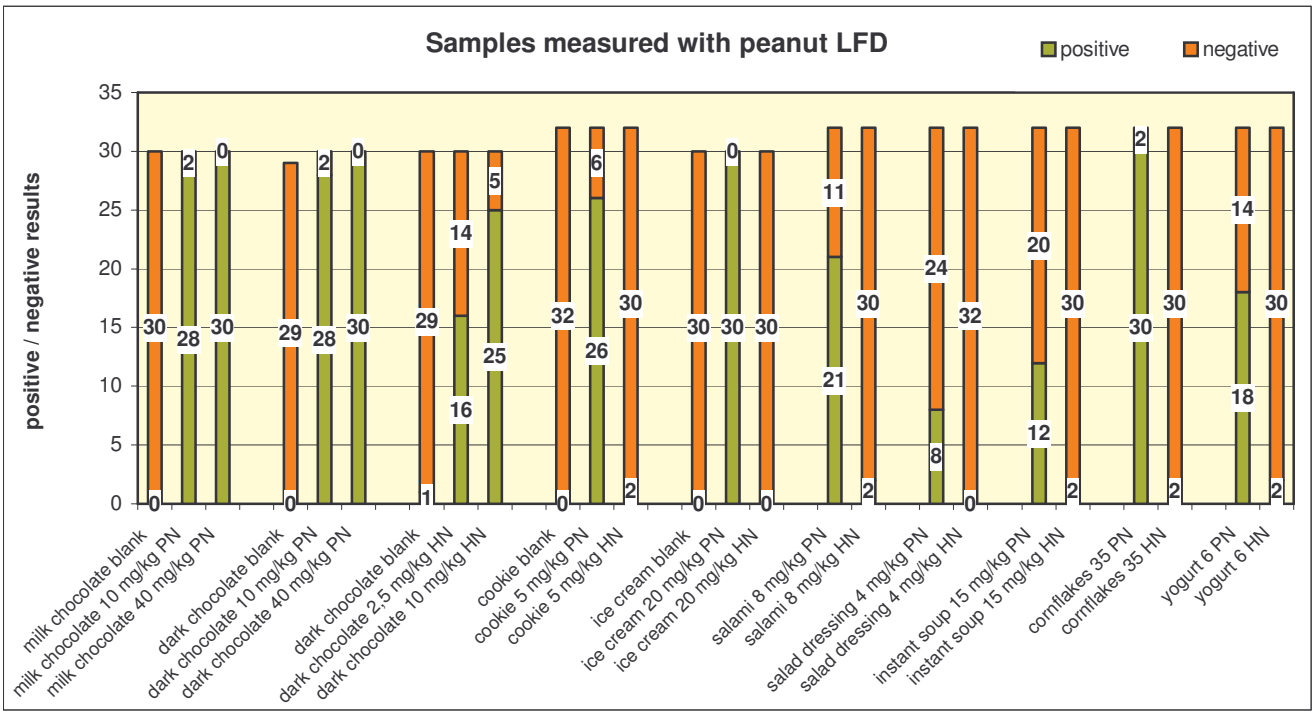


Figure III.41: Results for samples measured with peanut LFD

The mentioned peanut contamination of the dark chocolate used for in house spiking with hazelnut was detected measuring the hazelnut spiked dark chocolate samples with peanut LFD: dark chocolate spiked with 2.5 mg/kg hazelnut (16 positives for peanut out of 30) and dark chocolate spiked with 10 mg/kg hazelnut (25 positives for peanut out of 30).

All the peanut spiked samples were found positive for peanut with exclusion of salad dressing, 4 mg/kg peanut (8 positive results out of 32) and instant soup, 15 mg/kg peanut (12 positive results out of 32). The mentioned protein derogating effects caused by the acidic pH of the salad dressing matrix (as ascertained before, see 4.5.3) was regarded as responsible for the low number of positive results. The underestimation in case of the instant soup sample was interpreted as matrix effect influencing the performance of the test due to the high salt concentration of the extract applied undiluted.

The number of positive measurements was low (though still more than 50%) for the samples salami, 15 mg/kg peanut (21 positives out of 32) and yogurt, 6 mg/kg (18 positives out of 32). Reason for this low number of positive findings were again matrix effects due to the rather high salt content of salami (similar to instant soup) and protein derogation due to the acidic pH value (pH 4.5) of the yogurt matrix (similar to salad dressing).

4.6.2 Results hazelnut LFD

The results for measurements of all samples with hazelnut LFD are summarized in Figure III.42.

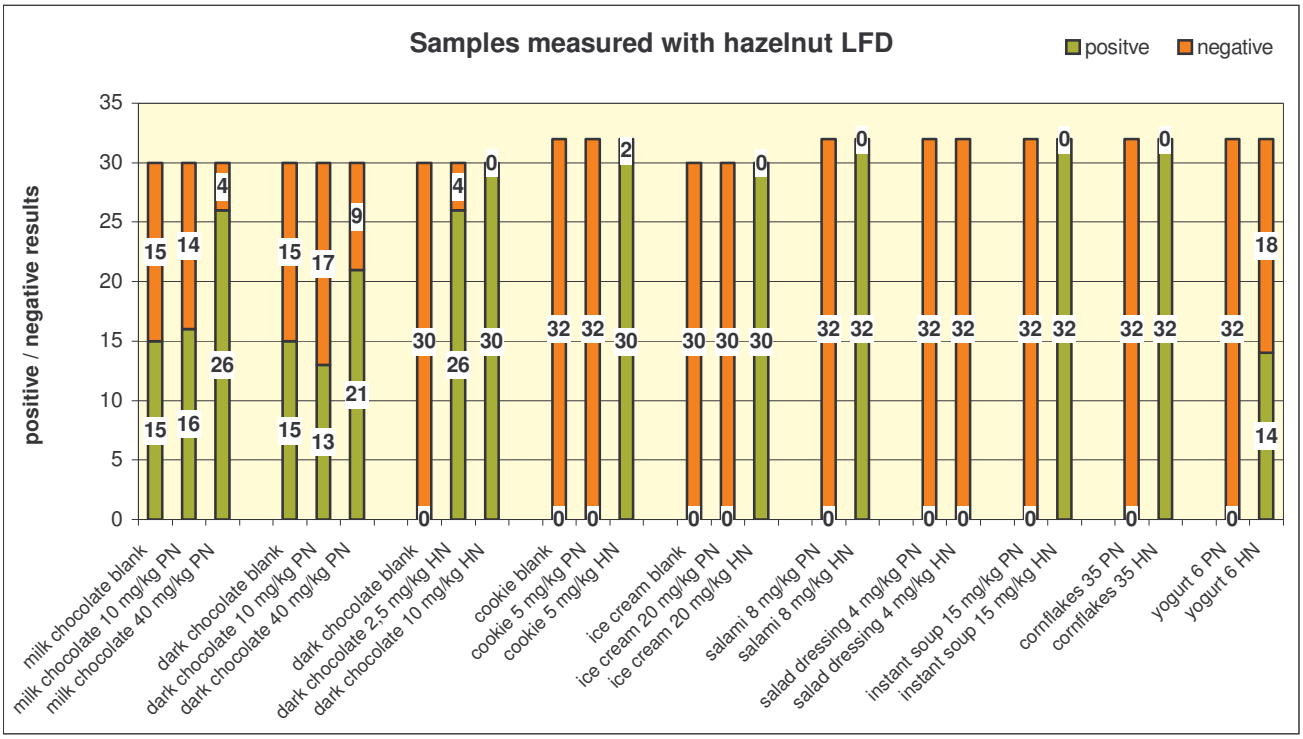


Figure III.42: Results for samples measured with hazelnut LFD

The hazelnut contamination of the commercial milk and dark chocolate samples spiked with peanut was detected measuring the peanut chocolate samples with hazelnut LFD: milk chocolate, blank (15 positives for hazelnut out of 30); milk chocolate spiked with 10 mg/kg peanut (16 positives for hazelnut out of 30); milk chocolate spiked with 40 mg/kg peanut (26 positives for hazelnut out of 30); dark chocolate blank (15 positives for hazelnut out of 30); dark chocolate spiked with 10 mg/kg peanut (13 positives for hazelnut out of 30); dark chocolate spiked with 40 mg/kg peanut (21 positives for hazelnut out of 30).

All the hazelnut spiked samples were found positive for hazelnut apart from salad dressing, 4 mg/kg hazelnut (0 positive out of 32) and yogurt, 6 mg/kg hazelnut (14 positives out of 32).

The reason for the underestimation of these samples was, as described above, a reduced spiking value due to the acidic pH of the matrices salad dressing and yogurt.

4.6.3 Statistical analysis of the LFD results

Statistical analysis of the LFD data obtained from the collaborative trial was carried out by a project partner (CSL). Sensitivity (the proportion of true positives identified by the test) and specificity (the proportion of true negatives identified by the test) with 95% confidence intervals were determined, see Table III.18 and Table III.19. Also accordance (agreement within laboratories) and concordance (agreement between laboratories) were calculated for the true positive and the true negative measurements, with 95% confidence intervals by the method of Langton, see Table III.20 and Table III.21.

Table III.18: Sensitivity of LFD measurements

	peanut LFD	hazelnut LFD
true positive total	156	180
true positive correct	129	125
sensitivity	82.7%	69.4%
lower 95% limit	76.8%	62.7%
upper 95% limit	88.6%	76.2%

Table III.19: Specificity of LFD measurements

	peanut LFD	hazelnut LFD
true negative total	144	120
true negative correct	144	120
specificity	100%	100%
lower 95% limit	100%	100%
upper 95% limit	100%	100%

Table III.20: Accordance and concordance for true positive LFD measurements

	peanut LFD	hazelnut LFD
accordance	75.95%	64.79%
lower 95% limit	72%	60.69%
upper 95% limit	82.26%	71.49%
concordance	70.28%	55.88%
lower 95% limit	62.74%	51.16%
upper 95% limit	78.13%	61.59%

Table III.21: Accordance and concordance for true negative LFD measurements

	peanut LFD	hazelnut LFD
accordance	100%	100%
lower 95% limit	100%	100%
upper 95% limit	100%	100%
concordance	100%	100%
lower 95% limit	100%	100%
upper 95% limit	100%	100%

4.6.4 Conclusion of the LFD results

The peanut LFD was able to detect peanut content in the food matrices milk chocolate, dark chocolate, cookies, ice cream, and cornflakes, the sensitivity of measurement was 82.7%, the specificity of measurement was 100%. The hazelnut LFD proofed capable to detect hazelnut in the food matrices dark chocolate, cookies, ice cream, salami, instant soup, and cornflakes, the sensitivity of measurement was 69.4% and the specificity was 100%. For the true positive samples the accordance of measurements was 75.95% for the peanut LFD and 64.79% for the hazelnut LFD, the concordance of measurements was 70.28% for the peanut LFD and 55.88% for the hazelnut LFD. For the true negative samples the accordance and concordance of measurements was 100% for both LFDs.

This means that all the blank samples have been identified correctly and there have been no false positive results with neither of the LFDs. The sensitivity of measurements was higher for the peanut LFD, also the performance in the collaborative trial was better with the peanut LFD, than with the hazelnut LFD.

Due to the protein derogating effect of matrices with an acidic pH value (like salad dressing and yogurt) that lead to falsification of the spiking value both LFDs were not suitable for the analysis of such matrices. For the peanut LFD additional matrix effects influencing the performance of the test were observed for samples with high salt content (like instant soup and salami). The hazelnut test was insensitive against high salt concentrations.

With both LFDs it was possible to detect peanut / hazelnut concentrations as low as 5 mg/kg in cookie matrix with an absolute majority of positive results in the collaborative trial. Therefore both assays proved to be rapid easy and sensitive tools for the detection of trace amounts of peanut / hazelnut in food.

4.7 Comparison of the interlaboratory ELISA and LFD results

None of the assays could be regarded as suitable tool for the determination of peanut / hazelnut in matrices with an acidic pH value (< pH 4.5) due to the protein derogating effect occurring during storage. Only the peanut LFD exhibited sensitivity against samples with high salt content. None of the assays, produced false positive results, all blank samples were correctly determined as negative. Due to the stability problems with the peanut spiked samples no valid comparison of results found with peanut ELISA and peanut LFD was possible. Measurements of the hazelnut spiked samples lead to congruent results with hazelnut ELISA and LFD. All the hazelnut spiked samples (with exclusion of matrices with an acidic pH) were found positive correctly with both assays.

4.8 Conclusion of the collaborative trial

The objectives of the study, to demonstrate the comparability of measured results between different laboratories when applying these new test kits to various food matrices containing traces of peanut / hazelnut, were met for the most part. The major drawback of the study was the lack of usable results for the peanut assays due to the instability of spiking value in the peanut samples. Though the problem was not apparent directly after spiking (acceptable recoveries were observed for peanut samples in the homogeneity study), preliminary storage experiments could have revealed the instability of the peanut spiking solution. Also the protein derogating effect of the matrices with acidic pH value (salad dressing, yogurt) could have been detected in preliminary storage experiments. Nevertheless these experiences are valuable inputs for similar studies in the future. There are several other reports on ELISA tests for the detection of peanut and hazelnut in food matrices [Yeung and Collins, 1996; Akkerdas et al, 2004; Drs et al, 2004; Poms et al, 2005], only few of them are fully validated. LFD tests for various analytes have been developed and also partly validated in interlaboratory evaluation studies [Hasegawa et al 2002; Danks et al, 2003; O`Keeffe, 2003]. However by now there have been no LFDs for the detection of peanut and hazelnut in food.
Anyway this study is the first attempt of a collaborative trial employing ELISA and LFD techniques for the detection of peanut and hazelnut on such a wide range of different food matrices. For both assays the agreement of results between the different participating laboratories was good, also the results compared for the two types of assays were congruent. Finally it can be concluded, that the developed assays represent rapid, sensitive and easy to use methods for the detection of trace amounts of peanut and hazelnut in food matrices as it was demanded for quality control, safety assurance and allergen monitoring.

5 FOOD SURVEY

The topic of the food survey was the analysis of approximately 40 pre-packaged food samples from 11 different countries regarding their content of peanut and hazelnut respectively. Therefore measurements with the peanut and hazelnut ELISA and LFD tests were performed. The objectives to be investigated were on the one hand the performance of the newly developed test kits on commercial food products and on the other hand the real content of peanut and hazelnut in the various products compared to the labelling on the package.

The results of the ELISA and LFD tests were concordant, a positive LFD result corresponded each with an ELISA result above the limit of quantification (LOQ for peanut and hazelnut ELISA: 2.5 ppm). Content of peanut and hazelnut was verified in all products were it was indicated as ingredient and also in a wide range of products were it was not declared.

A detailed list of the analyzed samples and the number and kind of products wherein undeclared contents of peanut or hazelnut were found is displayed in Table III.22.

Table III.22: Results Food Survey

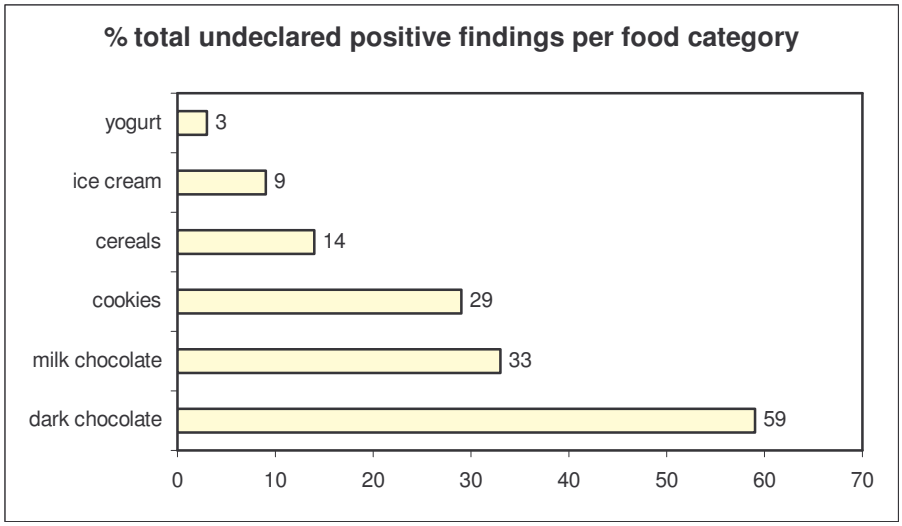
country	kind of sample	total number of samples	undeclared positive for peanut	undeclared positive for hazelnut
Austria	milk chocolate	15	1	-
	cookies	14	3	6
	cereals	4	-	-
	yogurt	5	-	-
Belgium	milk chocolate	9	1	-
	dark chocolate	10	1	2
	cookies	8	-	-
	cereals	10	-	1
Czech Republic	milk chocolate	8	1	4
	cookies	17	-	2
	cereals	12	4	2
France	milk chocolate	5	-	-
	dark chocolate	5	-	-
	cookies	9	-	3
	cereals	7	-	-
	ice-cream	4	-	-
	yogurt	6	-	-

country	kind of sample	total number of samples	undeclared positive for peanut	undeclared positive for hazelnut
Greece	milk chocolate	8	2	5
	dark chocolate	5	1	5
	cookies	11	1	2
	cereals	10	-	-
Italy	milk chocolate	3	-	2
	dark chocolate	4	-	4
	cookies	15	1	2
	cereals	6	-	-
	ice-cream	11	-	2
Norway	milk chocolate	9	-	1
	cookies	8	-	-
	cereals	11	-	2
	ice-cream	7	-	-
Portugal	milk chocolate	7	-	4
	dark chocolate	6	-	4
	cookies	11	-	3
	cereals	10	-	-
Slovenia	milk chocolate	11	-	4
	dark chocolate	2	-	2
	cookies	14	1	11
	cereals	8	-	3
	ice-cream	2	-	-
	yogurt	2	-	-
Spain	cookies	10	-	-
	cereals	7	-	-
	ice-cream	11	-	1
	yogurt	9	-	1
UK	milk chocolate	9	-	3
	cookies	13	-	2
	yogurt	7	-	-

5.1 Findings of undeclared nut content

Undeclared nut content was found in 25,3% of all the analysed food products, thereof 4.3% was peanut and 21% was hazelnut, in 2% of products both peanut and hazelnut was found. The majority of findings had a concentration below 10 ppm but there were also some results in the three-or four-digit range.
Undeclared hazelnut content is definitely more significant than undeclared peanut content, contamination with both nuts simultaneously is of minor relevance.

5.1.1 Undeclared positive findings per food category



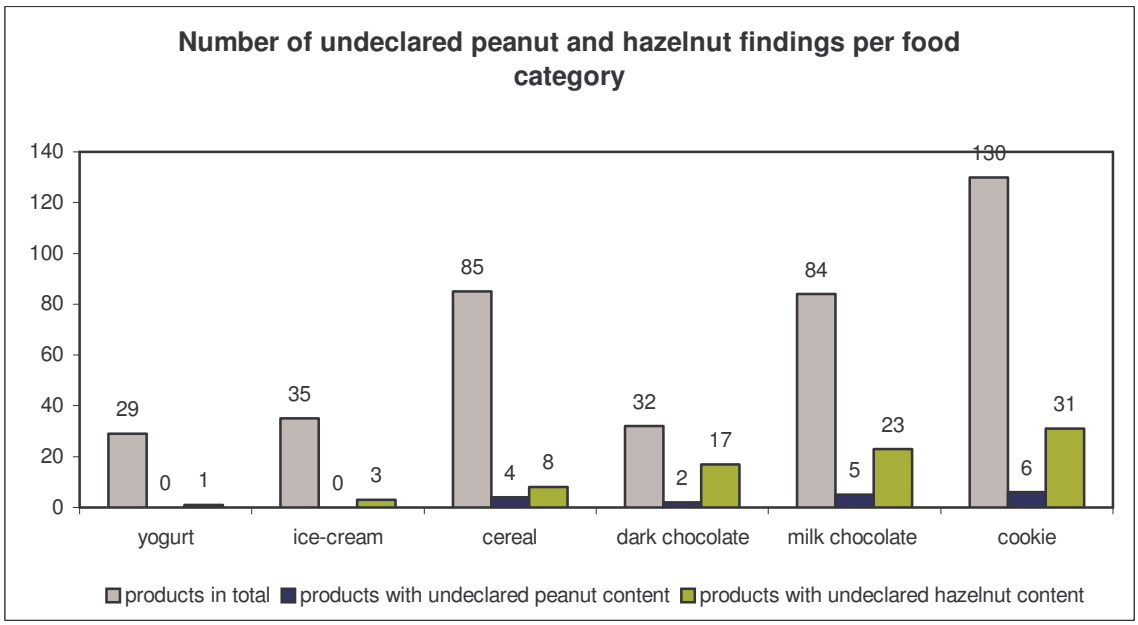
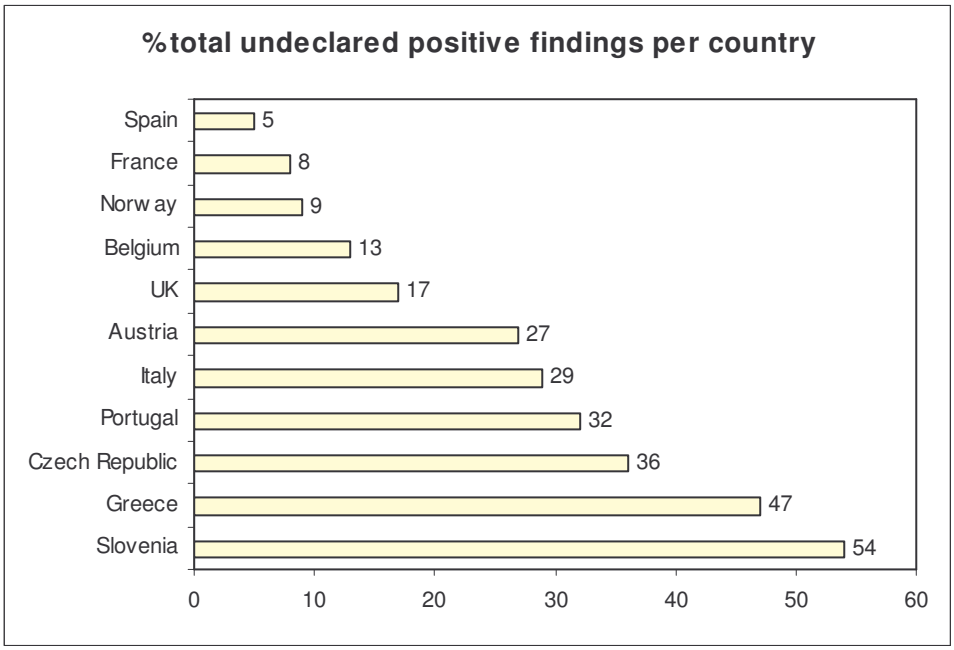


Figure III.43: Undeclared findings per food category

The food categories with the highest percentage of items with undeclared nut content in total are dark chocolate (59%), followed by milk chocolate (33%), and cookies (29%) as displayed in Figure III.43, upper table. Product classes with lower percentages of items with undeclared nut content are cereals (14%), ice cream (9%) and yogurt (3%). The cookie products with undeclared nut content often contained chocolate too, therefore it can be concluded, that milk – and dark chocolate are the matrices with the highest incidence of undeclared peanut or hazelnut content. The proportion of undeclared hazelnut findings is again substantially higher (see Figure III.43, lower table) as observed before.

5.1.2 Undeclared positive findings per country



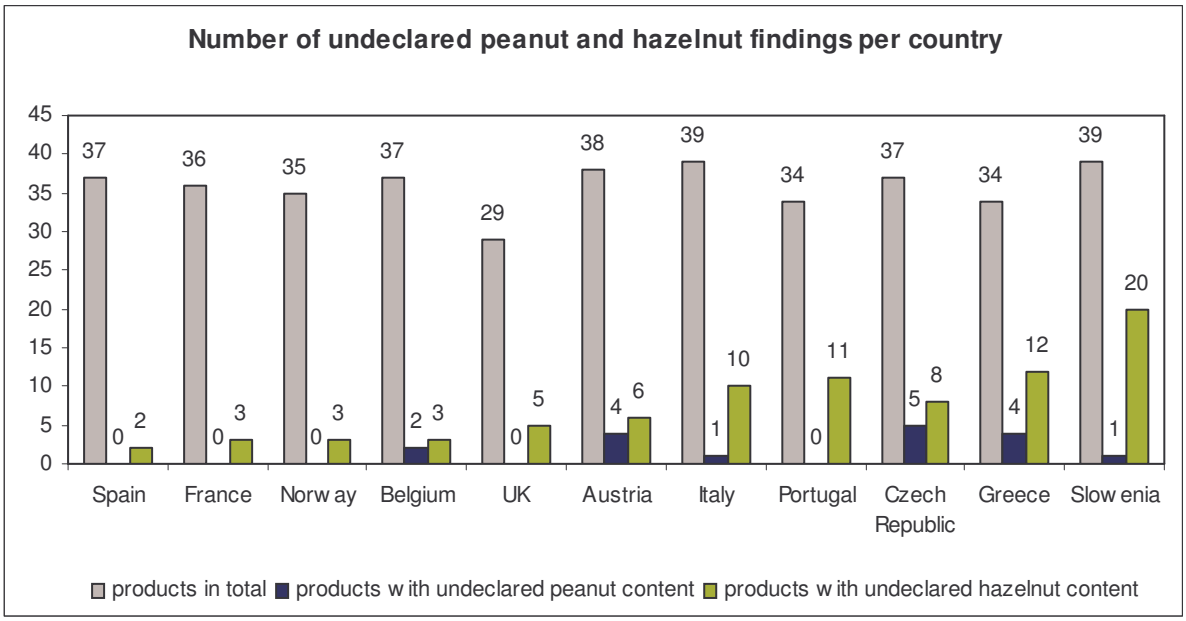


Figure III.44: Undeclared findings per country

The country with the highest percentage of products with undeclared nut content is Slovenia, the lowest percentage of items with undeclared nut content was found for the products from Spain (see Figure III.44, upper table). However Spain was the only country that did not send any chocolate products. As remarked before results for the other countries indicated, that products with chocolate matrix had the highest incidence of undeclared nut content. Therefore the absence of chocolate in the selection of the Spanish products might be the main reason for the low percentage of undeclared positive findings.
Again hazelnut accounted for the majority of undeclared positive findings.

5.2 False positive declaration

In some cases products were declared to contain traces of peanut or hazelnut but the measurements did not indicate any positive result. Products with such declarations and ELISA results below the limit of detection (LOD for peanut and hazelnut ELISA: 1.5 ppm) were defined as “false positive declared products”. Effectively these products might contain nut concentrations below the LOD of the test systems, nevertheless to allow an overview of tendencies percentages of false positive results for each country were outlined in Figure III.45. under the aspect that they only show approximate values.

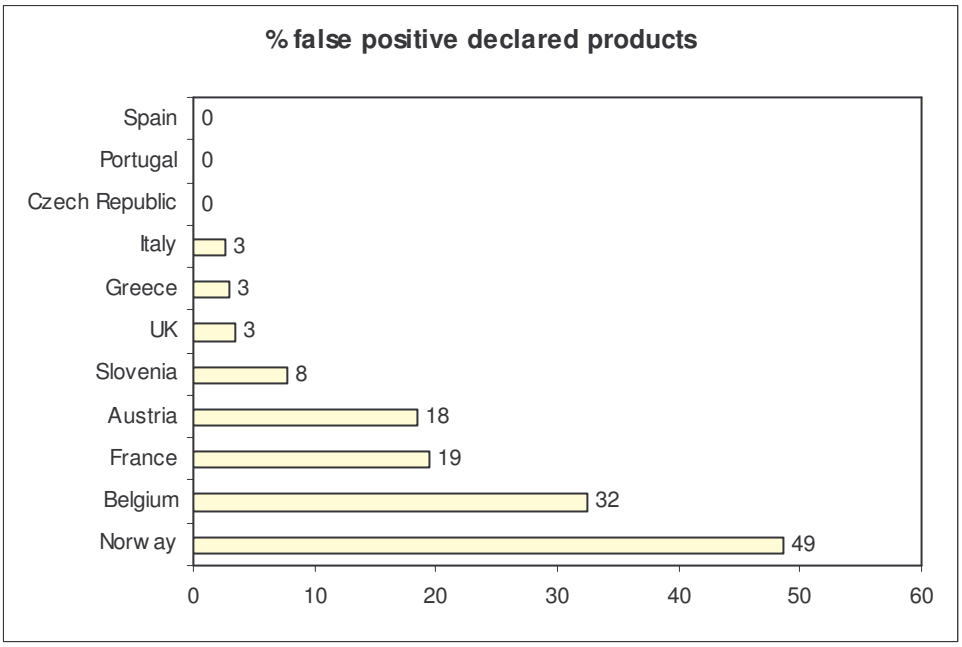


Figure III.45: False positive declared products

12% of total food products showed false positive declaration.
Consequentially those countries with a low percentage of undeclared positive findings have a high percentage of ”false positive declared” products.

Norway for example has the highest percentage of false positives (49%) and a comparatively low number of undeclared positive findings (see Figure III.44). For Spain, Portugal and Czech Republic no false positive declared products were found. Here it must be taken into account that the majority of products from these countries had no declarations concerning nut content at all.

5.3 Conclusion

The purpose of the Food Survey was the analysis of pre-packaged food products from different European countries regarding their content of peanut and hazelnut.
In doing so the performance of the newly developed ELISA and LFD kits on commercial food products was reviewed and the content of peanut and hazelnut in the various products compared to the labelling on the package.
The ELISA and LFD test kits proofed capable to detect peanut and hazelnut also in complex commercial food products. Declared contents of peanut and hazelnut were detected and the ELISA and LFD results accorded.
Comparing the detected contents of peanut and hazelnut with the declarations on the respective food products two kinds of discrepancies emerged, on the one hand there were declarations on products that did not contain peanut or hazelnut (“false positive declaration”), on the other hand several products showed undeclared contents of peanut and hazelnut.
12% of products in total had “false positive declarations”, 25.3% of total products contained peanut and hazelnut undeclared.
Recapitulating these results two modes of labelling policy can be observed:
1) < labelled products = < false positive declared products → > undeclared positive findings
→ < food safety
2) > labelled products = > false positive declared products → < undeclared positive findings
→ > food safety
reflecting the extent of awareness of the subject food allergy and the degree of food safety for allergic consumers in the respective countries.
The “false positive declarations” are less critical and appear mainly in countries with a high awareness of the subject food allergy. Food producing companies try to safeguard themselves from getting in conflict with the allergic consumer by indicating warnings on food products rather generously. However this strategy in the end leads to a drastically reduced choice of products for persons suffering from peanut and hazelnut allergy.
Products with undeclared contents of peanut and hazelnut on the other hand represent a serious risk for the sensitized consumer due to the possibility of unintended allergen intake.

Hence false declarations of any kind should be prevented and therefore fast and sensitive immunoassays are necessary, to audit the accurate degree of contamination at various steps during the food production process.

Food products with a special high degree of undeclared nut content were chocolate (milk and dark chocolate) and cookies. In general hazelnut was found in many more cases than peanut. This is most likely due to European consumption habits and is in accordance with literature [Groot et al, 1996].

IV FINAL CONCLUSION

FINAL CONCLUSION

A major objective of this thesis was the organisation, performance and evaluation of collaborative studies on the detection of peanut and hazelnut proteins in food with novel immunoasssays (ELISA and LFD). Additionally a food survey on pre-packaged food samples from 11 different countries was conducted with the new immunoassays. The development of these assays, the collaborative trial and the food survey were carried out within the framework of the EC founded project “AllergenTest”.
Besides, the labelling of peanut and hazelnut specific monoclonal and egg yolk antibodies to colloidal gold and fluorescent dye was optimised. Dipstick assays and microarrays were developed employing both types of antibodies and their performance compared.

For the performance of the collaborative trial samples were prepared by spiking nine, in part complex food matrices, with various amounts of milled peanut or hazelnut powder. The homogeneity of samples was surveyed. Eight laboratories from five different states within the European Union (Austria, Germany, Italy, Netherlands, U.K.) participated in the trial. Each participant received an identical set of samples and an appropriate number of test kits. Samples were extracted, measured in duplicate on ELISA and LFD, subsequently results were evaluated statistically.
Application of the new test kits on the various food matrices revealed that none of the 4 test kits is suitable for measurement of matrices with an acidic pH value (like salad dressing and yogurt) due to the protein derogating effect of that lead to falsification of the spiking value.
For the peanut LFD additional matrix effects influencing the performance of the test were observed for samples with high salt content (like instant soup and salami).
The hazelnut ELISA was successfully validated for the 5 food matrices dark chocolate, ice cream, salami, instant soup and cornflakes. Relative standard deviations for the validated matrices varied from 2%-10% (RSD_r) and 12%-50% (RSD_R) respectively, the recoveries ranged from 108%-215%. Unfortunately validation of the peanut ELISA in the collaborative trial was not possible due to instability of the spiking value in the sample material.
With the peanut LFD peanut content in the food matrices dark chocolate, milk chocolate, cookies, ice cream, and cornflakes, was successfully detected, the sensitivity of measurement was 82.7%, the specificity of measurement was 100%.
The hazelnut LFD proofed capable to detect hazelnut in the food matrices dark chocolate, cookies, ice cream, salami, instant soup, and cornflakes, the sensitivity of measurement was 69.4% and the specificity was 100%. For the true positive samples the accordance of measurements (agreement within laboratories) was 75.95% for the peanut LFD and 64.79% for the hazelnut LFD, the concordance of measurements (agreement between laboratories) was 70.28% for the peanut LFD and 55.88% for the hazelnut LFD. For the true negative samples the accordance and concordance of measurements was 100% for both LFDs.

Additionally the developed ELISA and LFD kits were employed in a food survey. Approximately 40 pre-packaged food samples from 11 different countries were analysed regarding their content of peanut and hazelnut respectively. The performance of the newly developed ELISA and LFD kits on commercial food products was reviewed and the content of peanut and hazelnut in the various products compared to the labelling on the package. The ELISA and LFD test kits proofed capable to detect peanut and hazelnut also in complex commercial food products.
25.3% of total products contained peanut and / or hazelnut and did not display any declaration on the package. 12% of products in total had “false positive declarations”, which means content of peanut / hazelnut was declared on the package, but products that did not contain peanut / hazelnut.

Concluding, the results of the internal validation of test kits, the collaborative trial and the food survey indicate that the 4 new developed test kits are appropriate for the determination of traces of peanut and hazelnut protein in various complex food matrices with the exception of matrices with an acidic pH value. Several similar test systems have been developed, nevertheless only a few of them are fully validated. Although there are some other reports on ELISA tests applied to various food products [Koppelman et al 1999; Drs et al, 2004; Akkerdaas et al, 2004] the collaborative trial in the present study was the first attempt where a peanut / hazelnut ELISA was applied to a wide range of different food matrices within a collaborative trial. LFD tests for various analytes have been developed and also partly validated in interlaboratory evaluation studies [Hasegawa et al 2002; Danks et al, 2003; O`Keeffe, 2003]. However by now there have been no LFDs for the detection of peanut and hazelnut in food.

Another topic of this work was the development of dipstick assays and microarrays for the detection of peanut and hazelnut in food. Monoclonal and egg yolk antibodies were used for the construction of the assays in a sandwich format. The microarray technology is a rather new field of research in general, application of antibody microarrays for the quantitative analysis of complex protein solutions is still quite uncommon [Angenendt, 2005]. This was the first attempt to use antibody microarrays for the detection of peanut and hazelnut proteins in food. Although egg yolk antibodies are an inexpensive, convenient and animal friendly alternative to antibodies derived from mammals, they are rarely employed in the development of fast immunoassays and immunoassays in general. This study was one of the first efforts to apply egg yolk antibodies for the detection of hidden allergens in food.

The application of egg yolk antibodies and the positive detection of peanut / hazelnut extract was possible in both test systems, when the egg yolk antibodies were combined with monoclonal antibodies in the sandwich format. However the assay formats with egg yolk antibodies were less sensitive compared to formats with monoclonal antibodies only.

A dipstick with egg yolk antibody for capture and monoclonal antibody for detection lead to weak signals for concentrations between 50-100 µg/ 10 ml nut, in contrast limit of detection of the dipsticks with exclusively monoclonal antibodies was 30 µg/10 ml for peanut extract and 10 µg/10 ml for hazelnut extract respectively.

In case of the microarray combinations with monoclonal antibody for capture and IgY as detection antibody enabled detection down to 10 µg/ 10ml nut compared to limits of detection of 0.6-0.8 µg/10 ml for peanut extract an 2 µg/10 ml for hazelnut extract respectively for the microarray formats with monoclonal antibodies exclusively.

The conclusions of this part of the thesis are, that egg yolk antibodies are presently not able to meet the performance of monoclonal antibodies in immunoassays, especially regarding sensitivity and selectivity, as previous studies have already indicated [Drs, 2004].

Therefore sandwich formats with monoclonal antibodies exclusively were chosen for the development of dipstick and microarray assays.

The limit of detection for the peanut dipstick was between 40-100 ppm for the matrices milk chocolate, cookies, cornflakes, instant soup, ice cream and yoghurt, the hazelnut dipstick had a limit of detection at 30 ppm for all of these matrices.

For the peanut microarray the limit of detection for the mentioned matrices ranged between 1-2.5 ppm, no such validation data was evaluated for the hazelnut microarray due to problems in reproducibility of performance of this assay.

Hence the microarray has the potential to reach lower detection limits compared with the dipstick however the dipstick format rather meets the requirements of a rapid and easy-to-use immunoassay, because of its short incubation times and the independence of laboratory equipment.

The microarray technology was for the first time successfully employed for the detection of traces in peanut and hazelnut in complex protein mixtures, although further improvements on reproducibility and background signals would be necessary to create a reliable test kit for the analysis of food products. The dipstick assays should be further optimised to achieve maximum sensitivity, which requires the availability of high affinity antibodies with minimum cross-reactivity. For both assay types the simultaneous detection of peanut and hazelnut proteins will be a major challenge for future studies.

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VI APPENDIX

Abbreviations

A	absorption
au	arbitrary units
ANOVA	statistical analysis of variance
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	bovine serum albumin
CMC	carboxymethylcellulose
EAST	enzyme-allergosorbent test
EDTA	ethylenediaminetetraacidic acid
ELISA	enzyme linked immunosorbent assay
FPLC	fast protein liquid chromatography
HRP	horseradish peroxidase
IgY	Immunoglobuline of class Y
LFD	lateral flow device
mAb	monoclonal antibody
MES	2-(N-Morpholino)ethanesulfonic acid
MW	molecular weight
NBT	nitro blue tetrazolium
pAb	polyclonal antibody
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMT	photomultiplier
ppm	part per million
RAST	radio-allergosorbent test
RIA	radio-immunoassay
RIE	immunoblotting
RSD	relative standard deviation
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TEM	transmission electron microscope
TMB	3, 3´,5, 5´-tetramethylbenzidine

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