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# Allergens and Molecular Diagnosis.

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**ABSTRACT**

The major eight allergens are known for their severe consequences, ranging from small respiratory illnesses to severe anaphylaxis. It is thus important to analyze which is a better molecular tool to detect the presence of an allergen. The paper summarizes the effects of food processing on allergens along with the molecular tools used to detect each of the major eight allergens. Finally, commercial testing kits are listed to show their Limit of Detection (LoD) and the time taken to receive the results.

**Keywords:** Allergens, Commercial Testing Kits, Molecular Tools, Biosensors.

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## INTRODUCTION

Allergy is defined as a moderate to severe immunological response to various factors like food, pollen, fur, dust, insects and so on. Among these allergens, food contributes to approximately 15% of allergenicity in individuals[1]. Globally, eight major allergens have been identified and nucleic acid and protein-based molecular assays have been developed for them[2]. The response towards allergenic substances differs according to race, culture, genetics and /or environmental factors[3]. Of the races, self- identified Africans and the African ancestry were found to be sensitive towards food allergens. The African ancestry was found to be more sensitized towards peanut allergens[4,5]. This paper details on the literature available on the allergen characteristics and the effect of processing methods on the allergens along with the molecular methods of detection of the eight major allergens.

## PEANUT ALLERGY:

Of all the major allergens, peanuts cause the most serious allergic reactions. At the same time, they are the most studied of all allergens, with molecular tools and quantification available for diagnosis [6]. Many people are found to be allergic to peanuts, and this number seems to be rising[7]. While atopy may be a risk factor for peanut allergy, isolated peanut allergy is more common, as the persistence of the same seems to prolong as compared to other allergens[8]. There are about 12 allergens recognized by the Allergome, and all major plant allergens are represented[9].Clinical manifestations of peanuts, as well as tree nuts, result in minor GI tract discomforts, respiratory symptoms and skin symptoms [10].

Peanut proteins are found to be more thermostable at low water levels and glycation and cross-linking reactions are known to increase the allergenicity activity[19]. Frying in vegetable oil and cooking in boiling water reduces the IgE binding capability of Ara H1, more than roasting (at 170◦C, 20 minutes). Another result is that the IgE binding capability of Ara H2 and Ara H3 reduces in fried and boiled peanuts as compared to roasted peanuts[11]. Ara H 2 is shown to have increased IgE activity after thermal processing. A study by Gruber et. al., (2005) showed that Ara H2 showed higher IgE reactivity after thermal processing [12].

ELISAs are commonly used to detect the presence of peanut, however, this test is set back by matrix effects and thermal processing methods [13]. A multiplex PCR detection method has also been made during the simultaneous detection of hazelnut and peanut, where the initial amount of sample was around 50 pg. The sample was amplified using a peptide nucleic acid (PNA) microarray [14]. Many types of PCRs were then made available for the quantification and detection of peanuts at very low limit-of-detection (LOD). These types of PCRs include quantitative PCRs, multiplex PCRs and other extended features of the same, for improved product detection [6]. DNA microarrays have been developed, for the purpose of a versatile, specific and sensitive detection of peanuts. The LOD of this tool is as low as 1µg/kg. If an individual is reported to be allergic to peanut, it is usually advised to avoid the whole group of nuts since around 20-40% of individuals who are allergic to peanut are also allergic to tree nuts. [15]

Surface plasmon resonance (SPR) optical biosensors can be used to substitute the use of ELISAs in the rapid detection of peanut allergies. For example, the presence of peanut could be detected in cookies and dark chocolate in the range of low mg/kg[16]. Light scattering biosensors were used for the diagnosis of peanut allergies using whole blood samples as the sample matrix, where the specific allergen, Peanut Ara H1 was the subject of study[17].

Other electrochemical and impedance based biosensors have been described, with impedance based biosensors showing great promise for the detection of the peanut Ara H1 protein, at the point-of-care due to its sensitivity, low cost, and maintenance[18]. Dot immuno-blotting seems to be a good semi-quantitative test for screening peanuts in food detection level being 2.5-3 mg/kg[20].

Mass Spectroscopy (MS) is also suitable for the detection of peanuts, provided that there is a reference material available. In the case of peanuts, by using synthetically produced isotopes, the limit of detection has been found to be around 5-50 mg/kg[21].Liquid Chromatography- Mass Spectroscopy (LC-MS) has also been regarded as a good alternative of detecting peanuts in food samples. A methodology of enzymatic digestion developed by Sealey-Voyksner et al.,(2016) is sensitive beyond limits of ELISA and is

capable of quantifying nuts- including 11 varieties of tree nuts and peanuts. Using a specific marker peptide minimizes issues of cross-reactivity and can be used for labeling purposes. [22]

A number of studies have been conducted to compare the molecular tools used to detect the presence of peanut. In the studies conducted, it was found out that the results from PCR and ELISAs are almost similar except for the slight variations found in ELISA due to cross-reactions. However, a complete comparison is tough to analyze due to the absence of a standardized reference material. Dipsticks, laminar flow assays (LFAs) are versatile and have a very low LOD, however, all of these characteristics are affected by the food matrix used and are preferable in the context of diagnosing the allergy, using a blood sample.

**TREE NUT ALLERGY:**

Tree nuts are another type of major allergen that must be avoided. These nuts include almonds, cashews, macadamia nuts, and pistachios. They fall in the category of the major food allergens. Individuals are usually sensitized to nuts as a result of exposure to non-pollen mediated food, except in the case of Hazelnuts (where the exposure of birch pollen could lead to allergies)[23]. Also, due to their fatal anaphylactic reactions, it is usually advised to avoid all types of nuts[15]. This cross-reaction is often hypothesized to be due to the taxonomic proximity, or due to the similar IgE binding sites seen in the allergens[24,25].

The allergenicity of tree nuts can change with the type of food processing. When subjecting hazelnuts to thermal processing, the allergenicity is almost completely removed. In a study conducted to analyse the IgE binding capacity in roasted and raw hazelnuts, it was seen that this binding capacity is greatly reduced in roasted nuts [26]. The same thermal treatment was shown to not affect other nuts, like almonds, walnuts, and cashews, and it was seen that a pH of 5.0 to 7.0 and a high temperature of 110° C is needed to denature the allergen found in Brazil nuts. Enzymatic treatments were also found to reduce the effect of the allergenicity in hazelnuts. It was seen that the extent of enzymatic treatment, for example, hydrolysis could alter the extent of allergenicity. Partial hydrolysis can only minimally affect the extent of allergenicity [27]. Many cases of cross-reactions have been observed in the detection of tree nuts and peanuts. Research has been going on to produce a more sensitive test,that can differentiate the nut allergens without any cross-reactions. A multiresidue enzyme immunoassay has been developed to differentiate between the markers of peanut, hazelnut, cashew and Brazil nuts. Run in a competitive, indirect fashion, the LOD was found to be of concentrations lower than 1µg/kg. [28] Other tests, like the DNA comet assay, was developed to differentiate between radiated and non-radiated samples of wheat and tree nuts. After the run of the assay, radiated samples indicated a stretch in the DNA towards the anode, whereas non-radiated sample was intact. [29]

LFAs and dipsticks are used regularly as screening tests.Many ELISA kits have been made available for the detection of tree nuts, with high sensitivity and detection levels as low as 0.1 mg/kg. All tests produce quantitative results and can present variability due to cross-reactivity. One drawback of this test is that the matrix used can heavily affect the validity of the test. [30]. Apart from these tests, MS and PCR-based methods are also available, presenting a higher sensitivity and a lesser cross-reactivity than ELISAs. [6]

**WHEAT:**

Wheat is a gluten-containing cereal and was first found to cause allergies in a bakery worker, causing Baker's asthma. This was due to the inhalation of wheat, which caused an IgE-mediated response, which eventually resulted in respiratory illnesses[31]. The glutenin and gliadins, that make up gluten are mostly the cause of allergic responses in individuals. Gliadin, the antigenic protein with specific peptide sequences have been known to cause the disease, or allergic reaction [32].Cross-reactivity between wheat and other cereals is a common problem as all cereals are taxonomically derived from the same family. Researchers believe that the route of sensitization may point to differences between cereals. In the example of Baker's asthma, the individual only becomes sensitized when he inhales the allergen, not through ingestion. Ingestion evokes a delayed response[33].There seems to be no change in the allergenicity properties of wheat even after food processing treatments like baking and microwaving [6] Studies towards finding specific peptide sequences to detect the presence of wheat allergen has been done, and a sequence, Gln-Gln-Gln-Pro-Pro has been found to exist in a gluten[34]. This can now be used in any preventive or diagnostic test to check the presence of

wheat[35]. Besides baker's asthma, has been found to contain lipid transfer proteins (LTPs) and this can also be used as a diagnostic tool [36].

Among all cereals, wheat is considered to be the most allergenic. ELISAs, other immunoassays, and PCR methods have been used in the detection of wheat. However, MS techniques are most extensively used for the detection of this allergen; but no LOD has been stated so far. The least amount of wheat that can elicit an allergic response is around 2.5 mg of wheat protein. [6] Biosensors, a faster tool to detect allergen presence has also been used to detect the presence of wheat. Chemiluminescence microarrays have been used to detect the specific IgE binding site of wheat. [37] Electrochemical biosensors, like quartz-crystal microbalance (QCM) have been studied to detect the presence of gliadin in wheat matrices. [38]

**SOY ALLERGY:**

Soy is consumed largely in the continents of Asia, Europe, and the USA. The soy products consumed range from soy milk to fermented products like miso, tempeh, and tofu. It is often used as a substitute for milk and looked as a cheap protein source for vegetarians. Soy, like the allergens mentioned above, also triggers an IgE-mediated immune response but have not resulted in an extreme immune response such as those resulted from peanuts. [39] However, those individuals who are allergic to peanuts have severe reactions while consuming soy products as well. Soybeans contain at least 19 IgE-binding protein fractions and have been documented in the Allergome. There are instances of serological cross-reactions in soy allergic individuals in relation to peanut, peas, beans, and wheat flour. However this does not correlate to clinical cross-reactivities. Food processing like thermal, fermentation and high hydrostatic pressure reduce the IgE-binding capacity in soybeans. [6]

As the above allergens, the most common tools used to detect the presence of soy are ELISAs, PCR and MS. Soy processing and high cross-reaction affect the detection of ELISA. The analysis of DNA is more robust, specific and selective for the detection of soy. MS is also a suitable tool to detect the presence of soy, however, it is not suited for the detection of multiple samples. Biosensors have also been developed of very low LODS of less than 0.1% of the protein.

**MILK ALLERGY:**

Cow's milk allergy can be defined as an adverse immunological reaction to the proteins present in cow's milk. With less than 5% of preschoolers, less than 1% of children and less than 0.5% adults affected by the allergy, it can be concluded that it is not prevalent. [40]

Although milk contains around 25 different proteins, IgE hypersensitivity can be divided into reactions due to casein and non-casein proteins. IgE reactivity to caseins present in the milk results in immediate cutaneous, respiratory and gastrointestinal reactions and in extreme cases even systematic anaphylaxis[41].Non-IgE mediated CMA does occur but is difficult to diagnose[42].

Competitive ELISA is used to detect bovine serum albumin, casein, β-lactoglobulin with a limit of detection (LOD) ranging from 0.1 to 5 mg/kg. Polyclonal antibodies are favoured in this case for multi-allergen detection. A competitive fluorescence immunosorbent assay was developed for the detection of α-lactalbumin in dairy products. The assay used monoclonal antibodies bioconjugated with CdSe/ZnS quantum dots [43]. Efficient detection of α-lactalbumin, β-lactoglobulin, and bovine serum albumin can be possibly done by a laser induced fluorescence detector[44].The LC-MS/MS is a valuable confirmatory method to support the ELISA results. Enzymatic (Tryptic) digestion of cow's milk proteins followed by LC-MS/MS, facilitates their identification, characterization, and quantitative determination. Tryptic peptides of β-lactoglobulin and β-, αS2- and κ- casein were selected as markers for quantification. (LOD values 0.2- 0.5 mg/kg)[45].DNA-based assays are not so useful due to the low amounts of DNA present in the product, however, it is a robust method to detect allergens in pre-treated samples[46].

Amongst methods available to-date, the ELISA technique has demonstrated to be the most sensitive and specific for the detection of milk proteins in food products. Keeping in mind the low DNA content in milk and less sensitivity of DNA-based detection of allergic milk proteins, DNA-based detection methods are

generally not preferred for milk allergens. Fast biosensor immunoassays that were recently developed have been described for the detection of residual immunogenicity of food products with a good sensitivity. Although such techniques have been described to be fast, repeatable and fully automated they do not allow a characterization of the immunoreactive milk proteins. Mass Spectroscopy does solve this problem.

**EGG ALLERGY:**

Hen's egg allergy is the second most common allergy in children, next to cow's milk allergy[47]. The egg white portion is more allergic than the egg yolk as the majority of the allergens are present in the egg white portion. The egg white allergens include around 24 proteins of which orosomucoid (which comprises of 10% of the egg white) is the most allergenic [48]. Serological and clinical cross-reactivity with other bird eggs such as turkey, duck, goose, seagull, and quail are common in hen's egg allergy, making these eggs unsafe for the majority of egg-allergic individuals[49]. Chicken serum albumin(Gal d 5) has been found to be responsible for this cross-reactivity[50]. On thermal processing, the accuracy of detectability of egg allergens in food matrix decreases, on account of the reduced recognition of the modified native protein by antibodies and/or the decreased solubility of the proteins [51].

ELISA kits are usually used for the egg allergen analysis and they generally, target ovalbumin and orosomucoid. Yeung et al. (2000) developed an ELISA assay based on polyclonal antibodies. The assay was specific to whole egg proteins with a LOD of 0.2 mg/kg [52]. A sandwich ELISA assay which used ovalbumin and dehydrated egg white solids as antigens with a LOD of 1 mg/kg [53] has also been developed and applied to numerous foods.

Several optical-based biosensors have been described for the detection of ovalbumin. An optical resonance enhanced absorption (REA)-based immuno- chip sensor indirect and sandwich assay formats using antibodies functionalized with gold nanoparticles has been proposed as a rapid colorimetric method for detecting ovalbumin and ovomucoid in foods[54]. The biosensor gave reproducible and selective results with a LOD of 1 ng/mL, enabling high-throughput screening.

An optical planar waveguide array platform has also been developed for the detection of multiple allergens, including ovalbumin, using fluorescence sandwich immunoassays with a LOD of 25 pg/mL in the buffer and of 1.3 ng/mL (13 ng/g) in pasta[55].

Although the accuracy of detection of egg proteins by MS and ELISA methods is affected by the matrix, thermal processing, and extraction conditions, these methods are more reliable than DNA-based methods. DNA-based methods should not be used for the detection egg proteins in foods firstly, due to the low content of DNA in eggs. Secondly, due to the fact that egg DNA cannot be distinguished from chicken DNA and this may lead to misinterpretation of the data obtained while analyzing complex food mixtures.

**FISH ALLERGY:**

Fish is a valuable source of essential amino acids, polyunsaturated fatty acids and lipid soluble vitamins for human beings. However, fish is considered one of the eight most common allergenic foods which are collectively considered to be responsible for about 90% of food allergic reactions[56].Route of fish allergen exposure is not only via gastrointestinal tract during ingestion, but also via the respiratory system and skin contact during inhalation of cooking vapours and manual handling, respectively.

In a study conducted in Japan, a sandwich enzyme-linked immunosorbent assay (ELISA) was developed for the determination of the major fish allergen, parvalbumin, in processed foods, by usage of a polyclonal antibody raised against Pacific Mackerel parvalbumin. The limit of detection (LOD) was estimated to be 0.23 mg fish protein/kg food and limit of quantification (LOQ) were 0.70 mg fish protein/kg food. The developed sandwich ELISA showed 22.6- 99.0 % reactivity to parvalbumins from various species of fish. Further, inter-laboratory validation of sandwich ELISA demonstrated good recovery results (69.4- 84.8%) and the repeatability and reproducibility relative standard deviations were satisfactorily low. (≤ 10.5%) Thus, it was judged to be a reliable tool for detection of parvalbumin in processed foods[57].

PCR-based assays can be used to detect and differentiate fish DNA of closely related fish species[6].Conventional PCR has been used for detection of parvalbumin DNA of Pacific mackerel (*Scomber japonicas*)[58].It has also been used for specific detection of salmonid fish and Atlantic herring in processed foods, with LODs of 0.02 mg DNA /µl and of 10 pg DNA/µl, respectively[59,60].A real-time PCR assay which identifies eight fish species in food was developed by using PCR for amplification of fish parvalbumin introns and the multianalyte profiling (xMAP$^{TM}$) technology with probes targeting species- specific sequences. The LODs for the eight fish species ranged from 0.01% to 0.04%[61]. Mass Spectroscopy (MS) can be used for the identification of fish parvalbumin in several processed foods. Parvalbumin is extracted, purified and digested with trypsin in High-Intensity Focused Ultrasound (HIFU) equipment. Nineteen selected peptide biomarkers are separated by LC and monitored by Selected MS/MS Ion Monitoring (SMIM) in a linear ion trap mass spectrometer[62].

In the case of fish allergy, the chance of occurrence of cross-reactivity is very high. Usage of MS for allergen detection may help us overcome the cross-reactive problem which takes place in case of immune-assays. However, ELISA, MS, and PCR have been used successfully to detect parvalbumin and DNA to different fish species in foods.

**SHELLFISH ALLERGY:**

Shellfish mainly includes crustacean and molluscs. Allergy to crustacean shellfish, which includes shrimp, prawns, lobsters and crabs seem to affect school-aged children and adults predominantly. The allergens responsible for crustacean allergies include tropomyosin, arginine kinase, sarcoplasmic calcium-binding protein (SCBP), myosin light chain(MLC), troponin C and triosephosphate isomerases[63].

Tropomyosin (Pen a 1, Pen I 1, Met e 1) is the major shrimp allergen, identified in *Penaeusaztecus, Penaeusindicus* and *Metapenaeusensis*, respectively. Around 80% of shrimp allergic subjects, produce IgE antibodies which are reactive to tropomyosin. In a crustacean allergic individual, the probability of cross-reactivity to another crustacean species has been estimated to be 75%[64]. Although tropomyosin is the allergen most frequently involved in cross-reactions among crustaceans[65], SCBP (sarcoplasmic calcium binding proteins) also shows high sequence identity among crustaceans and also contributes to serological cross-reactivity[66,67].

Monoclonal antibody (Mab) sandwich ELISA was the first method developed for the quantification of major shrimp allergen tropomyosin Pen a 1 of brown shrimp (*Penaeusaztecus*). The method was sensitive,(LOD 1 ng/ml), reproducible and suitable to detect Pen a 1 like molecules in extracts from other crustaceans like crab and lobster. However, it was not tested for detection of other shellfish in cooked foods[68].Rabbit antisera as the capture reagent and a biotin-labelled mAb as detector reagent was used to develop a sandwich protein chip assay. The assay could quantify shrimp allergens in food matrices[69]. The protein chip assay, although cross-reactive with allergens from other crustaceans, had LOD of 0.054 mg tropomyosin/kg and LOQ of 0.096 mg tropomyosin /kg.

With a PCR-restriction fragment length polymorphism (PCR-RFLP) method, detection and species identification of crustacean DNA was achieved. The specificity of the method was demonstrated by analysing shrimp, crab, lobster and crawfish. The LOD was < 0.1 % for shrimp in a raw meat mixture[70].Mitochondrial genes were targeted by two real-time PCR assays which aimed to detect penaeid shrimp and blue crab. Due to the high copy number of mitochondrial genes, these assays were highly sensitive[72].The assays were tested using shrimp and crab meat spiked into several types of foods at levels ranging from 0.1 to 106 mg/kg and analysed either raw or cooked. Thermal processing of foods had little effect on the assay performance. LODs were between 0.1 and 1 mg/kg.

In a cell-based electrochemical biosensor for the quantification of shrimp tropomyosin (Pen a 1) [73], rat basophilic leukemia (RBL-2H3) mast cells encapsulated in type I collagen were immobilised on a self-assembled L-cysteine/gold nanoparticle(AuNPsCys)-modified gold electrode and pre-sensitised by specific anti-shrimp tropomyosin IgE. In the presence of the antigen, mast cells exhibit morphological changes which indicate degranulation, thus inducing dose-dependent impedance signals which can be detected by EIS. The impedance value increased with the concentration of purified tropomyosin, with a LOD of 0.15 µg/mL.

Due to the existence of a huge number of crustaceans, all of which possessing the major allergen, tropomyosin, the chances of cross-reactivity in detection methods is very high. Cross-reactivity may take place not only amongst crustaceans but also with other seafood which may include molluscs and fishes. DNA-based methods can discriminate among crustaceans by targeting specific nucleotide sequences, while commercially available ELISA kits targeting tropomyosin are generally not able to differentiate between shrimp and crab species because of the high homology of the tropomyosins. Thus, DNA-based detection methods prove to be superior to immunological methods for detection of crustacean allergens in the food matrix.

**RECENT TRENDS:**

Newer techniques involve simultaneous analysis of multiple allergies for rapid detection of the allergens. For example, a multi-method for the detection of seven allergenic foods (egg, milk, soy, hazelnut, peanut, walnut and almond) implied extraction of the allergenic proteins from the food matrix, digestion with trypsin and selection of the marker peptides. Peptides were separated by HPLC and analyzed with a LOD of 50 µg/g [74]. Two quantitative hexaplex real-time PCR systems for the simultaneous detection and quantification of 12 allergenic proteins in food was developed in 2012 [75]. The two tests showed good specificity and sensitivity (LOD of at least 0.01 % for all allergenic ingredients) in mixed foods. Detection time is biosensors is reduced by the integration of ELISA with microfluidics analysis. The sensor was proved to detect wheat and Ara H 1 allergic proteins, and was found to have a quicker response time as compared to ELISA [76]. The advantage that comes with biosensors is that very minute concentrations can be analyzed with biosensors [77].

Commercial testing kits that estimate the quantity, as well as the presence of the allergen in the food sample, are also available, as shown in Table 1. The analysis of food samples is different from that of a blood sample, as the presence of an allergen in a blood sample would not be as easily detectable in a food sample. The most commonly used tool is ELISA, which reinforces the fact that so far, it is the best molecular tool to detect allergens. With the rapid advancements in the components of biosensors this tool seems to hold promise in food sector due to its versatility.

**Table 1: Table of Allergens**

| Allergen Name | Biochemical Name | Allergen | Common Foods | Commercial Testing Kit | Type of Tool | LOD (ppm) | Time for results (minutes) |
|---|---|---|---|---|---|---|---|
| **Peanut** | *Arachishypogaea* | Ara h 1 through Ara h 17 | Candies, cereals, chocolates | NeogenBiokits Peanut Assay; | ELISA | 0.1 | 30 |
| | | | | RIDASCREEN FAST Peanut | | 0.13 | 30 |
| **Tree Nuts** | Includes Hazelnuts, Pecan nuts, Cashew, Pistachio, Walnut | Car i 1, 2 and 4 Cor a 1,2,6,8 to 14 Ana o 1 to 4 | Bread, cookies, cereal, baked goods | MonoTrace ELISA testing kit | ELISA | < 1 | 30 |
| | | | | AllerSnap | Rapid Swab Test | 3µg | 15 |
| **Egg** | Egg white and yolk [78] | Gal d 1 to 6 | Mayonnaise, Bread, Bakery items, Pastry | Imutest Egg Allergy Test | ELISA | N/A | 30 |
| | | | | AgraStrip Egg | Lateral Flow Device | | N/A |
| **Wheat** | *Triticumaestivum*[79] | Tri a 12 to 45 | Rusk, Semolina, Couscous, Flour | Morinaga (MloBS) | ELISA | 0.25 | N/A |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Fish** | All types of species of fish | Lep s 1, Pon l 4,7 | Canned Tuna, Salmon, Cod, Mackerel | SureFood (R-BioPharm) | PCR | <0.4 | N/A |
| | | | | Fish-Check (Bio-Check) | ELISA | <1 | N/A |
| **Soybean** | *Glycine max* | Gly m 1 to 8 | Soy milk, soy ice cream, soy sprouts, miso, natto | Soya-Check (Bio-Check) | ELISA | <0.7 | 60 |
| | | | | SureFood (R-BioPharm) | PCR | <0.4 | N/A |
| **Shellfish** | Includes crustaceans, molluscs, echinoderms | Art fr 5, Cha f 1 | Seafood-shrimp, crab, clams, oysters | Lateral Flow Crustacean Assay | Lateral Flow | 10 | 10 |
| **Milk** | Allergens are whey and casein proteins | Bos d 4 to 12 | All milk and dairy products | RIDEASCREEN FAST Casein (R-BioPharm) | Sandwich ELISA | 0.12 | 30 |

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