Review:

Determination of Hidden Allergens in Foods by Immunoassays

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SUMMARY

Hidden food allergens present a potential threat to allergic individuals. In Europe mandatory labelling of the most important food allergens is in preparation (Amendment of EU Food Labeling Directive). On the other hand there are only a few validated methods for the detection and quantitation of minute amounts of allergens in foods. Immunological methods can involve either human IgE or animal antisera. Dot-immunblotting and SDS-PAGE / immunoblotting are sufficient for qualitative detection of food allergens, while Rocket-immunoelectrophoresis and Enzyme-linked immunosorbent assays (ELISA) are applications to quantitate hidden food allergens. The performance of the methods such as their sensitivity, specificity, limit of detection, recovery and reproducability are reviewed in detail.
(Internet Symposium on Food Allergens 2002, 4(1):1-18)

KEYWORDS

hidden allergens
food manufacture
food labeling
immunodiffusion
immunoblotting
immunoelectrophoresis
ELISA

[Introduction] [Sources of Hidden Allergens] [Amounts] [Methods of Determination] [Common Criteria] [Rocket-Immunoelectrophoresis] [Dot-Immunoblot] [SDS/PAGE-Immunoblot] [Competitive-ELISA] [Sandwich-ELISA] [RAST/EAST-Inhibition] [RAST vs ELISA] [Commercial Test-Kits] [Frequency of Undeclared Allergens] [Conclusions] [References] [Abbreviations]

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INTRODUCTION

The problem of hidden food allergens has been recognized for decades (Miller 1978). In most cases hidden food allergens may induce only mild symptoms in allergic subjects, but tragically even fatal events have occurred after inadvertent ingestion (Sampson 1998, Wüthrich 2000, Bock 2001). In Canada and the USA the food authorities frequently publish alerts on recalls of food products which may contain most severe food allergens not declared on the labels. Labeling of food products for the presence of food allergens is at present the most effective way to enable food allergic individuals to avoid the ingestion of hidden allergens. Therefore the aim of allergen determination in foods is of major concern for both the food industry and the food allergic consumer, and testing foods for the presence of allergens should have a definite place in the HACCP (hazard analysis and critical control point) plans and allergen control plans of food manufacturers (Deibel et al. 1997, Hugget & Hischenhuber 1998).

Only recently the FAO/WHO and the European Commission proposed a list of allergens which have to be labelled on prepackaged foods regardless of the amounts present. The allergen lists are based on the prevalence and severity of the related allergies. The stability of these food allergens, their allergenic potential and frequency in processed foods should be considered as well (Bousquet et al. 1998, Yeung et al. 2000). The Codex Alimentarius standard includes milk, eggs, fish, crustaceae, peanut, soybean, tree nuts, and wheat (gluten-containing cereals), while the European proposal additionally includes sesame seeds (Table 1). The food allergens to be included should be subject to a continuing scientific evaluation. For example celery is not included although the scientific criteria for inclusion have been fulfilled recently (Ballmer-Weber et al. 2000). Currently both sets of labeling regulations do not cover allergen contamination of food products by "cross-contact".
US-Attorneys called for reforms in food labelling and processing in a recent Citizen Petition to the U.S. Food and Drug Administration (2000). The petition demanded a symbol on the label to alert consumers that the product in the package contains allergens such as peanuts, tree nuts, milk, eggs, fish, crustaceans, molluscs, wheat or soybeans; declaration when allergenic ingredients are used even in small amounts that are currently designated as "insignificant levels"; a toll-free hotline where consumers can obtain reliable food ingredient information, and food industry guidelines to prevent the migration of allergenic ingredients from one product to another during food processing and preparation.

For these reasons the detection and determination of hidden allergens in foods is becoming more and more important. There is clearly a need for analytical methods which are highly specific and sensitive in detecting even trace amounts of allergens. These methods need to be rapid, robust, reliable, and cost-effective. This review gives a short overview of circumstances leading to the presence of hidden allergens in foods. After discussing the amounts of hidden allergens in foods which can elicit allergic symptoms, the analytical methods for the detection of food allergens are introduced in detail. A brief explanation of the principle of

Table 1: List of food allergens to be labelled on prepackaged foods

FAO/WHO Standard (Codex Alimentarius Commission 1999)	**Amendment of Labelling Directive** (Proposal from the European Commission 2001)
Milk	Milk
Hen's Egg	Hen's Egg
Fish	Fish
Crustaceae	Crustaceae
Peanut	Peanut
Tree Nuts	Tree Nuts
Soybean	Soybean
Wheat	Wheat
	Sesame Seed

each detection method is followed by some selected applications. It should be noted that the cited methods have been selected on the basis of sufficient limits of detection and successful application to authentic food samples (for a recent review including a broader range of applications see Besler 2001). Assays for the determination of wheat proteins (gluten / gliadins) are not included. These methods were recently reviewed by Denery-Papini et al. (1999).

SOURCES OF HIDDEN ALLERGENS IN FOOD PRODUCTS

Circumstances of food manufacture which result in the presence of hidden allergens in foods include many potential sources (Deibel et al. 1997, Hugget & Hischenhuber 1998). Global trade and transport often makes it extremely difficult to exclude the presence of certain allergenic compounds. Major reasons for the occurrence of hidden allergens in processed foods are:

- **Cross-contact**, which is a problem arising from using the same equipment for the production of foods containing a specific allergenic compound and for the production of foods not containing this compound (shared equipment).
- **Carry-over** of an allergenic compound may occur during food production, for example if inappropriate rework containing an allergenic ingredient is used.
- **Changes of the formulation** of a product without appropriate changes on the label.
- **Incomplete or incorrect lists of ingredients**
- The raw materials may contain **unknown ingredients**
- **Misinterpretation** of common names or ingredients could be derived from allergenic sources which are not indicated on the label
- **Exemptions of labelling** in the labelling regulations. For example ingredients of a compound which constitutes less than 25% of the food product do not have to be labelled. (The so-called 25%-rule will be deleted according to the proposed amendment of the EU labelling directive.)

AMOUNTS AND THRESHOLDS OF HIDDEN FOOD ALLERGENS

Only in a minority of allergic events involving the ingestion of food products did it prove possible to quantitate or even identify the allergenic source. Some cases where the allergenic source was determined are given in Table 2. The detected food allergens include peanut, hazelnut, milk, and egg. Ingested foods were a dry soup, chocolate, cookies, a fruit sorbet, icecream, a sausage and pasta. Generally the ingested amount of protein ranged from 10 to 100 mg. In only two cases was the ingestion of lower amounts described. The first case involved the hidden presence of hazelnut protein in a chocolate. 700 µg of hazelnut protein were reportedly ingested. The other event occurred after ingestion of 120-180 µg of whey proteins in a fruit sorbet. On the basis of ingestion of 100 g of a respective food the lowest concentrations of hidden allergens were about 1.2-1.8 mg/kg and 7 mg/kg, while the concentrations ranged from 100 to 1000 mg/kg in the other reports.

Taylor et al. (2002) identified considerable data related to the threshold doses for peanut, cow's milk, and egg, analyzing clinical files; only limited data were available for other foods, such as fish and mustard. However, the authors concluded that the estimation of a threshold dose is very difficult and a standardized protocol for clinical experiments to allow determination of the threshold dose should be developed.

The lowest doses eliciting allergic symptoms in DBPCFC studies were 4 mg of peanut, 6 mg of codfish, and 50 mg of egg white (Hourihane et al. 1997, Hansen & Bindslev-Jensen 1992, Norgaard & Bindslev-Jensen 1992). Short-lived, subjective symptoms occurred after ingestion of 100 µg peanut protein. While severe, systemic reactions were induced by ingestion of 5 mg peanut protein (Hourihane et al. 1997). Assuming an ingestion of 100 g of an offending food, a concentration of at least 50 mg/kg peanut protein should be detectable in processed foods with respect to severe allergic reactions.

Most recently Morisset & Moneret-Vautrin (2001) proposed threshold levels of clinical reactivity to food allergens evaluating a standardized placebo-controlled oral challenge protocol. In this study cases of severe food allergy corresponded to positive oral challenges with cumulative reactive doses of less than 6.5 mg of

egg protein, 32 mg of milk protein, 16 mg of peanut protein, and 12 mg of sesame protein. On the basis of an ingestion of 100 g of an offending food the authors demand assay detection limits of 65 mg/kg for egg proteins, 300 mg/kg for milk proteins, and 165 mg for peanut proteins in foods. However 0.8% of 125 egg allergic patients, 1.7% of 59 milk allergic patients, and 3.9% of peanut allergic patients reacted to even lower cumulative doses. For these patients the assays should be more sensitive (10 mg/kg for egg protein, 30 mg/kg for milk protein, and 24 mg/kg for peanut protein, respectively).

Table 2: Ingested amounts of hidden allergens reportedly eliciting allergic symptoms

Hidden Allergen	Amount of Protein	Ingested Food	Reference
Peanut	45 mg	Dry Soup	McKenna & Klontz 1997
Hazelnut	700 µg (Corylin)	Chocolate	European Commission 1998
Hazelnut	50 mg (Corylin)	Cookies	European Commission 1998
Milk	120-180 µg (Whey Proteins)	Fruit Sorbet	Laoprasert et al. 1998
Milk	60 mg (Caseins)	Sausage	Malmheden Yman et al. 1994
Milk	10 mg (Caseins)	Soy-based Icecream	European Commission 1998
Hen's Egg	10 mg (Ovalbumin)	Pasta	European Commission 1998
Hen's Egg	100 mg (Ovalbumin)	Cookies	European Commission 1998

ANALYTICAL METHODS FOR THE DETECTION OF FOOD ALLERGENS

Nearly all food allergens are proteins or glycoproteins with a molecular mass ranging from about 10 to 70 kDa. Immunological methods have been applied for the characterization of food allergens since they were first identified. The most common methods for the detection of food allergens are summarized in Table 3. Immunoassays involving human IgE antibodies are mainly used to characterize the allergenic properties of a protein, while immunoassays using animal antisera detect certain proteins used for the immunization of the animal during antibody production, but not specifically an "allergenic protein" or "allergen".

The detection of allergens by human IgE-antibodies include radio-allergosorbent test (RAST) inhibition or enzyme-allergosorbent test (EAST) inhibition methods. These methods are variations of the RAST or EAST applications usually used for the characterization of patient's sera determining specific IgE-levels. SDS-PAGE immunoblot techniques can be used for the identification and characterization of major and minor food allergens. 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 sera is usually limited. Moreover, multiple sensitivities and/or cross-reactivities to more than one allergenic food may be present in human serum-IgE.

Detection methods involving antibodies from rabbits, mice, goats, sheep, or chicken include immunodiffusion techniques, rocket-immunoelectrophoresis, dot-immunoblotting, SDS-PAGE immunoblotting, and enzyme-linked immunosorbent assays (ELISA-Techniques). With the exception of immunodiffusion techniques, which are not sensitive enough, these methods are used for the detection and in some cases for the quantitation of food allergens. The ELISA techniques are the most promising tools for the determination of hidden allergens in foods.
Detecting DNA from allergenic sources is just at the beginning of its development. Only very few applications of PCR-reactions for the detection of allergens, namely hazelnut and wheat, have been published (Koeppel et al. 1998, Holzhauser et al. 2000). PCR methods are not further discussed here (for a brief discussion of PCR-based methods see Besler 2001).

Table 3: Analytical methods for the detection of food allergens

Detection of Allergen	Detection of Protein	Detection of DNA
❐ Immunoassays involving Human IgE Antibodies	❐ Immunoassays involving Antibodies from Rabbits, Mice, Goats, Sheep, or Chicken	❐ Encoding for a Specific Protein
❐ RAST / EAST-Inhibition ❐ SDS-PAGE / Immunoblotting	❐ Immunodiffusion ❐ Rocket-Immunoelectrophoresis ❐ Dot-Immunoblotting ❐ SDS-PAGE / Immunoblotting ❐ ELISA	❐ PCR-Reaction

COMMON CRITERIA FOR IMMUNOASSAYS

Some general recommendations must be considered in performing immunoassays. The sample preparation is always a most critical step. An analytical method can only be as good as the sample preparation is. An important characteristic is the extraction efficiency, depending on the food matrix to be analysed. Acceptable recoveries for ELISA methods vary between 70 and 120% with coefficients of variation (CV) of less than 20% (Lipton et al. 2000).
The sensitivity and limits of detection and quantitation, respectively, should meet the requirements of detecting even trace amounts of allergens in foods. As mentioned above, detecting amounts as low as 1-100 mg/kg are required as limits of detection for some food allergens. Furthermore an immunoassay should be specific. Therefore cross-reactivities should be excluded or well-characterized, respectively. The antibody specificity depends, for example on the purity of the used immunogen (e.g. crude protein extract or purified protein) and its similarity to other proteins. Therefore antibody specificity must be tested. In order to minimize cross-reactivities antisera can be preabsorbed with related food items. For example anti-hazelnut corylin antibodies preabsorbed against various nuts and anti-peanut antibodies preabsorbed against soybean, white bean, and marzipan (almonds) are commercially available (Holzhauser et al. 1999a, 1999b). Moreover, antisera must be capable of detecting allergens in processed foods. Thus antibodies raised with native food protein extracts may not be or may be less reactive to food proteins denatured by various treatments during food processing. This can be circumvented by raising antibodies with protein extracts from pre-treated foods such as roasted peanuts or hazelnuts.

The need of a thorough quality control even when a commercial test kit is used is demonstrated by Keck-Gassenmeier et al. (1999), who employed a commercial ELISA test kit for the determination of peanut protein in dark chocolate. They showed that the extraction method supplied by the test kit manufacturer was not sufficient to detect trace amounts of peanut protein in dark chocolate. By the simple addition of 10% fish gelatine to the extraction buffer the recovery rates improved from 2-3% to 63-89% for amounts as low as 2 mg/kg. The authors attributed the striking improvement of the recoveries to tannin-binding properties of fish gelatine. Interestingly the investigation of milk chocolate revealed no difference for both extraction buffers (with and without fish gelatine) which was probably due to the higher amount of milk proteins and lower amount of cacao (tannin). Furthermore the different results of spiking dark chocolate with peanut proteins or peanut butter underlined the importance of analysing recoveries under almost real-life conditions.

Similarly the limits of detection may differ for different food matrices. Blais & Phillipe (2001) demonstrated a 10 fold variation of the limit of detection of hazelnut protein investigating nine different foods. In this study the lowest limit of detection was found for a cake mix (0.12 mg/kg), while the highest detection limits were found for almond and fruit bars (both 1 mg/kg).

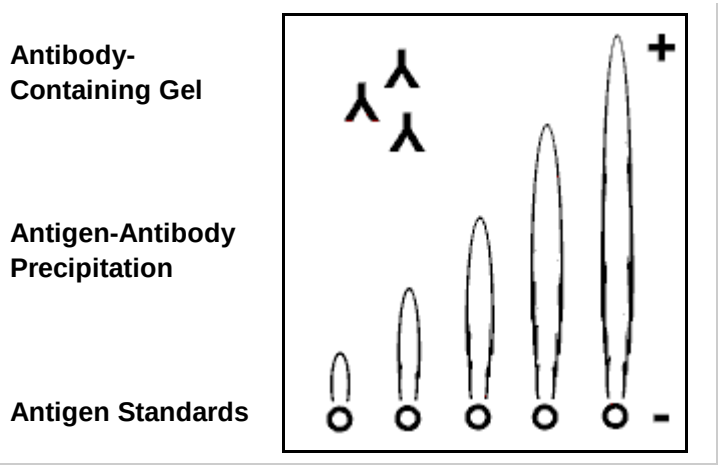

Figure 1: Principle of rocket-immunoelectrophoresis

ROCKET IMMUNOELECTROPHORESIS

Principle

Rocket-immunoelectrophoresis employs an antibody-containing gel (Figure 1). The standard or sample proteins (antigens) migrate according to their electrophoretic mobility until antigen-antibody-complexes precipitate in the gel. Rocket-shaped precipitates are build at a constant antigen / antibody ratio. The height of the rockets is proportional to the amount of antigen applied.

Applications

The presence of undeclared allergens was detected by rocket-immunoelectrophoresis in various food products (Table 4). Egg, hazelnut, milk and peanut proteins could be analyzed with a detection limit of 30 mg/kg. The sensitivity or range of detection was 25-420 µg/mL using Coomassie brilliant blue for staining of gels (Malmheden Yman et al. 1994).

A more sensitive application was described by Holzhauser & Vieths (1998). The detection of peanut proteins was improved by a staining method involving an enzyme-labeled anti-rabbit IgG antibody. The sensitivity ranged from 20 to 1440 ng/mL, resulting in a superior limit of detection of 2.5 mg/kg.

Major disadvantages of rocket-immunoelectrophoretic applications are the rather uneasy and time consuming handling of gel preparation and immunostaining procedures.

Table 4: Applications of rocket-immunoelectrophoresis for the detection of food allergens

Food Allergen	Cross-Reactivities	Applications	Reference
a) **Egg** (Ovalbumin) b) **Hazelnut** (Corylin) c) **Milk** (Caseins) d) **Peanut** (Protein) *Sensitivity:* 25-420 µg/mL	not available *Antisera:* rabbit Ab (a, b, c), sheep Ab (d)	**Samples:** a) Meat Balls, Pasta b) Chocolate c) Ice Cream, Chocolate, Lollipop, Sausage, Hot Dog, Recombined Ham, Meringue d) Cake **Limit of Detection:** 30 mg/kg	Malmheden Yman et al. 1994
Peanut (Protein) *Sensitivity:* 20-1440 ng/mL (Peanut Protein)	No (20 Legumes, Nuts, and other Ingredients tested) *Antiserum (in Gel):* rabbit Ab	**Samples:** Candy, Chocolate Products, Cornflakes, Ice Cream, Muesli, Rice Cracker **Limit of Quantitation:** 2.5 mg/kg **Recovery:** 85-101% **CV:** <5%	Holzhauser & Vieths 1998

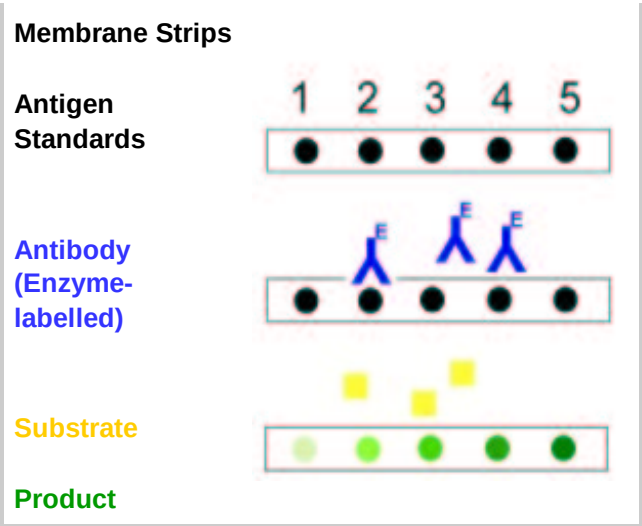

Figure 2: Principle of dot-immunoblotting

DOT-IMMUNOBLOTTING

Principle

In dot-immunoblotting the standards and samples are spotted onto membrane strips. Specific detection is achieved by incubation with enzyme-labeled antibodies which bind to the target antigens. The spots are visualized by addition of a substrate which is transformed by an enzymic reaction into a colored product. The intensity of the spots is proportional to the amount of antigen.

Applications

Recently a dot-immunoblotting application was described for the detection of peanut proteins in various foods (Blais & Phillipe 2000). This method is capable of detecting amounts as low as 2.5 mg/kg. Despite the fact that no quantitation was performed, the method allows simple and inexpensive screening of food samples.

Table 5: Applications of dot-immunoblotting for the detection of food allergens

Food Allergen	Cross-Reactivities	Applications	Reference
Peanut (Protein) *Sensitivity:* 30 ng/mL (Peanut Protein)	No (Chick Pea, Lentils, Red Kidney Beans, Hazelnut, Brazil Nut tested) *Antiserum:* chicken Ab (IgY)	**Samples:** Almond Butter, Bars, Chocolate Products, Cookies, Ice Cream, Potato Chips **Limit of Detection:** 2.5 mg/kg	Blais & Phillippe 2000

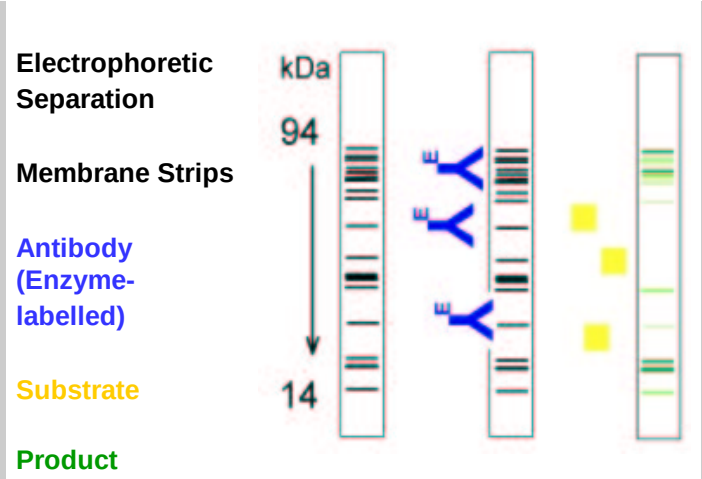

Figure 3: Principle of SDS/PAGE-immunoblotting

SDS/PAGE-IMMUNOBLOTTING

Principle

Samples and standards are separated in SDS-Polyacrylamid-Gelelectrophoresis according to their molecular mass. Afterwards the separated bands are transferred onto a membrane and detected with enzyme-labeled antibodies as described for dot-immunoblotting. This method allows the detection and identification of individual proteins or allergens.

Applications

Most recently an SDS-PAGE / immunoblot application for the qualitative detection of almond and hazelnut proteins in chocolates was described by Scheibe et al. (2001). The sensitivity of the method was about 200 ng/mL, resulting in a limit of detection of 5 mg/kg. Schäppi et al. (2001) detected the major peanut allergens (Ara h 1, 2, 3, and 4) in cereal bars, corn crackers and potato snacks. The content of undeclared peanuts ranged from 0.05 to 0.5% in the samples.

Table 6: Applications of SDS/PAGE-immunoblotting for the detection of food allergens

Food Allergen	Cross-Reactivities	Applications	Reference
a) **Almond** b) **Hazelnut** *Sensitivity:* 200 ng/mL	No (Hazelnut, Almond, Milk, Cocoa, Peanut) *Antisera:* rabbit pAb	**Samples:** Chocolates **Limit of Detection:** 5 mg/kg	Scheibe et al. 2001
Peanut *Sensitivity:* -	No IgE-binding cross-reactivity to other food allergens *Antisera:* human IgE	**Samples:** Cereal Bars, Corn Crackers, Potato Snack **Limit of Detection:** 5-50 mg/kg	Schäppi et al. 2001

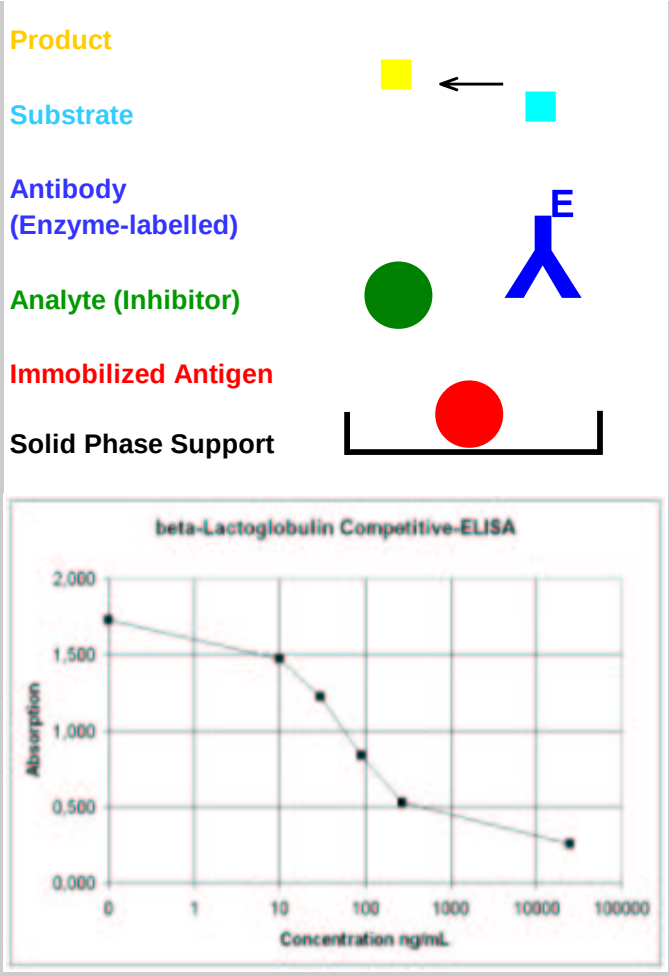

Figure 4: Principle of Competitive-ELISA

COMPETITIVE-ELISA

Principle

Enzyme-linked Immunosorbent Assays are most frequently performed in 96-well microplates or in 8-well strips. The competitive ELISA involves immobilized antigens bound to the solid phase. If no sample antigen is present the enzyme-labelled antibody shows maximal binding to the solid phase bound antigen, resulting in high absorption of the colored product formed. Binding of the enzyme-labelled antibody is inhibited by increasing amounts of antigen. The standard curve shows the typical sigmoid shape. In this example the standard curve of beta-lactoglobulin, a whey protein, is shown.

Applications

Applications of the Competitive-ELISA are shown in Table 7. The tests for the detection of hazelnut and peanut proteins used polyclonal antisera from rabbits, while the ELISA for the determination of beta-lactoglobulin compared a polyclonal rabbit-antibody and a monoclonal mouse-antibody.

The hazelnut-ELISA was performed in the range of 5 to 1000 ng/mL with a detection limit of 1 mg/kg (Koppelman et al. 1999). The recovery from samples like chocolate, cookies, and cake ranged from 67 to 132%. Significant cross-reactivities were observed for several nuts and peanuts. A similar assay performance was described for the Peanut-ELISA by Holzhauser & Vieths (1999a). Only a slightly poorer sensitivity and limit of detection were observed.

A more sensitive Peanut-ELISA was described by Yeung & Collins (1996). The sensitivity was between 1 and 63 ng/mL, resulting in a detection limit of 0.4 mg/kg. No cross-reactivities were observed to 22 tested legumes, nuts, and other food ingredients.
Mariager et al. (1994) determined beta-lactoglobulin in cow's milk and infant formulas comparing a polyclonal antibody with a monoclonal antibody. The polyclonal antibody offered a 3 to 4 fold broader range of detection and a 30 fold lower limit of detection.

Table 7: Applications of Competitive-ELISA for the detection of food allergens

Food Allergen	Cross-Reactivities	Applications	Reference
Hazelnut (Protein) *Sensitivity:* 5-1000 ng/mL	Walnut, Cashew, Almond, Brazil Nut, Peanut, Pine Nut *Antiserum:* rabbit pAb	**Samples:** Chocolate Products, Cookies, Cake, Milk Flavour **Limit of Detection:** 1 mg/kg **Recovery:** 67-132%	Koppelman et al. 1999
Peanut (Protein) *Sensitivity:* 1-63 ng/mL	No (22 Legumes, Nuts, and other Ingredients tested) *Antiserum:* rabbit pAb	**Samples:** Chocolate Bars, Cookies, Ice Cream, Mixed Nuts and Seeds, Pasta Sauces **Limit of Detection:** 0.4 mg/kg **Recovery:** 68-90% **CV:** 2-22%	Yeung & Collins 1996
Peanut (Protein) *Sensitivity:* 24-1000 ng/mL	Walnut, Pinto Bean *Antiserum:* rabbit pAb	**Samples:** Cashew, Chocolate, Nut and Chocolate, Raisin, Coconut Cookies, Amarettini, Cereal Bars **Limit of Detection:** 2 mg/kg **Recovery:** 84-126% **CV:** <15%	Holzhauser & Vieths 1999a
Cow's Milk (beta-Lactoglobulin) *Sensitivity:* a) 0.1-1000 ng/mL b) 4-50 ng/mL	not available *Antisera:* a) rabbit pAb (against heat treated beta-Lactoglobulin) b) mouse IgG mAb	**Samples:** Whole Milk, Infant Formulas (ready to use) **Limit of Detection:** a) 0.08 µg/L b) 3.2 µg/L **CV:** <33%	Mariager et al. 1994

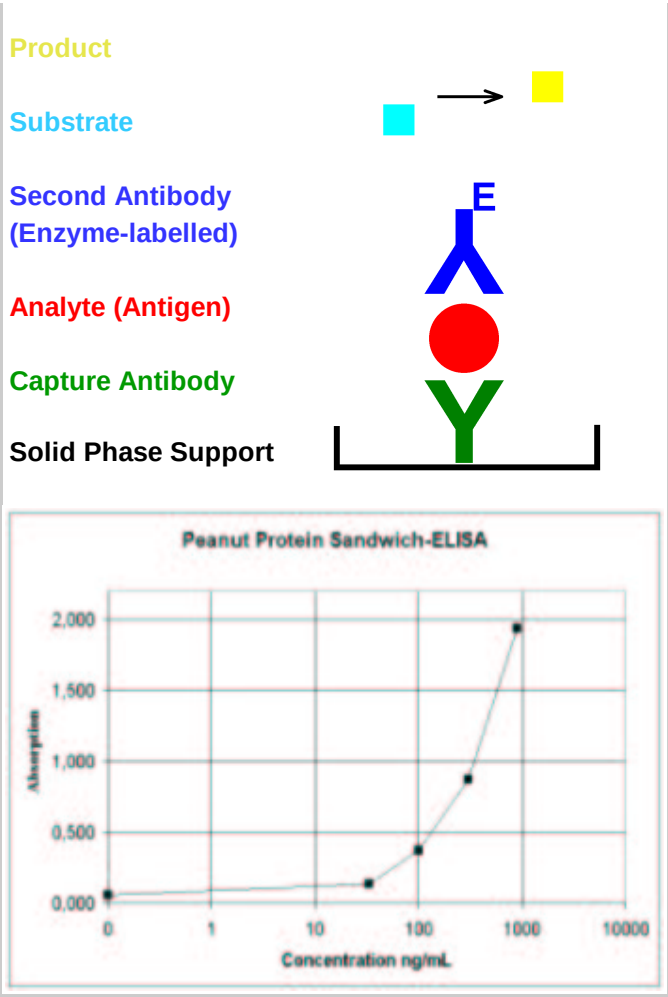

Figure 5: Principle of Sandwich-ELISA

SANDWICH-ELISA

Principle

For the detection of proteins, sandwich ELISA is the most common type of immunoassay performed. This format involves an immobilized capture antibody on the microplate wells (Figure 5). After adding the standard or sample solution antibody-analyte binding occurs. A second, analyte specific, labeled antibody is added and also binds to the analyte, forming a "sandwich". Then a substrate is added, reacting with the enzyme and producing a colored product. The absorption is directly proportional to the concentration of the analyte. The curve shows the peanut standards of a commercial ELISA-Test-Kit.

Applications

Table 8 shows applications of Sandwich-ELISA. The Almond- and the Hazelnut-ELISA involved rabbit and sheep polyclonal antisera as capture and secondary antibodies, respectively, while the Peanut-ELISA used an unlabeled and an enzyme-labeled rabbit polyclonal antiserum. For determination of almonds a sensitivity of 100 ng/mL and a limit of detection of 1 mg/kg was achieved.

However, several seeds and nuts gave significant cross-reactivities (Hlywka et al. 2000).

The sensitivity of the Hazelnut-ELISA ranged from 1 to 600 ng/mL, resulting in a detection limit of 2 mg/kg (Holzhauser & Vieths 1999b). Tolerable amounts of cross-reactive pumpkin seeds, walnut, and cashew (not interfering with the detection of hazelnut protein) were determined. It seems very useful to know the amounts of cross-reactive sample ingredients which can be tolerated by the assay. So it can be estimated whether the test is applicable for to a certain sample containing interfering ingredients or not. The peanut application gave a detection limit of 0.1 mg/kg (Koppelman et al. 1996). The sensitivity ranged from 5 to 1000 ng/mL. Cross-reactivities were observed for almond and cashew.

Tsuji et al. (1993, 1995) developed a Sandwich-ELISA for the determination of the major soybean allergen (Gly m Bd 30K). They used two monoclonal antibodies as capture and secondary antibody, respectively. Within the range of 140-700 mg/kg, Gly m Bd 30K was detected in various food products, while it was not detected in fermented soybean products such as miso, shoyu, and natto.

Hefle et al. (2001) described a Sandwich-ELISA for the detection of egg white in various pasta products. Interestingly the most sensitive ELISA-format was achieved using a capture antibody raised against egg white and a detection antibody specific for ovalbumin. The limit of detection was 1 mg/kg whole egg in the sample.

Table 8: Applications of Sandwich-ELISA for the detection of food allergens

Food Allergen	Cross-Reactivities	Applications	Reference
Almond (Protein) *Sensitivity:* 100 ng/mL (Almond Flour containing 21% Protein)	Sesame Seed, Black Walnut, Macadamia, Pistachio, Brazil Nut, Hazelnut, Cashew *Capture Antibody:* rabbit pAb *Secondary Antibody:* sheep pAb	**Samples:** Cereals, Chocolate, Dairy Foods, Confectionary Items **Limit of Detection:** 1 mg/kg (Almond) **Recovery:** 86-100%	Hlywka et al. 2000
Hazelnut (Protein) *Sensitivity:* 1-600 ng/mL	Pumpkin Seed, Walnut, and Cashew (tolerable amounts of 10, 20, and 50%, respectively) *Capture Antibody:* rabbit pAb *Secondary Antibody:* sheep pAb	**Samples:** Chocolates, Chocolate Products, Muesli **Limit of Detection:** 2 mg/kg **Recovery:** 67-132% **CV:** <15%	Holzhauser & Vieths 1999b
Peanut (Protein) *Sensitivity:* 5-1000 ng/mL	Almond, Cashew *Capture Antibody:* rabbit pAb *Secondary Antibody:* same Ab, labelled	**Samples:** Cookies, Chocolate Bars and Candy, Sate Sauce **Limit of Detection:** 0.1 mg/kg **Recovery:** 35-75%	Koppelman et al. 1996
Soybean (Gly m Bd 30K) *Sensitivity:* 10-500 ng/well (2-200 ng/well for reduced and carboxymethylated allergen)	No cross-reactivity to other soybean allergens *Capture Antibody:* mice mAb *Secondary Antibody:* mice mAb (both raised against reduced and carboxymethylated allergen)	**Samples:** Soy Milk, Tofu, Kori-Dofu, Yuba, Meat Balls, Beef Croquettes, Fried Chicken, Fermented Soybean Products **Range of Detection:** 140-700 mg/kg **CV:** 4-17%	Tsuji et al. 1993, 1995
Egg White (Ovalbumin) *Sensitivity:* not available	Portobello Mushroom, Basil Leaves (no cross-reactivity to other selected pasta ingredients) *Capture Antibody:* goat pAb (anti-Egg White) *Secondary Antibody:* rabbit pAb (anti-Ovalbumin)	**Samples:** Several Pastas **Limit of Detection:** 1 mg/kg (Whole Egg)	Hefle et al. 2001

RAST / EAST-INHIBITION

Principle

RAST or EAST inhibition represent a kind of Competitive ELISA employing human serum IgE antibodies. A solid phase bound antigen is involved which binds specific human IgE (Figure 6). Standard or sample analytes inhibit IgE binding to the solid phase bound antigen. An enzyme-labeled antibody is used to detect the bound human IgE antibodies. The substrate-enzyme reaction gives a colored product. The standard curve in Figure 6 shows the inhibition of IgE-binding to the major hen's egg allergen ovomucoid (self-inhibition compared to deglycosylated ovomucoid).

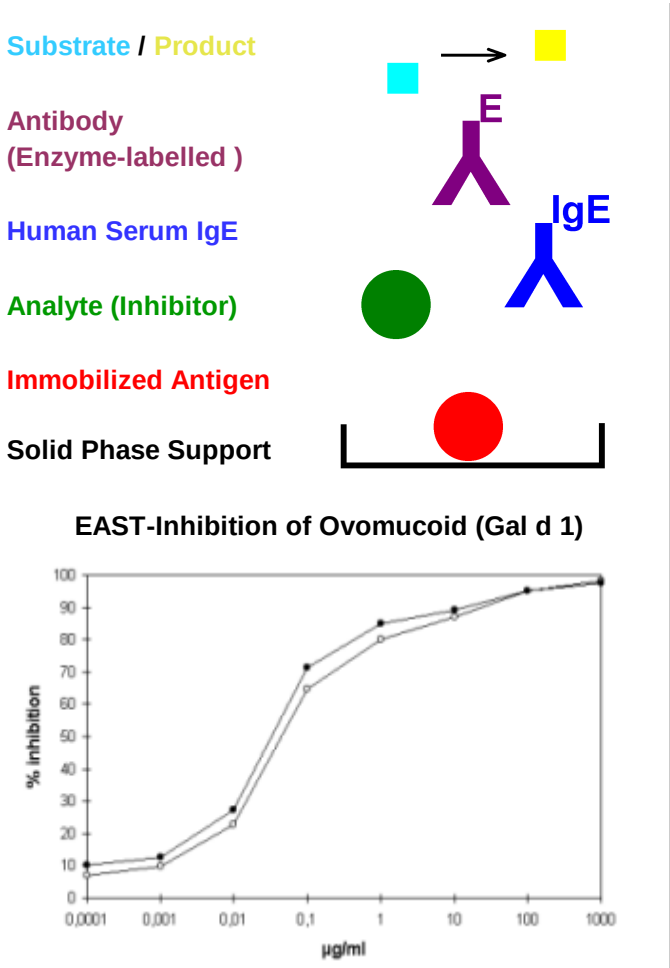

Figure 6: Principle of RAST or EAST-Inhibition

Applications

RAST / EAST inhibition applications are seldom used to quantitate allergens in foods (Table 10). One example is the detection of alpha-Lactalbumin in baby food and food quality lactose (Frémont et al. 1996). The standard curve gave a range of detection from 100 ng/mL to 10 µg/mL, resulting in a limit of detection of 1 mg/kg in the samples.

The other applications shown in Table 10 were not used for the determination of hidden allergens. The hazelnut RAST inhibition was used to compare the performance with a Competitive ELISA format (Koppelman et al. 1999), while the peanut RAST inhibition was used to compare the allergenic potential of different peanut varieties (Koppelman et al. 2000).

The major drawback of RAST or EAST inhibition with respect to quantitation is its reliance on non-standardized human sera whose amounts are often limited. Furthermore, variable specificities of human IgE antibodies hinder the use in a wider range of analytical laboratories. In addition commercial solid-phases of food allergens can vary considerably in IgE-binding activities.

These limitations prevent commercial applications to quantitate food allergens by RAST / EAST inhibition (Taylor & Nordlee 1995).

RAST/EAST inhibition has been applied for qualitative allergen detection and for the assessment of allergenic potencies in a wide range of food products, e.g:

- Detection of codfish allergens in surimi, a Japanese food product imitating shrimps, and pizza toppings by RAST inhibition (Helbling et al. 1992, Mata et al. 1994).
- IgE-binding potencies of hypoallergenic infant formulas in comparison to cow's milk proteins (Oldaeus et al. 1991).
- Assessment of the allergenic potencies of protein extracts from a wide range of peanut containing food products such as peanut flour, roasted peanuts, peanut butter, and hydrolyzed peanut protein (Nordlee et al. 1981), or crude, neutralized, and refined peanut oil (Olszewskiet al. 1998) in comparison to peanut protein extract.
- The allergenic potencies of various soybean products such as raw soybeans, sprouts, acid- hydrolyzed sauce, tofu, hydrolyzed vegetable protein, tempeh, miso, and mold-hydrolyzed sauce were characterized by RAST inhibition (Herian et al. 1993).
- Characterization of heat and hydrolytic stability of hazelnut allergens by EAST inhibition (Wigotzki et al. 2000 a, b).

Table 10: Applications of RAST or EAST-inhibition for the detection of food allergens

Food Allergen	Cross-Reactivities	Applications	Reference
Cow's Milk (alpha-Lactalbumin) *Sensitivity:* 100 - 10000 ng/mL	not available *Antisera:* human IgE	**Samples:** Baby Food, Food Quality Lactose **Limit of Detection:** 1 mg/kg	Frémont et al. 1996
Hazelnut (Protein) *Sensitivity:* 30-1000 ng/mL	Walnut, Cashew, Pecan Nut, Pistachio *Antisera:* human IgE	**Limit of Detection:** 6 mg/kg	Koppelman et al. 1999
Peanut (Protein) *Sensitivity:* approximately 50-300 ng/mL	not available *Antisera:* human IgE	**Samples:** 13 Different Peanut Samples **Relative Allergenicity:** Comparison of 50%-Inhibition **CV:** 10%	Koppelman et al. 2000

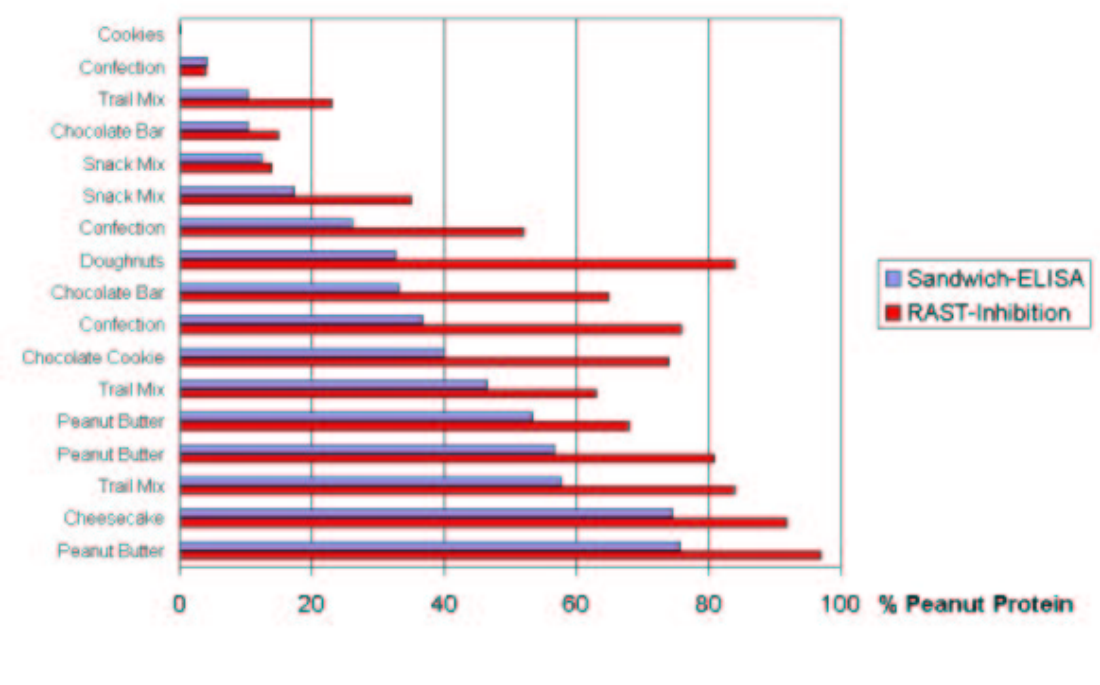

Figure 7: ELISA versus RAST: Determination of peanut protein (data from Hefle et al. 1994)

ELISA VERSUS RAST / EAST

Figure 7 shows the results of the determination of peanut protein by a Sandwich-ELISA (the violet bars) as compared to RAST-Inhibition (the red bars) (Hefle et al. 1994). A significant overestimation of peanut content by RAST-Inhibition was demonstrated in 15 of 17 different food samples. The major cause of overestimation is probably a high degree of cross-reactivities of the human IgE antibodies to other food ingredients than peanuts. A pooled serum from about 10 patients was used in this study. It is most likely that these patients had some concomitant IgE-sensitizations.

This example reflects the major disadvantage of RAST / EAST inhibition. As mentioned above it is difficult to obtain standardized antisera. Human sera are often limited. Furthermore every patient serum has a different individual pattern of IgE-specificities.
Therefore RAST or EAST inhibition is seldom used for the determination of allergens in foods, but it is an ideal tool for the characterization of IgE-binding properties reflecting the allergenic potential of crude protein extracts, purified food allergens, allergenic activities of different varieties and various processed foods.

ELISA TEST KITS

Table 11 gives an overview of commercially available Test-Kits with sufficient limits of detection for the determination of food allergens. To date there are ELISA-Test-Kits available for egg, milk, peanut, and wheat.

It is obvious that Tests for many other important food allergens are not available. For the screening and quality control of food products it recommended to use standardized, evaluated Test-Kits to obtain reproducible and precise results minimizing the risk of false negative and false positive results, respectively.

Table 11: Commercially available ELISA Test Kits

Food Allergen	Limit of Detection	Trademark / Company
Egg, Milk, Peanut	10 mg/kg	Veratox / Neogen
a) Peanut b) Wheat Gluten	a) 0.5 - 2 mg/kg b) 20 / 200 mg/kg	BioKits / Tepnel BioSystems ELISA-TEK / ELISA Technologies
a) Egg White (Ovalbumin) b) Milk (beta-Lactoglobulin) c) Peanut d) Wheat (omega-Gliadin)	a) 5 mg/kg b) 5 mg/kg c) 2.5 mg/kg d) 5 mg/kg	Ridascreen / R-Biopharm
a) Milk (Caseins) b) Milk (beta-Lactoglobulin) c) Peanut d) Wheat (omega-Gliadin)	a) 25 mg/kg b) 25 mg/kg c) 0.5 - 2 mg/kg d) 10 mg/kg	Transia (Tepnel BioSystems, Diffchamb S.A.)

FREQUENCY OF HIDDEN FOOD ALLERGENS

Undeclared Allergens in Foods

Unfortunately, up to now, there are no systematic studies on the frequency of hidden allergens in foods. There have been only very few investigations which analyzed more than a few samples. These samples are most probably food samples suspected to contain a certain allergen, meaning these studies may not be representative for the investigated food products as a whole.
But nevertheless, the data listed in Table 12 indicate that a significant number of foods contain undeclared allergens. 43% of 28 analyzed chocolates and chocolate products and mueslis contained undeclared amounts of hazelnut protein (Holzhauser & Vieths 1999b). In another study 58% of 26 similar food samples contained undeclared hazelnut protein (Koppelman et al. 1999).
Undeclared peanut proteins were detected in 29% of 17 samples (Holzhauser & Vieths 1999a). While Schäppi et al. (2001) detected undeclared peanut proteins in 5 of 7 products (cereal bars, corn crackers, potato snacks).
In a recent study 83 chocolates supposed to be free of almond and hazelnut were analyzed (Scheibe et al. 2001). Almond was detected in 61% and hazelnut in 72% of samples, respectively.

Table 12: Frequency of Hidden Allergens in Foods not Declared on the Label

Food Samples*	Undeclared Allergen	Percentage	Reference
28 Chocolates, Chocolate Products, Muesli	Hazelnut	43 %	Holzhauser & Vieths 1999b
17 Roasted Cashews, Chocolates, Nuts and Chocolate, Raisin and Chocolate, Coconut Cookie, Amarettini, Cereal Bars	Peanut	29 %	Holzhauser & Vieths 1999a
26 Chocolate Spreads, Bars, and Cookies, Muesli Cookie, Cake	Hazelnut	58 %	Koppelman et al. 1999
83 Chocolates	Almond Hazelnut	61 % 72 %	Scheibe et al. 2001

* Please note: samples may not be representative for the kind of foods investigated.

Foods Labeled as Being Free of Allergens

In contrast, food products labeled as being free of a certain allergen contained significantly less frequently hidden allergens. But nevertheless, again, a significant number of samples was contaminated with hidden allergens (Table 13).

In the case of egg, 1.3% of 319 samples contained egg protein. Milk proteins were detected in 2.3% of 838 samples, and wheat in 5.2% of 1583 samples. These results demonstrate the difficulty of producing "allergen free" products.

It should be noted that the samples and detection methods were not indicated. Therefore the majority of samples could be samples suspected to contain the related allergen.

Table 13: Frequency of hidden allergens in foods labeled as being free of the respective allergen

Food Samples*	Labeled as being free of	Percentage	Reference
319 (not specified)	Egg	1.3 %	Standing Committee for Foodstuffs 1997
838 (not specified)	Milk	2.3 %	Standing Committee for Foodstuffs 1997
1583 (not specified)	Wheat (Gluten)	5.2 %	Standing Committee for Foodstuffs 1997

* Please note: samples may include complain samples not be representative for the kind of foods investigated.

CONCLUSIONS

At present immunoassays are the method of choice to determine hidden food allergens. Suitable immunological methods for the detection of trace amounts of allergens in foods are the rocket immunoelectropheresis, with a sensitivity of less than 5 µg/mL; SDS/PAGE- and dot-immunoblot applications, with sensitivites in the range of 30 to 200 ng/mL, and ELISA methods with sensitivities of approximately 0.1 to 100 ng/mL. Immunodiffusion techniques usually have an insufficient sensitivity, in the range of 10-20 µg/mL. In summary:

- Immunoassays are specific, sensitive, and rapid methods (usually 2 to 4 hours) to detect and quantitate even trace amounts of allergens in food products.
- Standardized (commercial) ELISA-Test-Kits are available for egg, milk, peanut, and wheat proteins only.
- Test-Kits for soybean, hazelnut (and other tree nuts), sesame seed, celery, fish and shellfish are not available at the moment.

- Furthermore there is a need for reliable and cost-effective screening methods which can rapidly detect minute amounts of food allergens.

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[Summary] [Abbreveations]
