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Development of Analytical Methods for the Detection of Various Food Allergens

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Preface

This work is done from January 2007 till June 2010 under the supervision of Ao. Univ. Prof. Dr. Margit Cichna-Markl at the Department of Analytical Chemistry, University of Vienna, Währingerstraße 38, 1090, Vienna Austria.

To My Parents who have always sacrificed their wishes over mine

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1 INTRODUCTION

People often encounter ill effects of food intake. The term adverse reaction to food applies to any clinically abnormal response induced by food.[1] Food allergy, an adverse reaction to food, is now recognized as a significant food safety issue. Usually harmless dietary proteins of either animal or plant origin are common elicitors of food allergic reactions in susceptible humans evoking a range of symptoms and are thus gaining prominence as a problem in clinical medicine.[2, 3]

This chapter is divided into four sections; the first section deals with various aspects of food allergy, the second section focuses on its management and treatment, the effects of food processing on the allergenicity of food are discussed in the third section and the last section deals with the legislations, analytical methods to verify correct declarations and some food allergens in detail.

1.1 Food allergy and various aspects

1.1.1 Food allergy and its causes

Food allergy is an abnormal response towards specific foods and specific proteins present within. Such hypersensitive responses mediated by the immune system occur in sensitized individuals only.[3-6]

It is the food allergen that is responsible for the allergenicity. Food allergens are proteins and at times the products of protein breakdown such as the peptide fragments that retain the allergenicity of the native protein are also considered as allergens.[7] Food allergens are generally glycoproteins of molecular weight ranging from 12 to 30- 40 kDa or polymers of those proteins.[7]

There are two major unanswered questions related to the food allergy associated research. One being, what makes an individual allergic to completely harmless food but not another individual of perhaps even the same family. The other question is about the traits of that particular food that renders the allergenic potency in comparison to other foods. Various studies are being carried out to investigate this.[8-10]

To maintain health, a properly functioning and well balanced immune system is essential. Food allergy is a response of the immune system. One of the theories used to explain food allergy is that since the World War II, prevalence of infectious diseases like mumps, tuberculosis, measles and several others has decreased and the life style has become more hygienic. All these factors have resulted in immune maturity that is either impaired or delayed. Therefore, overly hygienic living conditions have led to defective immune maturation which results in sensitization towards completely harmless food.[11]

Despite the fact that food allergy poses a serious health problem and is considered the fourth most important public health problem by the World Health Organization (WHO), there is still no effective treatment other than total avoidance of the food allergen or some rescue medication in case of accidental ingestion.[2, 5]

1.1.2 The difference between food intolerance and food allergy

A misperception of food allergy exists in general public; this is mainly due to the fact that food allergy is misunderstood and often intolerance is proclaimed as allergy. It is therefore important to differentiate between the two. True food allergies or hypersensitivities differ from other forms of adverse reactions to food which are more appropriately termed as food intolerances as any abnormal reaction due to them does not involve the immune system.[6] Food intolerance can also be of a toxic or pharmacological origin.[12] Allergies, on the other hand, represent an abnormal immunological reaction of great magnitude towards allergens. This reaction triggers the production of allergen-specific IgE antibodies (or IgA or T cells).[13]

1.1.3 Prevalence of food allergy

The exact prevalence of food allergies is unknown since every diagnosis is not based on double-blind placebo controlled food challenge (DBPCFC) test; instead, diagnosis is often made on the perception of allergic symptoms.[14, 15]

The prevalence of allergic diseases has increased gradually since the 1960s, especially in the first world countries. Despite the difficulties encountered in obtaining substantial population based data on the prevalence of food allergies, epidemiological studies published over the last 10 to15 years indicate that a significant percentage of the population suffers from food allergies and this percentage is continually on the increase while the exact cause remains unknown.[15-18] As a result

it has become an important health problem all over the world.[18] It is also gaining popularity with media and general public leading to increased awareness but the perception of the frequency of allergic reactions in general public is still far less than the actual incidences of food allergy.[19] Evidence suggests that the prevalence of IgE mediated food allergies is higher in infants and young children in comparison to the adult population.[18] Adverse health effects due to food allergic reactions occur in about 1-3% of the population and about 4-6% of children.[5, 13, 15, 20, 21]

1.1.4 The physiology of the immune response

The factors leading to a reaction after the allergen intake are of significance. Food allergy consists of two separate phases, first sensitization where no symptoms occur, and later elicitation with clinical manifestations. When an allergen is ingested, digestive enzymes act on it, it gets degraded to a certain extent and is absorbed in the gut mucosa. Specialized cells of the immune system deal with it and finally present it to the immune cells that are responsible for producing an immune response. In allergic patients, there is a resulting synthesis of specific antibodies of the immunoglobulin E (IgE) class by lymphocytes. The specific IgE antibodies circulate and bind to the membrane of blood basophils and tissue mast cells. Subsequent contact of the immune system with the same allergen will result in the production of more specific IgE that bind to basophils and mast cells and once the density of IgE antibodies bound to the mast cell and basophiles is sufficient, the allergen molecule binds with specific IgE. This triggers the allergic reaction and the release of pharmacologically active mediators like histamine, prostaglandins and leukotrienes causes the onset of the various symptoms associated with allergy.[5, 7]

1.1.5 Diagnosis of food allergy

An accurate and reliable clinical diagnosis is critical in food allergy as it helps patients against unnecessary and at times potentially health threatening diets and unnecessary exclusion diets imposed by misdiagnosis and also allows adherence to dietary suggestions for the allergic consumer.[1, 2] The diagnosis tends to ascertain a reliable link between the clinical history of an adverse reaction to food, as proclaimed by the patient, and the immunological basis of the reaction.[1] The diagnosis is based on several indirect tools that utilize the properties of the blood serum; the blood of the patients contains white blood cells and IgE antibodies.[5] Skin tests or *in vitro* assays are usually employed since these try to link the clinical reaction with the IgE

mediated pathophysiology. These diagnostic methods indicate the presence of food specific IgE antibodies but do not diagnose food allergy itself. A positive controlled food challenge gives the conclusive verification of the clinical relevance between the reported history and the detected food specific IgE.[1] These methods are discussed in detail in the following text.

1.1.5.1 Skin tests

The skin test was introduced by Blackley as the intradermal form of allergen skin testing more than 100 years ago and the prick test was described by Lewis and Grant in 1924. Since it is fast and cheap and due to the readily available results, skin testing is still the primary tool for the diagnosis of food allergy in daily practice.[1, 22] In a skin prick test, the skin is punctured through a drop of a glycerinated food extract or the test is conducted by first puncturing the native food and then the skin for the prick - to - prick technique. A skin prick test (SPT) reaction is considered to be positive in case of a mean wheal diameter of 3 mm or a wheal area of 7 mm^2, respectively.[1, 23]

1.1.5.2 *In vitro* tests

The *in vitro* determination of specific IgE is more time consuming and expensive and in general, renders comparable results to the SPT, nevertheless it has its own advantages which includes quantification of food specific IgE antibodies. This allows keeping track of the course of allergic disease in each patient.[1, 23]

1.1.5.3 Oral challenge tests - Double - blind placebo - controlled food challenges

Charles May is given the credit for initiating the use of double - blind placebo - controlled oral food challenges (DBPCFCs), in routine clinical and research aspect.[22] The DBPCFC is acknowledged as a gold standard in food allergen diagnosis, it also is the most credible and reliable method to confirm or dismiss the occurrence of an adverse reaction to a food in older children and adults.[23] It is however not employed for individuals with a history of anaphylactic shock. A standardized procedure has not been developed as yet; the challenge is performed by administration of increasing doses of the food under consideration. This is usually done by doubling the dose every 15-30 minutes until a maximum dose is reached, that is the normal daily serving. It is important that the first dose is below the individual threshold level.[1]

1.1.6 Symptoms

Allergic reactions to food encompass a broad spectrum of symptoms which range from mild ones like a skin rash to as severe as being potentially life threatening or even fatal in extreme cases.[3, 6, 24] Even the intake of minute amounts of food allergens is sufficient to elicit allergic reactions in sensitized individuals. In general, IgE mediated immediate hypersensitivity reactions occur rapidly and are often severe, fatalities have also been associated with them.[13, 18] The time observed for allergic reactions to appear varies from a few minutes to 2 hours following food ingestion or inhalation.[1] Elaborating further, a number of target organs may be affected and the symptoms manifested include digestive disorders, respiratory symptoms, circulatory symptoms and skin irritations and the reactions include urticaria, angioedema, rhino conjunctivitis, laryngeal edema, asthma, oral allergy syndrome, vomiting, diarrhea and systemic anaphylaxis.[13] The symptoms associated with food allergy are discussed below.

1.1.6.1 Cutaneous reactions (skin symptoms)

Skin symptoms, also referred to as the cutaneous reactions, are the most common clinical manifestations of food allergy along with the symptoms of the gastrointestinal tract.[25] Acute and generalized urticaria, with or without angioedema, is the most common occurrence. It is frequently observed in combination with symptoms of other target organs, but may be present as the sole demonstration of an allergic response.[1]

1.1.6.2 Oral allergy syndrome

Oral allergy syndrome (OAS) is a type of urticaria which is confined to the lips and oropharyngeal mucosa. The symptoms associated with the OAS include the pruritus of the lips, tongue, palate, ear and throat. These symptoms may also be coupled with a mild angioedema at the same sites and generally appear within 5 to 15 minutes following food intake. Even though food can trigger OAS, the syndrome is more commonly observed in patients allergic to pollen in association with allergy to fresh fruits, nuts or vegetables, or cross reactivity between homologous proteins present in pollen and plants.[1]

1.1.6.3 Gastrointestinal symptoms

Symptoms of food allergy induced by the gastrointestinal tract incorporate nausea, vomiting, abdominal pain and diarrhoea. These symptoms sometimes become apparent solely but more often are evident in combination with other target organs.[1]

1.1.6.4 The respiratory tract and food hypersensitivity

In addition to the above mentioned symptoms, respiratory tract symptoms may also be attributed to food allergy. These symptoms are uncommon, do not occur in isolation and encompass a broad spectrum. These include nasal congestion, rhinorrhea, sneezing, nose and throat itching, coughing and wheezing. Asthmatic reactions and acute bronchospasm caused by food allergy constitute a more worrisome and dramatic group of clinical manifestations with high risk since they are common during fatal and near fatal food allergic reactions.[1, 25, 26]

1.1.6.5 Anaphylaxis

Anaphylaxis is the severest manifestation of food allergy, can be fatal and has been established as a medical emergency.[19] It is an immunologically mediated event that occurs within minutes of exposure to a particular foreign substance even if the intake is in trace amounts.[27] It is caused by massive release of mast cell mediators that may involve multiple organ systems. The patients may also develop other symptoms like urticaria, pruritus, vomiting, diarrhoea, angioedema, laryngeal edema, bronchospasm, abdominal cramps, cardiac arrhythmias, hypotension and shock.[1] An approved definition does not exist, however, anaphylaxis may be defined as an acute, rare, life threatening generalized or systematic hypersensitivity reaction regardless of the mechanism or it may be defined by a set of more specific criteria especially involving severe respiratory or cardiovascular symptoms.[3]

Although fatal allergic reactions have been known for over 4500 years, anaphylaxis was not fully identified until this century. The term “anaphylaxis” finds its origin in a classic study by Portier and Richet in 1902 where they elucidated the rapid death of several dogs that they attempted to immunize against the toxic sting of the sea anemone. Since the reaction represented the opposite of their intended “prophylaxis” they contrived the term “anaphylaxis”, that is without or against protection. Several studies have indicated the main cause of anaphylaxis to be food hypersensitivity. The first contemporary information on food anaphylaxis in humans was

published by Golbert and colleagues in 1969. They described 10 cases of anaphylaxis following ingestion of various food assortments including legumes, fish and milk.[19]

1.1.6.5.1 Prevalence of anaphylaxis to food

A large variety of food stuffs has been reported to induce anaphylactic reactions and food anaphylaxis is the dominant cause of anaphylaxis as its occurrence is even greater than that of a bee sting.[3] The list of food stuffs that may do so is almost unlimited since in theory, any food protein is able to trigger an anaphylactic reaction.[27] Foods that are likely to induce an anaphylactic reaction include fruits, legumes, tree nuts, peanuts, fish, shell fish, cow's milk, egg, seeds and cereals or grains. Anaphylactic symptoms associated with food allergy occur in several patients, specially children and adolescents and the cases of anaphylaxis treated in the emergency department are increasing.[19] However, there are no exact prevalence figures of food anaphylaxis. One reason for unreliable data for its prevalence is the absence of an approved definition of anaphylaxis.[3]

1.1.7 Geographical aspect of food allergy

The affect of a particular region on the prevalence of food allergy cannot be ignored since it has been observed in certain populations. Food allergy is determined by the complex interactions of allergen exposure and the individual susceptibility. The allergen exposure is considered to be an important determinant since a lot depends on eating habits. In an area where a food is frequently consumed, the risk of allergy to that food is also greater than in areas where it is rarely eaten.[7] The various foods that are common elicitors of an allergic reaction vary from USA to Canada to the European Union.[6] This can be elaborated by the following examples; fish allergy has been reported to be more dominant in areas where it is largely consumed, as in Norway, Portugal and Japan. Similarly, shrimp allergy is reported to be more prevalent in southern states of USA. Peach allergy appears to be more frequent in countries where it is common part of food, for example, Spain, Portugal and Italy.[7] Also, peanut allergy is the most frequent food allergy in the United States and in England but it is not so widespread in other European countries. However, it has also been observed that with time, various allergies are spreading rapidly and becoming more common in those parts of the world where they were previously rare.[3]

1.1.8 Cross reactivity

Cross reactivity refers to allergic reactions towards multiple food stuffs due to the presence of proteins that share common or similar epitopes.[7] Several such cross reactions have been observed and reported. Cross reactivity between pollen and food stuffs also exists, frequent cross reactions are observed between birch pollen and hazelnut and apple and also between pollen of mugwort and celery.[7]

At times, a significant association exists between sensitization to certain fruits and vegetables along with sensitization to other foods belonging to either the same botanical family or to a different one. Clinically this phenomenon has been explained as “cluster of hypersensitivity”.[28] Several clusters have been observed and in 1984 Eriksson reported various clusters based on a long list of case studies.[28] These clusters include apple and pear, kiwi fruit and avocado, potato and carrot, parsley and celery and also stone fruits in combination with apple and celery, as well as hazelnut, walnut, Brazil nut, almond with desert almond.[28] Several other clusters have also been reported, these include celery, carrot, mugwort and spices; apple, carrot and potato; fennel and celery; cherry and apple; melon, water melon and tomato; fennel, carrot and celery; lettuce and carrot; tomato and peanut; celery, cucumber, carrot and watermelon.

1.1.9 Threshold doses

In toxicology the term threshold dose refers to the lowest quantity capable of eliciting a reaction. Likewise, in food allergy a threshold dose refers to the lowest amount of allergens that can trigger an allergic reaction. Any amount below it will not trigger a reaction and is therefore safe to consume.[7] The threshold dose that provokes symptoms is quite low and for IgE mediated food allergies it varies considerably ranging from a few milligrams to as much as eight grams or more.[18] It is generally assumed that the severity of the reaction is proportional to the degree of sensitization and the level of intake of the offending food. Clinical data shows that the threshold dose level, also termed as minimal eliciting dose (MED), vary among people and also within the individual over time.[6] Generally, it is believed that food allergic patients are at risk after consumption of food containing allergenic contaminates at > 10 µg / g food.[20]

1.1.10 Psychological considerations of food allergy

Food plays a significant role towards the social and physical development of an individual; it is no surprise that various psychological disorders are associated with food. Among various problems like anorexia, obesity, bulimia and many more, food allergy also has a significant role to enact.[24]

The most common psychological affect is anxiety or stress; food allergy may also become a continuous source of stress to the patient as well as to the entire family because of the endless vigilance necessary to avoid a reaction.[24] The stress posed by food allergy not only affects the sensitized individual and his family emotionally, it also limits family activities such as eating out as it is associated with a high risk factor. A vast number of problems arise because a considerable majority of the populace is still ignorant of the severity of the problem and do not comprehend the risk associated with ingestion of allergens. Despite the increasing awareness a lot of people still do not believe food to be life threatening in any way therefore, a sensitized individual or his family may encounter problems when interacting with such people. They might even try to feed the antigen containing food just to prove their point. Due to this allergic children may also have problems at school and for a highly sensitive child, parents may decide to home school the child to reduce the risk of exposure to offensive food. Another aspect is the development of certain eating habits after a reaction by the sensitized individual. This may include eating only certain things and avoiding all other food out of fear of a reaction which may lead to malnutrition. After an incidence of a reaction the patient may also become withdrawn, fearful and mistrust others for their food and parents of such individuals feel guilty and may lose confidence in their parenting skills. Thus psychologically both the patient and their families are affected since all the above mentioned factors have a huge impact on the personalities.[29]

1.2 Allergy management and treatment

1.2.1 Allergy management

A relationship between food ingestion and its reaction is clearly observed in certain cases, an acute IgE mediated reaction following food ingestion is one such instance. Allergy management then becomes considerably important for individuals susceptible to such reactions.[24] A comprehensive management plan for food allergy must encompass all aspects and include

adequate instructions required for avoidance of such offensive food and a written emergency plan of action for accidental ingestion.[1]

1.2.1.1 Allergen avoidance

Strict and complete avoidance is the key to prevent an allergic reaction and the only proven therapy.[1, 24, 30] Despite its importance, it has proved to be a challenge difficult to achieve. Various reports of such incidences exist in literature when an individual, fully aware of his allergic disposition, accidentally ingested the allergen containing food. As minute amounts of allergen can cause calamitous results, providing adequate information to the patient regarding the allergen holds great importance that cannot be denied. Awareness must be raised in patients concerning the different terms used for that particular food in the ingredient list and also information about the foods more likely to contain them.[1, 24]

1.2.1.2 Elimination diet

Since strict avoidance is the only way out for an allergic person, elimination diets are recommended. These tend to eliminate the offensive allergen from the diet of the individual. To recommend an appropriate diet, correct diagnosis with an accurate identification of the allergen is crucial. A significant clinical problem concerns the people suffering from allergic reactions to multiple foods, sharing homologous proteins. In such cases, where several foods need to be avoided, elimination diets may result in eating disorders and malnutrition. In such cases dietician supervision is imperative, particularly for growing children.[1, 12]

1.2.1.3 Label reading

For allergen avoidance, label reading is a critical step, thus affected individuals and their families depend on ingredient labels of commercial products to ascertain the safety of their food. An error in this process can arise due to incorrect labeling or erroneous reading of the label by the consumer and can have drastic results.[1, 31] It is therefore vital to read the label each time a product is purchased since different batches of the same product may have different ingredients. To read the label more than once, as a precautionary measure while purchasing a product will help minimize the chance of error in label reading.[1, 24] Another critical aspect of label reading has been reported by Joshi et al. in a study where they found out that percentage of people who were successfully able to identify the offending food on the ingredient list was very low since

most people were not aware of all the technical or scientific terminologies used. Examples of such incidences would be the presence of milk protein in a product; often labeled as “whey” or “ammonium caseinate” or the case of eggs, where the presence can be indicated as “albumin” or “globulin”.[31] Thus, to minimize the risk, patients must improve their awareness of the offending food and its terminology.

1.2.1.4 Allergy management outside the home

Most of the severe food allergic reactions occur outside home therefore eating out should be either avoided or carefully monitored to avoid any hidden allergens. It can also be avoided while travelling for long hours, even on a flight by carrying home cooked meals rather than relying on the meals served since most flights do not ensure allergen free food. Besides these precautions, friends and colleagues should be well aware of how to react in case of a reaction.[12, 32]

1.2.1.5 Allergen management in the food industry and associated issues

Consumer complaints concerning the presence of undeclared allergen at hazardous levels indicate that unresolved issues exist with allergen management systems in the industry. The food industry has taken numerous initiatives concerning several issues to meet the requirements of food allergic consumers. A brief overview of accidental presence of allergens and related issues follows.[16]

1.2.1.5.1 Raw materials

On the industrial scale, the first step where contamination may occur is the raw material. The supplier has often little or no knowledge of the formulation of ingredients used as raw materials. To overcome this, regular audits and training of the supplier may help.[16]

1.2.1.5.2 Manufacturing processes

The presence of undeclared allergen in a product can be due to the multi step manufacturing and factors associated with each step. These include inadequate cleaning, lack of physical separation at cross over points in production lines, carry-over of allergens from shared storage equipment or from maintenance tools. To overcome these problems, thorough cleaning, inclusive of disassembling of the equipment, different storage facilities and maintenance equipment for specific allergenic foods should be observed.[16]

1.2.1.5.3 Equipment design, effective training of employees and labeling

Equipment design can pose as an important issue; either because it is hard to clean because of the difficulty in dismantling that may result in allergen accumulation which cannot be readily detected by inspection.[16] Training of the employees is also critical since often wrong people are trained or not enough people are provided with adequate training.[16] Since avoidance is the key for survival, effective and correct labeling is essential to achieve a high health protection level and to guarantee complete information to consumers.[33] As most consumers are aware of their specific sensitivities, a complete labeling of ingredients will minimize the chances of a reaction.[33] Instead of using old labels, new labels must be used each time because if the labeling is not reviewed each time, ingredient declaration could be inaccurate.[16]

1.2.1.5.4 Cross contamination

Cross contamination is another serious issue; it is the contamination of allergen free food products with traces of food allergens through the production process. As a precaution, food manufacturers often label their food products with "may contain" a certain allergen. This drastically reduces the range of food products suitable for allergic consumers.[13] Contamination in bakeries is quite likely, especially when fresh products are placed in close proximity without any packing. Avoiding such high risk foods will definitely help minimize the risk of accidental ingestion of the allergen.[34]

1.2.2 Treatment of a food – allergic reaction

1.2.2.1 Rescue medication

Although most allergic individuals with severe food allergy are aware of their allergic disposition and the causative food, avoiding food allergens is often complex and the possibility of accidental ingestion of allergens cannot be ruled out, in fact it is quite frequent.[20] Therefore having a rescue medicine is of vital importance. All patients and their close acquaintances should be trained for an early recognition and treatment of an allergic reaction. Epinephrine and adrenaline are the key drugs for treatment of severe or potentially severe food allergic reactions. Patients must carry self injectable adrenaline and should be trained to inject it intramuscularly.[1, 35]

1.2.2.2 Immunotherapy

Current methods of allergy management, complete avoidance of the offensive food and rescue medication are certainly not the cure.[2] A curative treatment is an essential requirement for food allergic patients suffering from severe anaphylactic reactions that cannot follow elimination diets because of exposure to hidden allergens and a risk of health threatening or even fatal reactions. Since for respiratory and insect venom allergies, specific allergen immune therapy (IT) emerged as an effective treatment it can be expected that it could possibly be a therapeutic option for food allergies. From recent studies it can be expected that in future new strategies, such as sublingual and oral immunotherapy, will be effective in reducing sensitivity to allergens.[36]

1.3 Food allergens and effects of processing

1.3.1 Allergens in foods

Considerable efforts have been taken so as to identify the attributes of non-toxic food proteins that evoke an IgE mediated allergic response in sensitized patients. Several allergens have been identified and allergen data bases have also been set up. Food allergens seem to be restricted to a small number of protein families.[37] Proteins are consumed daily in enormous variety through plant and animal sources. In an atopic individual, there is a high risk of an allergic reaction as soon as the protein is ingested. Allergenicity of any food is rarely due to a single protein, it is mainly due to an allergen repertoire comprising several different proteins.[7] According to structure and sequence criteria these proteins are classified into different protein families, one such database classifying proteins into families on the basis of sequence homology is Pfam database.[38] Allergenic proteins are found in not more than 29 of the total of *ca.* 4000 Pfam families with only three dominating plant food allergen protein families/superfamilies that have been identified. These include the prolamin (including 2S albumins and LTPs), the cupin family and the Bet v 1 family (PR 10 family). These along with profilins account for more than 65% of all plant food allergens.[38, 39]

1.3.2 The effects of food processing on allergens

Factors affecting allergenicity of food include the food matrix and its effect on protein stability as well as food processing techniques which can either increase or decrease the allergenic activity.[37] Recent research has highlighted this important aspect of processing on allergenicity of

food.[38, 40] There is a general agreement that depending on the biochemical and physical specificities of the allergenic proteins in a certain food, the impact of a given technology on the allergenicity of food is variable.[39, 41] During thermal preservation of several fruits and vegetables their allergenicity is reduced or even rapidly lost for a vast majority of sensitized consumers. This is because consumers may suffer from the oral allergy syndrome which is provoked by the PR 10 proteins. These are relatively unstable to heating since several epitopes, the protein region recognized by an antibody, are conformational and more vulnerable to heat treatment than the linear epitopes.[38, 39]

1.3.2.1 Thermolabile proteins - unfolding conformational epitopes

The Bet v 1 superfamily of plant food allergens is the major group of proteins involved in the pollen and fruit or vegetable cross reactivity. In general Bet v 1 homologues are labile proteins and during cooking, the conformational epitopes are destroyed as a consequence of unfolding, their IgE reactivity and ability to elicit a reaction is also reduced by such food processing.[42]

1.3.2.2 Thermostable proteins – undergoing limited folding and aggregation

Some food proteins unfold to a limited extent, resulting in partially folded molten globule structures which may aggregate and form other structures within foods. The 11S and 7S globulins are significant plant food allergens belonging to the cupin super family with a tendency to form large thermally induced aggregates. These globulins are stable, the thermal transition of 7S globulins is around 70 – 75 ºC while 11S unfold at temperatures above 94 ºC. However, only minor changes are observed in the protein secondary structure even on heating to such temperatures. As a result of this structural stability of the globulins their allergenicity also shows remarkable thermostability.[42]

1.3.2.3 Thermostable proteins – resisting unfolding and refolding on cooling

There are certain proteins that are stable even in denaturing conditions such as high temperature and pressure or low pH and high concentrations of chaotropes, which are capable of disrupting macromolecular structures such as proteins; these include guanidine hydrochloride or urea. The prolamin superfamily is an example for such proteins.[42] Thus food processing has no effect on such families of proteins.

1.3.3 Options for processing to minimize allergenicity in foods

1.3.3.1 High pressure processing of food

Few studies concerning the influence of high pressure treatment on allergenicity have been published. High pressure processing has been used to reduce the allergenicity of food stuffs in some cases. For instance, allergenicity of rice was reduced using high pressures of 100-400 MPa. Under such conditions, a considerable amount of proteins was released which on analysis by SDS-PAGE and immunoblot, proved to be the major rice allergens.[39]

1.3.3.2 Removal of allergens

Separation technology can also be employed to reduce food allergenicity. Ultra filtration, which has been used to prepare infant formulas in combination with proteolysis can be used to remove large immunologically active allergen fragments.[39] In foods where allergens are expressed in specific tissues only, tissue removal will reduce the allergen load of the resulting processed food.[39]

1.3.3.3 Enzymatic treatment

Allergenicity can be reduced via enzymatic treatment in two ways. One of these is the proteolytic modification of the epitopes. In this case the epitopes are targeted in such a way that the biological functioning of the allergenic protein is not affected. Ascertaining sufficient contact between protease and allergen and proper combination of the two can help reduce allergenicity. Proteolytic processing and using the information on epitope structure and suitable proteases have been successfully applied to decrease the allergenicity of wheat flour gluten and soy.[39] Ensuring adequate epitope or allergen contact with the enzyme, enzymatic processing of raw materials may also reduce the allergenicity.[39]

The other method is the enzymatic oxidation and cross-linking; it is presumed that the oxidatives affect proteins allergenicity. Sufficient contact between allergen and oxidising enzyme is essential, enzymes include the polyphenol oxidases and peroxidases. This is associated with some effects of the oxidation reaction like decolouration or changes in taste.[39]

1.3.3.4 Other novel technologies

Other techniques include gamma irradiation and pulsed electric fields and acoustic treatment. So far, there are no reports of successful attempts to reduce allergenicity by means of gamma radiation. It has been proved to be unsuccessful in reducing the allergenicity of celery allergens.[39]

1.4 Legislations, important allergens and analytical methods

1.4.1 Legislations concerning food allergy

The allergenic potential of a broad range of food stuffs has been identified. It is particularly important for an allergic patient to know the exact composition of food especially concerning the presence of potentially allergenic constituents. Several initiatives have been taken by public health authorities and subsequently legislations have been formulated. Until recently, labeling of ingredients that were less than 25% of the finished product was not compulsory in Europe as indicated in Article 6 of EC Directive 2000/13 of the European Parliament. However, due to strong consumer requests for better information about food composition and for consumer protection, the European Commission has issued EC Directive 2003/89 as an amendment to EC Directive 2000/13, effective since 2005 that deals with ingredient declarations on food stuffs. Annex IIIa of this guideline includes a list of food ingredients and products thereof classified as possible allergens or leading to possible intolerance. In addition, any ingredient used in production and is present in the finished product, whether in its original form or altered, must be indicated on the label with clear reference to the name of this ingredient.[33] Sesame and its products are on the list as well as lupine and its products, which have been added in 2006 to the list.[13] Poppy has yet to make its way to this list since allergy to it is very rare although anaphylactic reactions to poppy have been reported. With the requirement of food allergen labeling in the EU and the US the awareness of food allergies is not only increasing among consumers but also among food processors and regulatory agencies.[10]

Keeping in mind the severity and prevalence of the three afore mentioned food stuffs, sesame, poppy and lupine, the following text discusses in detail their plant physiology, uses and allergenic potential.

1.4.2 Sesame

Sesame being an herbaceous plant of the family Pedaliaceae is an annual self pollinating plant with an erect, pubescent, branching stem, and is 0.60 to 1.20 m tall. The principal species found in commerce is *Sesamum indicum*.[43] Sesame (*Sesamum indicum* L., Pedaliaceae) is a very old crop and is probably the most ancient oilseed cultivated by mankind. It is thought to have originated in Africa but it has also been characterized to be a highly prized oil crop of Babylon and Assyria about 4000 years ago. Sesame has historically been associated with various regions, the Chinese used sesame oil for light and to make soot for ink blocks. Another example is of African slaves who brought sesame seed to America; they used the term "benne seeds" for it and thus sesame became a popular ingredient in Southern recipes. The English term "sesame" traces back to the Arabic term of *simsim*, the Coptic *semsem*, and the early Egyptian term of *semsent*.[44] Sesame is still being used worldwide with different names according to the region of production, in some areas like China, Mexico, South and Central America it is known as "sesamum", in South India and Burma it is termed as "gingelly". In regions of Sierra Leone, Guinea and West Africa it is called "benniseed" whereas it is termed as "sim-sim" in Middle East and "till" in East and North Africa.[45] China, India, Sudan, Mexico and Burma are the major producers of sesame seeds in the world by contributing to approximately 60% of its total world production. In Burma, it is the major source of edible oil for local consumption.

1.4.2.1 Nutritional value of sesame

Sesame is used in the form of whole or crushed seeds or as sesame oil since it is a highly valued source of edible oil as well as protein and the crop holds great importance owing to its high content of excellent quality oil. The sesame oil, with a mild pleasant taste, is a natural salad oil which is easily digested and is stable to oxidative stress, for these reasons it is acknowledged as healthy for consumption.[45, 46] The chemical composition of sesame shows that it is an important source of oil containing 42–54% oil and 22–25% protein.[45, 47] It was also reported to have 13.5% of carbohydrates, 5.3% of ash and 5.2% of moisture. Sesame constitutes an inexpensive source of protein, fat, minerals and vitamins in the diets of rural populations, especially children. Products derived from sesame have been recommended for young children in societies of the Mediterranean region, because of their high nutritional value.[43] The edible parts of sesame seeds consist of the embryo. The embryo of the sesame seeds is used to make sesame butter-like that is

called “tehineh” which is a popular food in the Middle East. Another popular sesame food is “Ogerie”, it is used in Sierra Leone, West Africa, and is used in bakeries, confectionaries and in the formulation of baby food. The embryo is also used in the making of sesame oil. Literature has various reports concerning the health benefits associated with the consumption of sesame including weight gain control, prevention against cardiovascular diseases and protection against ageing.[45]

1.4.2.2 Protein composition of sesame

The amino acid composition of the sesame seeds is unique and unusual among the oilseed proteins owing to the high content of sulphur containing amino acids that is, methionine and cysteine, and low content of lysine. Due to its amino acid profile, sesame has been recommended as a protein supplement for legumes. Furthermore, sesame is also a rich source of niacin, folic acid, vitamin E, calcium and phosphorus. Major proteins present in sesame seeds are storage proteins such as albumins that constitute 8.9% of the proteins, globulins (67.3%), prolamins (1.3%) and glutelins (6.9%). The major proteins of sesame include the water insoluble 11S globulin which constitutes 60-70% of the total seed proteins and the soluble 2S albumin which constitutes approximately 25% of the total seed proteins. The two are conventionally termed α-globulin and ß-globulin, respectively.[47,48] The existence of a 7S globulin as a minor constituent of the total storage proteins in sesame has briefly been reported in several investigations. The 7S globulin constitutes approximately 5% of the total sesame protein.[47]

1.4.2.3 Sesame allergy and its prevalence

Sesame seed allergy is a common cause of food allergy in many countries, including Israel, Japan, the United States, and various European countries.[49] The incidence of sesame seed allergy in adults and children has been increasing for the past several decades and has been documented in several countries, including Great Britain and Australia.[50, 51] During recent decades, the use of sesame seed has spread to North America and Europe. The increasing prevalence in European countries could represent 2–4% of total food allergies.[52-55] Its prevalence in Israel is more common than in North America and Europe which can be attributed to the early exposure to sesame in Israel where it is often recommended to children in their first year of age.[56] In a more complex study investigating 4078 Australian children, the sensitization rate to sesame seed was found to be one third to that of the peanut sensitization rate and higher than the sensitization rate

to any tree nut.[57] The increasing consumption of foods containing sesame seeds and sesame oil is paralleled by an increase in reported sesame-induced allergic reactions.[43, 58, 59] The increasing consumption of sesame seed containing foods is due to the products which have come into the market due to increasing trends of vegetarianism and other products such as the international fast food and bakery products, where sesame seeds are generally used for toppings on breads, like hamburger buns, bagels, bread sticks, and other baked goods.[57] Sesame is not only used in edible products but also in cosmetics and pharmaceutical products, and allergic reactions to cosmetics/toiletries have also been reported.[59] This increase in sesame-induced allergic reactions called for additional studies on the characterization and identification of the specific sesame allergens.[43]

1.4.2.4 Clinical manifestations of sesame allergy

Ingestion of sesame seeds and sesame oil by sensitized individuals has various clinical manifestations. Sesame seed allergy can result in either a delayed-type hypersensitivity reaction or an immediate-type systemic immunoglobulin E–mediated response. The clinical picture includes characterization by atopic dermatitis, oral pruritus; angioedema of the lips, tongue, and uvula; generalized erythema or urticaria; rhinitis, respiratory distress, asthma, nausea, vomiting, weakness, or hypotension. Hypersensitivity reactions tend to be more severe in nature, often resulting in an anaphylactic reaction in severe cases.[57, 60] Many sesame allergic patients are also allergic to tree nuts or peanuts, which is not surprising given the high level of antigenic cross reactivity observed between sesame seeds and tree nuts.[51]

1.4.2.5 Identifying sesame allergy

To identify sesame allergic individuals is not an easy task. The DBPCFC (double-blind, placebo-controlled food challenge) is the gold standard for the diagnosis of sesame allergy. Moreover, in many instances, it is perhaps the only way of establishing a link between a clinical reaction and exposure to sesame allergens. However, oral challenges on patients with a history of anaphylaxis are generally not performed due to the severity of the reactions triggered by sesame. Generally SPTs and *in vitro* tests are the only procedures used to identify sesame allergic individuals.[61]

1.4.2.6 Allergens in sesame

The major allergens of sesame seeds have already been identified: these include a sulfur poor 2S albumin Ses i 1 with the molecular weight of 10 kDa, a sulfur rich 2S albumin, Ses i 2, with the molecular weight of 7 kDa, a 7S vicillin-like globulin that is Ses i 3 with a molecular weight of 45 kDa,[51, 57, 62, 63] two oleosins with a molecular weight of 17 kDa and 15 kDa called Ses i 4 and Ses i 5, respectively [55] and two 11S globulins called Ses i 6 and Ses i 7.[49]

1.4.2.7 Threshold doses

Sesame allergens are known to be very potent, causing particularly severe reactions in sensitized persons with a high risk of life threatening anaphylaxis.[54, 56, 64, 65] Exceptionally minute amounts of sesame are capable of eliciting a severe allergic reaction in highly sensitized patients. Extremely sensitive subjects were reported to react to doses as low as 30 mg crushed sesame seeds and 1 mL sesame oil.[61]

1.4.3 Poppy plant

The poppy plant (*Papaver somniferum*) is a member of the botanical family Papaveraceae and an annual herb. It originated from the coast of the black sea and is cultivated extensively in many countries, especially in Asia and Central and South America. It has also been grown all over Europe for centuries and is the source of both poppy seeds and opium.[66, 67]

1.4.3.1 Uses of poppy

Papaver somniferum is the source of both poppy seeds and opium. The commercially available seeds are widely used as ingredients for various cuisines.[66, 67] Poppy seeds and poppy oil are used both for cooking and flavoring. The commercially available seeds are widely used throughout Europe as ingredients especially for poppy cakes and poppy rolls. Crushed and sweetened poppy seeds are a common filling for certain crepes, strudels, and pastries.[67, 68] In addition to food, poppy seeds are sometimes used for coloring of, for example, antitussive syrups.

1.4.3.2 Allergenicity of poppy

Despite the widespread use of poppy, allergic reactions as a consequence of consumption are rare.[68] Seeds can induce immediate-type allergic reactions and IgE-mediated sensitization to poppy seeds, if present, is usually associated with severe clinical symptoms.[66] Since sensitized patients can develop severe anaphylactic symptoms, it is important to recognize the

hypersensitivity to poppy seed.[67] Positive prick tests with raw and baked poppy seed are indicative of the thermostability of the relevant antigen.[69]

1.4.3.3 Symptoms

Allergic reactions to poppy are rare but severe and associated symptoms may affect the skin, mucosa, and bronchial and gastrointestinal tracts. The clinical symptoms reported range from itching around the mouth and lips and nausea to anaphylactic reactions after consumption of poppy.[69] Another fact of significant importance is that a reaction to poppy seed can also be caused by inhalation and not only by oral exposure to the allergen. This further confirms that poppy seed allergy is usually severe.[68]

1.4.3.4 Allergens in poppy

The allergens identified and that have so far been isolated include Pap s 17 kDa, which is also a Bet v 1 homologue, a Pap s 34 kDa and a Pap s profilin.[66] In an instance, where the sera of 11 poppy seed-allergic patients was studied, specific IgE from the sera of 10 patients bound to a 45 kDa protein, of 4 bound to a 34 kDa protein and of 5 to a 17 kDa and to a 14 kDa protein and 3 to a 5 kDa protein. The 40 and 45 kDa allergens were glycoproteins and contained IgE binding carbohydrate moieties. Homologues of pollen allergens that are cross reactive were detected in poppy seed extract. These included Bet v 1 and profilin.[66]

1.4.3.5 Cross reactivity

It was observed, in the reported cases, that poppy seed allergy coincides with various other food allergies.[69] Immunological cross reactivity was recognized by skin prick tests (SPT) and RAST inhibition experiments. Cross-sensitizations or cross reactivity between poppy seeds and other food stuffs such as sesame, hazelnut, rye grain and kiwi fruit have been described. [66, 68] Allergological workup also revealed a novel cross-sensitization with buckwheat.[67]

1.4.3.6 Reported cases and increasing prevalence

The increasing prevalence of poppy seed allergy is evident from various reported cases of individuals suffering from allergic symptoms after poppy ingestion. In one instance, a 52-year-old patient suffered from epigastric pain, angioedema, and respiratory distress and required emergency care, all a few minutes later of poppy cake consumption. Skin prick test confirmed the poppy seed allergy.[69] In another case a 17-year-old boy suffered a reaction soon after eating a

poppy seed cake. The allergic reaction comprised of acute abdominal pain followed by diffuse urticaria and a very low blood pressure. After administration of adrenaline and corticosteroids the condition improved. A skin prick test against poppy proved to be highly positive.[68] Another report deals with a case of a woman, who complained swelling of the throat after ingesting ice cream with poppy seed topping.[70] In yet another incidence a 17-year-old unconscious female was admitted to the intensive care unit and was reported to suffer from an anaphylactic reaction with nausea, heat sensation, and abdominal pain ten minutes after having a bite of poppy cake.[67] All above mentioned cases are indicative of the increasing prevalence of poppy seed allergy. In another study dealing with food hypersensitivity in Sweden, Denmark, Estonia, Lithuania, and Russia poppy seeds were reported to show some slight symptoms associated with food allergy. Among these countries Sweden showed the highest prevalence of poppy seed allergy.[71]

1.4.4 Lupine

The genus *Lupinus*, commonly known as lupine, belongs to the Leguminosae family and subfamily *Papilionaceae*, it is a legume comprising of a wide range of over 450 species. Among the legume family, the second largest family of seed plants,[72] lupine is an interesting one due to various reasons. It can be cultivated under a variety of climatic conditions, representing a more easily available and therefore cheaper protein source than other legumes. It also has high protein content, comparable to that of soybean, about 35% of the dry weight and the lowest presence of anti nutritional compounds and relatively low oil content.

Several species have been cultivated in the Andean highlands and Mediterranean region and it is widely grown as a flowering plant with usual garden species being poisonous. However, varieties with low alkaloid contents are of agricultural importance and are used for human and animal consumption. These include *Lupinus luteus* which is yellow lupine of South America and the Central Europe region, *Lupinus albus* that is white lupine, a variety of the Mediterranean countries and *Lupinus angustifolius* or blue lupine cultivated in Australia.[73, 74] The afore mentioned varieties are known as sweet lupines and have been cultivated for centuries for domestic animal feed and also for human nutrition, mainly in several parts of Australia, Europe, and South America.[75] Blue and yellow lupine seeds are mostly used for feed, while the white lupines are primarily grown for food uses.[76, 77]

1.4.4.1 Uses of lupine

Being similar to soy in protein and amino acid content, lupine has proved to be an alternative to soy as a source of vegetable protein.[78] In addition, recent studies show various health benefits associated with lupine, they are capable of lowering cholesterol and glucose levels, or specifically are capable of lowering plasma cholesterol and triglyceride concentrations in hyper cholesterolemic laboratory animals.[75, 79] Moreover, lupine may provide a useful alternative for individuals wishing to substitute animal with plant proteins for cardiovascular disease prevention.[80]

Roasted lupine seeds have been used as snack food in Mediterranean countries for years. Since the introduction of lupine flour as an ingredient in wheat flour in 1990, lupine flour has been used as a substitute or as an additive to other flours and lupine consumption has become more widespread since then owing to its nutritional and food processing qualities.[73, 74, 81, 82]

Lupine is commonly added to wheat flour; and sweet lupine flour is used in baked goods and health foods in Europe. It may be added to breads, bread rolls, biscuits, pastas, jams, sauces, dietetic products, various meat products as well as milk and soy substitutes.[80, 83]
Products with about 10% of lupine flour show good consumer acceptance. A survey of 112 commercial samples from the Norwegian market showed that lupine has become a common ingredient in food products.[73] Due to abundant protein content lupine flour is a popular ingredient in vegetarian, lactose and gluten free products for people with lactose intolerance or celiac disease.[82]

1.4.4.2 Lupine allergy

With the increasing consumption, it has become clear that lupine seeds also contain allergenic proteins and the incidences of lupine allergy have increased too.[83] Some individuals experience allergic reactions upon ingestion or inhalation of lupine products, with symptoms ranging from rashes and nausea to anaphylaxis.[81] Lupine flour has been reported as a causative of allergic reactions and since 1994, a number of cases of immediate-type allergy to lupine flour-containing products have been published. In 2002 in France, lupine products were the fourth leading cause of anaphylactic food reactions, after peanuts, nuts, and shellfish.[82] Furthermore, the inhalation of lupine flour could be an important cause of allergic sensitization in exposed workers and might give rise to occupational asthma and food allergy.[80]

Lupine allergy may arise as a result of cross reactivity in people who are already allergic to another member of the Legume family, the most common incidences occur in peanut allergic patients. It may also arise by primary sensitization as lupine allergy can occur as a separate entity, without evidence of clinical or laboratory cross reactivity to other legumes.[72-74, 78, 84, 85]

1.4.4.3 Symptoms

Clinical symptoms, reported post lupine ingestion or inhalation are similar to those reported for other inhalant or food allergens. Symptoms include a vast range like throat tingling, cough, wheeze, urticaria, atopic dermatitis and oral allergy-like symptoms confined to the oral cavity have also been reported. Lupine allergy is also capable of eliciting anaphylactic reactions.[74, 86, 87] Ingested doses of lupine flour reported to have triggered clinical reactions range from 265 to 1000 mg, but the lowest dose triggering reactions has not been established.[74]

1.4.4.4 Allergens

The major allergens of the Lupinus species are storage proteins, the conglutins, they comprise of four fractions called α, β, γ and δ-conglutin.[88] It has also been presented that a major cross reactivity against peanuts is found in the γ-conglutin section of lupine.

It is believed that the major IgE-binding protein of lupine is located in the region between 43-45 kDa and immunoblots indicated that the most distinctly reactive band had a molecular mass of 43 kDa. Other possible major allergens of lupine flour have not been characterised in detail. In 2005, Magni and colleagues, using two-dimensional electrophoresis, reported that two lupine proteins, conglutin gamma (2S albumin) and 11S globulin, strongly reacted with the sera of their lupine-sensitised patients.[74, 89]

1.4.4.5 Reduction in allergenicity

Lupine allergens are relatively stable and there is no definite indication that technological treatments alter the allergenic potential of lupine when studied under various conditions of pressure, temperature autoclaving and microwave heating. Immunoblotting and SDS-PAGE confirmed that neither microwave treatment nor cooking reduced IgE binding capacity. However, reduction in allergenicity has been reported after autoclaving lupine seeds at 138 °C for 30 minutes.[74, 80, 88]

1.4.4.6 Cross reactivity with peanut and other legumes

Since legumes, peanut in particular and to some what a lesser extent, soybean, are well known allergenic foods, it is not surprising that lupine poses to be an allergenic food as well. Serologic cross reactivity between other members of the legume family and lupine is common.[72] Although lupine allergy has been reported to occur by primary sensitization, it is more often the consequence of cross reactivity in patients with an existing allergy to other legumes such as peanut, soybean, lentils, green beans, chick peas or peas. Several studies indicate that the risk of cross reaction between peanut and lupine is quite high in peanut-allergic patients and the incidence of peanut-lupine cross allergy is rising.[73, 80, 82, 83, 88, 90-93] It has also been estimated that 30% or 40% of peanut-allergic individuals react to lupine.[81, 84]

1.4.4.7 Prevalence

The prevalence of primary allergy to lupine in the general population is unknown and currently seems to be much rarer in non peanut-allergic atopic subjects.[94] It is likely to be dependent on local eating habits and lupine consumption appears to be increasing in several European countries. The risk is higher in peanut allergic individuals due to the potential cross reactivities and they represent about 0.7-1.5% of the European population. The possibility of under-reporting of allergic reactions to lupine cannot be excluded and further studies are needed to establish the prevalence of allergic reactions to lupine in peanut allergic individuals as well as in the population of allergic individuals.[74]

The first case of lupine allergy was reported in 1994 when a 5-year-old sensitive to peanut developed allergic symptoms after ingesting pasta fortified with lupine flour. Since then, various cases of lupine allergy amongst all age groups of people have been reported with all manifestations associated with a reaction including contact urticaria and respiratory symptoms including anaphylaxis.[73, 82, 90, 95, 96]

Due to the increasing incidences of lupine associated allergic reactions, the presence of lupine materials in foods has to be labeled in the European Union starting from December 23, 2008, according to Commission Directive EC 2006/142 [73]

1.4.5 Analytical methods – significance and requirements

Food allergies are now acknowledged as a serious and significant health and food safety issue. It is not only a matter of consumer concern, but it is also in the economic interest of food

manufacturers to be able to give assurance regarding allergen absence in food products. Hence developing analytical tools is necessary for the reliable, specific, sensitive and accurate detection and quantification of food allergens in food stuffs. It is also crucial to improve the robustness of the already available analytical methods.[13, 18] Despite all the precautionary measures that the food industry invests in like extra cleaning, changes in equipment design and the label stating "may contain traces of...", a probability of unintended consumption of food allergens still exists. This may be due to issues related with incoming raw material and so absolute guarantees concerning the absence of unwanted contamination are unlikely and so detection of residues of allergenic components is also important.[20] Hence, the requirements that an analytical method must meet for the detection of allergenic proteins in food are twofold. One, the method should have sufficient sensitivity to detect trace amounts of offensive proteins and two, appropriate specificity for the detection of allergenic proteins in complex food matrices as well as in processed foods. Additionally it is important that the methods are fast and robust with a certain amount of ease of handling so that the use is not limited to only highly trained personnel.[33]

The threshold dose, above which allergic reactions are elicited, is difficult to assess due to its variability. However, there is a general consensus that the detection limits of analytical methods for food products must be between 1 and 100 mg of allergenic protein per kg of food (ppm). Commercially available test kits for the detection and quantification of allergens in food products mostly make use of ELISA or PCR technology that fulfil this criterion and have limits of detections in the range of 0.1-10 mg/ kg.[13, 33]

1.4.6 Methods for analysing allergenic proteins

Detection is not an easy task since the allergens are present in minute amounts in complex and often highly processed food. There are various review articles that cover the latest developments in food allergen analysis.[15, 97, 98]

In the past decade or so of allergy based research various possibilities have proved useful in food allergen detection. At present, for allergenic protein analysis, immunoanalytical methods and PCR fulfil most of the prerequisites for the analysis concerning sensitivity and specificity. The former being easy to handle relies on antibodies for the detection of proteins with LODs in the range between 0.1 and 10 mg / kg. The latter involves nucleic acid amplification with LODs below 10 mg / kg.[33]

The protein based methods employed for detection purposes include ELISA, SDS-PAGE and immune sensors which are either optical, electrochemical or mass sensors. Real-time PCR methods have already been published for the detection of hazelnut[99, 100], peanut[101, 102], lupine[73, 103], sesame[51, 63, 104] or the simultaneous determination of sesame and hazelnut in food.[105] ELISAs have already been developed to detect several potential allergens such as hazelnut[106-108], peanut[109, 110], walnut[111], soy[112, 113] and lupine.[85, 114, 115] Up to now, only a few papers report the development of dipsticks[116, 117] or immuno sensors[118] that allow the detection of allergenic food. An overview of commercial immunoanalytical methods for the detection of allergenic food is given in a recent review article.[13]

1.4.7 Brief overview of published methods for food allergen detection

Holzhauser and Vieths (1999)[108] published a sandwich ELISA for the detection of traces of hazelnut protein. For the detection of native and roasted hazelnuts polyclonal anti-hazelnut antibodies from rabbit were used. The limit of detection was found to be 60 ± 50 ppb hazelnut protein in the sample. To determine the recovery 4 different food matrices, a coconut cookie, a yoghurt cereal bar, an almond candy cream chocolate bar and a homemade whole milk chocolate were spiked with hazelnut in concentrations from 0.001 to 10%. The obtained recoveries were in the range from 67 to 132%. To determine the cross reactivity a total of 39 different food ingredients were tested and no cross reactivity was detected.

Akkerdaas et al. (2004)[20] developed a sandwich enzyme-linked immunosorbent assay to detect pepsin resistant hazelnut proteins in food products. To obtain anti-hazelnut polyclonal antibodies rabbits were immunized with either pepsin-digested hazelnut or untreated hazelnut. Less than 1 μg hazelnut in 1 g food matrix could be detected. To determine the recovery of hazelnut chocolate samples were spiked with 0.5-100 μg hazelnut/g chocolate and the mean recovery of 97.3% was observed. Ten plant foods were tested for cross reactivity, the highest cross reactivity was found for peanut (0.034%) followed by walnut (0.017%), then almond (0.008%) and lastly sesame (0.002%).

Rejeb et al. (2003)[119] developed a competitive enzyme-linked immunosorbent assay (ELISA) to detect hazelnut by using polyclonal antibodies generated against a protein extract of roasted hazelnut by rabbits. The limit of detection was found to be 0.45 µg of hazelnut protein per gram sample. Recovery results were generated by spiking blank matrixes at four levels ranging from 1,

2.5, 5 and 10 µg/g of hazelnut protein. The recovery was found to be between 64-83% in chocolate and 78-97% in other matrices. No cross reactivity was observed for the 39 different food stuffs that were tested.

Drs et al. (2004)[106] presented an indirect competitive enzyme-immunoassay for the detection of hazelnut in food. Hens were immunized with a protein extract of roasted hazelnut and polyclonal antibodies were isolated from the egg yolk and purified. For the determination of the limit of detection of the ELISA an extracted blank cookie matrix was spiked with hazelnut protein solutions with different concentrations. The LOD was found to be 10 μg of hazelnut protein/L with an average recovery of 128%. The crude antibodies showed cross reactivity to almost all food stuffs tested whereas the immunoaffinity purified antibodies proved to be more specific and showed cross reactivity with beans (8.09%), sunflower seeds (6.26%), poppy seeds (5.36%) and chickpea (3.42%). The cross reactivity was below 3% for chestnut, Brazil nut, green pea, sesame, barley, wheat, yeast, rolled oats and corn.

Holzhauser and Vieths (1999)[110] developed an indirect competitive ELISA for the detection of hidden peanut proteins. The LOD was 2 ppm peanut protein (μg/g food), at this concentration the recovery of peanut protein was 143%. At peanut protein concentrations > 13 ppm the recovery was in the range from 84 to 126%. Thirty food ingredients such as legumes, nuts and stone fruits were tested for cross reactivity. None of the tested food samples showed cross reactivity to the antibodies except walnut and pinto.

Holden et al. (2005)[85] published a sandwich ELISA for the determination of lupine in food making use of polyclonal anti-lupine capture antibodies produced in rabbits and a biotinylated conjugate of the same antibody for detection. The LOD of the sandwich ELISA was 0.1 µg of lupine protein/g sample, taking the sample dilution into account. Furthermore, it was found that the LOD of the assay varied in the different blank food matrixes. In pasta and vegetarian sausage, the LOD in terms of lupine protein was 0.1 µg/g, in hot dog bread it was 0.2 µg/g, and in chocolate spread it was 0.4 µg/g. The recoveries obtained ranged from 60% to 116%. No cross reactivity was observed for bean, hazelnut, dried milk, or wheat flour. Minor cross reactivity was however observed for chick pea, peanut, pea, soy, and lentil, none exceeding a lupine protein concentration corresponding to 0.4 µg/g.

Holden et al. (2007)[115] developed a polyclonal-monoclonal antibody based sandwich ELISA for detection of lupine protein. The assay was sensitive to both native and processed proteins from *Lupinus angustifolius* and *Lupinus albus* and had a detection limit of 1 µg/g. Samples of lupine-free bread were spiked with extract of lupine flour at levels of 1, 100, and 1000 µg/g of bread sample. The recoveries for processed proteins from *L. albus* ranged from 78 to 112% in the polyclonal-polyclonal antibody assay and from 85 to 150% in the polyclonal-monoclonal antibody assay. However, the recovery for bread spiked with native lupine proteins from *L. angustifolius* was between 12 and 16% in the polyclonal-polyclonal antibody assay. In contrast, the recovery rate for lupine flour proteins was higher in the polyclonal-monoclonal antibody assay ranging from 44 to 88%. No cross reactivity was found in extracts obtained from 18 different food stuffs, however, the method tested positive for extracts from brown bean, walnut, linseed, curry, and tandoori masala. When these extracts were diluted to 100 µg of protein/g of sample, no cross reactivity was observed i.e., all extracts gave signals below the LOD.

Mohammed et al. (2001)[120] investigated if a biosensor was a feasible method to detect food allergens or not. The limit of detection improved with the higher antibody dilution since it increased the amount of antibody – antigen interactions. A detection limit of 0.7 µg peanut/mL extract was achieved. For an antibody dilution of 5000 the LOD was found to be 2.3 µg/mL, for the dilution of 10000 it was 1.5 µg/mL, for 20000 and 50000 the LOD was 0.7 µg/mL.

Piknovà et al. (2008)[121] published a real-time PCR method for the detection of the hsp 1 gene from hazelnuts; the detection was carried out with a TaqMan probe. The limit of detection was found to be 13 pg hazelnut DNA. In spiked pastry samples hazelnut could be detected at concentrations ≥ 0.01%. No cross reactivity was found with plants such as peanuts, walnuts, almond, pistachio nuts and cashews.

Hird et al. (2003)[101] developed a real-time PCR method for the detection of peanut. The primers and the probe were designed for the Ara h 2 gene. DNA extraction was carried out with different commercially available extraction kits. The PCR method allowed the detection of peanut in a biscuit which had been spiked with 2 ppm of lightly roasted peanut powder. For the 33 samples tested for cross reactivity no amplification of the peanut specific DNA sequence was observed.

Scarafoni et al. (2009)[103] published a real-time PCR method based on SYBR Green for the detection of lupine flour in food products. The amplification efficiency, determined by serial dilution of purified genomic leaf lupine DNA, was between 96% and 100%. The DNA extracted from lupine flour showed an amplification efficiency between 95% and 101%. The limit of detection was found to be 7 pg of lupine DNA that corresponds to 0.1% lupine flour in food. The primers did not show any cross reactivity to other foods.

Schöringhumer and Cichna-Markl (2007)[104] published a real-time PCR method for the detection of the gene coding for the Ses i 1 protein which is a major sesame allergen. A molecular beacon probe labeled with the reporter dye FAM and the quencher DABCYL was used. 17 samples that were tested did not show any cross reactivity. Serially diluting sesame DNA extracts up to 1:10000 yielded an amplification efficiency of 100.3%. To determine the limit of detection, a food matrix of crisp bread was spiked with sesame seeds in concentrations of 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01 and 0.001%. The efficiency was 86.4%. For sesame concentrations below 0.05%, C_t values over 35 were obtained.

Brzezinski (2007)[51] developed a real-time PCR method for sesame detection in food products. The amplification of the 66-bp fragment of the 2S albumin gene (Ses i 1) was detected by using a TaqMan probe. The limit of detection was 5 pg DNA that corresponds to 50 mg/kg sesame DNA in food products. No cross reactivity was found for seeds which are commonly used in bakery products, such as pumpkin, poppy and sunflower seeds.

Mustorp et al. (2008)[63] published a sesame specific PCR method for the gene encoding 2S albumin - a major allergen of sesame. The limit of detection of the developed method was 0.5 pg sesame DNA, the amplification efficiency was found to be 96% and the method did not show any cross reactivity towards a variety of plants that were tested.

Schöringhumer et al. (2009)[105] developed a duplex real-time PCR method for the simultaneous detection of sesame and hazelnut in food. Primers and probes were designed for the genes of two major allergenic proteins, Ses i 1 of sesame and Cor a 1 of hazelnut. Two TaqMan probes were used and were labeled with the reporter FAM and Cy5 and the Black hole quencher BHQ 1 and BHQ 2 for sesame and hazelnut, respectively. In spiked blank whole meal cookies sesame and

hazelnut could be detected until a concentration of 0.005% for both analytes. No cross reactivity was shown with 25 tested common food ingredients.

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2 AIM

Despite food allergy being the major cause of life - threatening hypersensitivity reaction, no therapy is available and strict avoidance of the allergen along with easy access to epinephrine injections are the only safe approaches that allergic individuals can undertake. Under current conditions, correct labeling of food ingredients is the sole way to help sensitized persons to avoid exposure to the allergenic proteins which are at times present only in trace amounts. Sesame and lupine are on the list containing 14 potentially allergenic foods which have to be declared according to the EU directive EC 2006/142. Poppy seed allergy, though severe is still rare and therefore there is no declaration requirement for labeling poppy, if present, as yet.

In view of legislations that have evolved, allergen detection in food and food stuffs at all levels is gaining importance. It is important to improve awareness amongst people and crucial for the food industry to be able to identify allergenic ingredients and to detect them in complex food matrices. Therefore, development of fast, reliable and robust analytical methods to detect potentially harmful allergens is absolutely vital.

Numerous analytical methods have already been developed for the purpose of allergen detection, amongst these, immunoassays and PCR methods play a significant role.

In light of the potential threat posed by food allergy to human health, and the significance of developing analytical methods for allergen detection, the aim of the present study was the development of analytical methods for three allergenic foods namely sesame, poppy seeds and lupine. The methods which had to be developed included a competitive ELISA and a quartz crystal microbalance (QCM) immuno sensor for sesame detection using anti-sesame polyclonal antibodies from a chicken. For this purpose the antibodies were to be prepared by injecting a 1 mg/mL sesame protein extract into a hen once a month. The isolation of the antibodies from the yolks of the eggs obtained seven days after the immunization injection was the next step. Besides these two methods a real-time PCR method for poppy seed detection was also to be developed. For all three methods, the competitive ELISA, the QCM immuno sensor and the real-time PCR, the development of the methods was to be followed

by optimization for the best conditions. As the validation of the developed methods is of vital importance, the LOD and the specificity of the developed method were to be determined. To demonstrate the applicability of the developed methods analysis of samples in complex matrices and commercially available food samples had to be carried out.

Besides the above mentioned methods, during the course of research, preliminary experiments for the development of an immuno-PCR method for the detection of lupine using anti-lupine antibodies from a chicken and a rabbit were also to be carried out. The antibodies in this case were to be produced by immunizing chickens and rabbits with 1 mg/mL of lupine protein extract once every thirty days. The antibodies were then to be isolated from the yolks of the eggs obtained in the case of chickens and from the serum of the rabbits.

3 THEORY

This chapter deals with the theoretical concepts employed for the purpose of the current research and is divided into six sections. These include the immune system (section 1) and different analytical methods such as enzyme linked immunosorbent assay, (ELISA) (section 2), immune sensors (section 3), the polymerase chain reaction, (PCR) (section 4) as well as a combination of ELISA and PCR, namely the immuno-PCR (section 5) and lastly gel electrophoresis (section 6).

3.1 The immune system

The fundamental role and purpose of the immune system is protection against infectious organisms and toxic products. The immune system incorporates extensive and elaborate range of mechanisms that identify and locate foreign cells, viruses or macromolecules, neutralize and ultimately eliminate them from the body. In the midst of these events it is certain protein molecules and cells circulating through the body that play a significant role.[1]

3.1.1 Defining certain terms associated with the immune system

Certain significant terms dealing with the immune system are defined and explained briefly; details of these will follow in later text.

An **antibody** is a molecule that binds to a known antigen; the term immunoglobulin is often used interchangeably for antibody although immunoglobulin refers to a group of proteins irrespective of whether or not their binding target is known.[1]

The terms **antigen** and **immunogen** are employed to express different attributes of a molecule. Any molecule that binds to an antibody is classified as an antigen. When a molecule is used to induce an adaptive immune response it is termed as an immunogen. **Immunogenicity** is not an intrinsic property of a molecule; it is only the ability to induce an adaptive response. Similarly **antigenicity** too is not an intrinsic property and is defined by a molecule's ability to be bound by an antibody.[1]

3.1.2 Non adaptive and adaptive immunity

The immune system encompasses two broad categories in which it works, namely adaptive and non adaptive immunity. Non adaptive immunity, mediated by cells that respond non specifically to foreign molecules does not improve with repeated exposure to the same foreign molecule. It includes phagocytosis by macrophages, secretion of lysozyme by lacrimal cells and cell lysis by natural killer cells. In contrast to this, adaptive immunity is directed against specific molecules mediated by cells called lymphocytes that synthesize cell surface receptors or secrete proteins, i.e. antibodies, which bind specifically to foreign molecules. Another distinguishing feature is that it is enhanced by reexposure to the same molecule.[1]

3.1.2.1 Classes of adaptive immunity

Adaptive immune response is further classified into two kinds, humoral and cell mediated. The humoral response, mediated by the B lymphocytes in alliance with the helper T cells, results in the generation of antibodies that bind to foreign antigens. Cell mediated responses on the other hand are typical for cytotoxic T lymphocytes and result in their binding to foreign or infected cells with subsequent lysis of these cells.[1]

3.1.3 Lymphocytes and the immune system

An immune response is a consequence of a complex series of events that involve the immunogen and lymphoid cells. The immune system comprises of more than 10^9 lymphocytes dispersed through the body, capable to respond expeditiously wherever needed. Lymphocytes are generated continuously from progenitor stem cells in the bone marrow and travel through the blood and lymphatic systems and accumulate in lymphoid organs, the lymph node and spleen in mammals. During the progression of the immune response, immunogens aggregate in one of the lymphoid organs that then becomes the focus of the response.[1, 2]

Several kinds of lymphocytes with varying functions have been identified. To illustrate their cellular functions they are classified into three basic types, the B cells, cytotoxic T cells and helper T cells. The B cells secrete antibodies and also carry a modified form of the same antibody on their surface acting as a receptor for antigens. Cytotoxic T cells carry out the

lysis of foreign or infected cells, and bind through T cell surface receptors to these targets. Helper T cells play a regulatory role of vital importance in controlling the response of B cells and cytotoxic T cells; these too have surface T cell receptors. Thus all three contain cell surface receptors that can bind antigens.[1, 2]

3.1.3.1 Clonal expansion of lymphocytes

The antibodies on the B cell surface bind the antigen, the B lymphocyte is activated to secrete antibody and later proliferation is activated. T cells respond in a similar fashion as well. This burst of cell division results in the clonal expansion of the antigen specific lymphocytes and this marks the first step in the development of an effective immune response. For this cell division, the primary site is in the lymphoid organs. For as long as the antigen remains, the lymphocytes activation continues, thus strengthening the immune response.[1]

3.1.3.2 Clonal selection governing properties of the immune system

Clonal selection determines certain properties of the immune system including the specificity and the tolerance.

3.1.4 Specificity of the immune response

The molecular basis of the specificity of the immune system comes from the phenomenon that the antibodies and T cell receptors bind to foreign molecules. The specificity of the immune response is controlled by a simple mechanism that is, one cell recognizes one antigen, and this is so as the entire antigen receptors on a single lymphocyte are identical. Antigen receptors are glycoproteins found on the surface of mature lymphocytes and by ensuring the synthesis of only one type of receptor within one cell, antigen specificity is adhered to. Although the kind of response of T and B lymphocytes differs, it is true for both.[1]

3.1.5 Tolerance and auto immune diseases

Since antibodies and cytotoxic T cells efficiently eliminate antigens, it is important for the immune system to distinguish between foreign molecules and normal components of the body. It is not common to generate an immune response against self or host molecules and this lack of response towards self is called tolerance. Tolerance is established and preserved by constantly eliminating any such lymphocytes that produce antibodies or T cells capable of

binding to host molecules by the process of clonal deletion which occurs in the thymus during the development of the immune system. Self reactive clones are specifically removed during their differentiation. Failure of this selection process results in an immune response against self. This causes a range of disorders collectively termed as autoimmune diseases.[1]

3.1.6 Antibodies

Antibodies are protein molecules or a large family of glycoproteins with key structural and functional attributes, produced by the host in response to the presence of a foreign entity in the body. They are in circulation throughout the blood and lymph where they tend to bind to the foreign antigens. Functionally, they are typified by their ability to bind to antigens as well as to specialized cells or proteins of the immune system.[1]

3.1.6.1 Structure of an IgG antibody

Structurally, antibodies are composed of a characteristic unit that can be envisioned as a Y shape. The most abundant, IgG antibodies, in the serum can be used to explain the structural features of antibodies since they consist of only one structural Y unit. The IgG molecules consist of three protein fragments; the two identical ones form the arms while the third fragment forms the base of the Y unit. Each arm contains a site that can bind to an antigen, thus rendering the IgG molecules bivalent.[1]

3.1.6.2 The heavy and light chains and protein domains and antigen binding sites

Figure 1 illustrates the structure of an antibody. The Y unit consists of four polypeptides, one identical set is known as the heavy chains and the other as the light chain. The heavy chains are approximately 55,000 daltons while the light chains are about 25,000 daltons.

One light chain combines with the amino–terminal region of one of the heavy chains forming the antigen binding domain, expressed as **Fab**, fragments with antigen binding site. The terminal regions with the carboxyl group of the two heavy chains fold together resulting in the **Fc**, fragment that crystallizes, domain that deals with immune regulation.

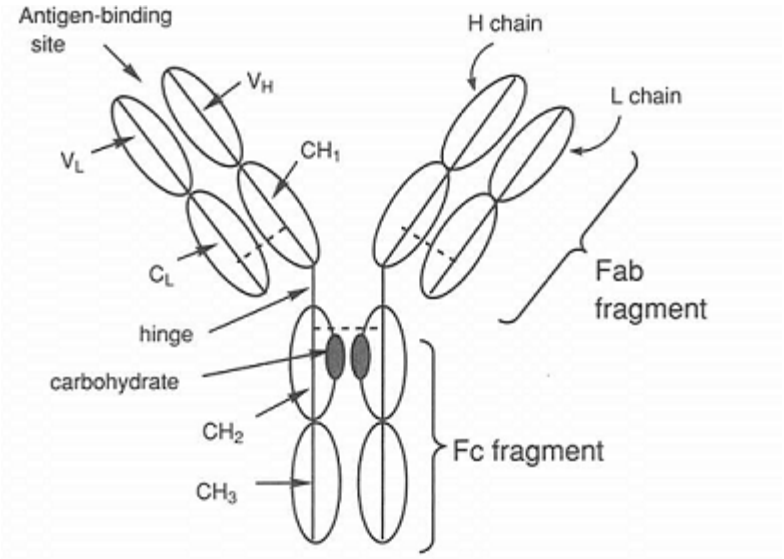

Figure 1: Antibody structure[1]

The region between the Fab and Fc fragments is the **hinge**; it allows lateral and rotational movements of the two antigen binding domains thus rendering freedom to interact with a large number of various antigen conformations. The four polypeptide chains are held together by disulfide bridges and non covalent bonds.[1]

3.1.6.3 Classes of antibodies and heavy and light chains

There are five classes of antibodies, the IgG, IgM, IgA, IgE and IgD. The distinguishing factors being the number of Y like units and the type of heavy chain found in the molecule. IgG molecules have heavy chains called γ chains, IgM's have μ chains, IgA's have α chains, IgE's have ε chains and IgD molecules have δ chains. The amino acid sequences found in the Fc fragment are responsible for these differences; this enables the antibodies to function in different types of immune responses and at particular stages of the maturation of the immune response. While there are five classes of heavy chains only two types of light chains, κ and λ, exist. An antibody will have one type of heavy chain and one type of light chain.[1]

3.1.6.4 Different regions in an antibody

Various regions are present in a Y unit; the details are presented in the following text.

3.1.6.4.1 Constant and variable regions in light and heavy chains

The length of the light chains is approximately 220 amino acids; a division in two results in two regions each being 110 amino acids long. A comparison of amino acid sequences from a number of light chains revealed that the amino-terminal half was heterogeneous, this region is called the variable (V) region and the carboxyl-terminal half is known as the constant (C) region. There are only two types of constant regions, one with κ and the other with λ light chains.

The IgG heavy chains are approximately 440 amino acids long with one variable and three constant regions each being 110 amino acids long. Heavy chains from different classes may have additional constant regions.[1]

3.1.6.4.2 Hyper variable regions

The variable regions of a heavy and light chain combine and form an antigen binding site. The structural basis for the large repertoire of binding sites is a result of the heterogeneity of the variable regions, this leads to an effective response. Heterogeneity is not random in the variable region; it is concentrated where the contact with the antigen occurs. Most of the variability occurs in three short regions of each chain, giving three hyper variable regions for both the light and heavy chain. These hyper variable regions lead to binding of the antibody to the antigen and are present on short loops that extend into the region that interacts with the antigen. They are referred to as the complimentary determining regions or CDRs since they are the actual binding site for the antigen.[1]

3.1.6.5 The antibody response

A series of interactions resulting from the presence of a foreign antigen between macrophages, T lymphocytes and B lymphocytes leads to an antibody response. The result of such a response is the production of a large number of antigen specific antibody molecules, formation of the antibody-antigen complex and its removal from circulation through phagocytosis by macrophages.[1]

3.1.6.5.1 Immunological memory and primary and secondary responses

The first encounter with a foreign antigen or the primary response results in a relatively weak reaction that results in non specific phagocytosis of the antigen. A certain sequence of events

amidst the primary response prepares the host for any successive exposure to the same antigen. The antigen is processed by the antigen presenting cells (APCs) and presented for an effective response. After an APC engulfs an antigen the phagocytic vesicle fuses with a lysosome, thus partially degrading the antigen. The fragments of the antigen on the cell surface of the APC along with a cell surface glycoprotein are found in a complex that is the major histocompatibility complex (MHC) class II protein. This complex of antigen fragment and class two protein is then bound by a receptor on the surface of a helper T cell known as T cell receptor. This step is essential for the differentiation of the B cell into the plasma cell.[1]

Some of the antigen specific lymphocytes remain in circulation even after the antigen elimination. These cells are capable of responding to any subsequent exposure to the same antigen consequently providing the cellular basis for immunological memory. Second exposure to the same antigen results in strong and rapid elicitation of a response, the secondary response. For both these responses specialized plasma cells are the main sites of antibody production and these are terminally differentiated B cells. The memory B cell from the B lineage is imperative in generating a rapid and vigorous secondary response. The ability to mount a strong secondary response is termed as immunological memory and has the potential to last as long as the animal lives. A classical primary and secondary response to an immunogen is a result of cooperation between all three types of lymphocytes.

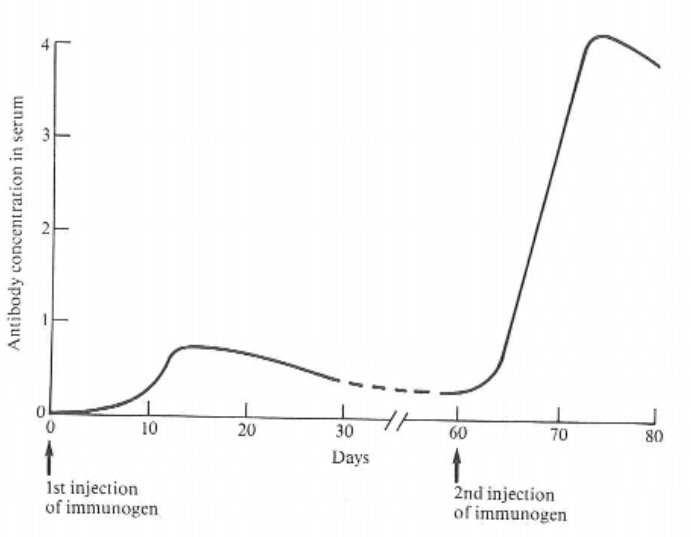

Figure 2: Classical primary and secondary response to an immunogen[2]

Figure 2 depicts the primary and secondary immune response. So a strong antibody response is a three step process; initially the antigen must bind to the surface antibody on the B cell which has the same antigen binding sites as the secreted antibody. Secondly, the processed antigen fragments must bind to class II proteins as this complex forms the basis of recognition by T cells. Thirdly, the antigen fragment and class II protein complex must bind to the T cell receptor to ensure that B cell stimulation by helper T cells is specific.[1, 2]

3.1.7 Production of antibodies and their use

The production of antibodies relies on *in vivo* humoral response of the animal. It is thus important that the production method for antibodies maximizes their potential affinity. Antibody production can be divided into three distinct stages: i) preparation of immunogen, ii) antibody production and iii) assessment of antibodies.[2] There are two kinds of antibodies, polyclonal and monoclonal. Polyclonal antibodies, generally with high affinity for the antigen are easier and uncomplicated to produce using highly purified antigens. Monoclonal antibodies require considerable investment in time and equipment but offer the ultimate advantage of providing the continuous production of a defined reagent, once they have been identified. They can also be produced using partially purified immunogen. Monoclonal antibodies by definition are more specific than polyclonal antibodies, however, this unique specificity can occasionally complicate matters.[2]

3.1.7.1 Immunogenicity and immunogenic trait of a molecule

Immunogenicity, the ability of a molecule to induce an immune response, is determined both by intrinsic chemical structure and the ability of the host animal to recognize the injected compound. In general, a compound must contain an epitope capable of binding to the cell surface antibody of a virgin B cell to be able to elicit a primary response and a strong secondary response. This binding is essential as it determines the specificity of the resulting antibodies. Many naturally occurring and synthetic compounds such as proteins, peptides, carbohydrates, nucleic acids and lipids can be successful immunogens. The second property of a molecule rendering the capability of an apt immunogen is the ability to promote cell to cell communication between B cells and helper T cells.[1, 2]

3.1.7.2 Phagocytosis and immunization

Since the first exposure to an antigen deals with the phagocytosis, techniques designed to optimize immunizations work by increasing the efficiency of phagocytosis. The factors that are taken into consideration include choice of an appropriate injection site, altering the form of the antigen, and using an adjuvant. The size and form of an antigen also determines the efficiency of phagocytosis, particulate antigens are phagocytosed with far more ease than the small soluble molecules. Therefore, the conversion of small soluble molecules into large particulate aggregates usually proves to be an effective approach to increase their immunogenicity.[1]

3.1.7.3 Immunizing animals

Only a limited number of sites are available to a researcher to intervene, manipulate and tailor the response to a particular antigen. The intervention is twofold, that is either antigen modification or alteration in injection conditions. The second class of intervention incorporates various factors such as the choice of animal, the dose and form of the antigen, the use of adjuvant, the route and number of injections and the period between injections.[1]

3.1.7.4 Antigen preparation

The aim of antigen preparation for the purpose immunization is to do so in a way that will procure the strongest and most appropriate response. While preparing an antigen, determining the level of antigen purity essential for starting the immunization schedule is of critical importance. This decision is based on the intended purpose of the antibodies, if specificity is essential, then the antigen has to be purified to homogeneity.[1]

3.1.7.4.1 Dose and form of the antigen

The dose and form of antigen are also important aspects. For determining the dose, two different criteria are taken into consideration, first the optimum dose required to achieve the strongest response, second the minimum dose likely to induce the production of a useful polyclonal antisera. The form of the antigen is also crucial; particulate antigens being better antigens than soluble molecules. The immunogen dose relationship suggests that there is a threshold concentration of immunogen which is required to be maintained for a sufficient time for the immune response to occur.

Generally it is possible to inject the large molecular weight proteins and polypeptides without further modification since they are naturally immunogenic. This also holds true for complex polysaccharides even though their natural immunogenicity may be a result of small amounts of protein associated with their structure. These large molecules usually have various epitopes, antigenic determinants, on their structure. Isolation and purification of these molecules sometimes leads to a disruption of their natural structure and hence an alteration in the configuration of the discontinuous epitopes.[1, 2]

3.1.7.5 Adjuvants

Non specific stimulators of the immune response are known as adjuvants; a wide spectrum of such immunological adjuvants exist.[2] Using adjuvants is essential to induce a strong antibody response to soluble antigens, though they may not always be required for particulate or whole cell antigens. Adjuvants incorporate two components; one is designed to form a deposit protecting the antigen from rapid catabolism. The second component needed for an effective adjuvant is a substance that will stimulate the immune response non specifically. The advantage of using adjuvants is twofold; first, they trap the antigen at a local deposit thus preventing rapid dispersal. Secondly they increase the rate of phagocytosis as they can stimulate the secretion leading to recruiting macrophages to the site of antigen deposition.[1]

3.1.7.6 Choice of animal

To meet the requirements of increased applications and quality control for antisera dealing with diagnositic purposes a solution is to produce the antisera in large animals from which a single bleed could provide a substantial volume of antisera that, if stored correctly, gives consistent specificity and reaction characteristics. Various factors determine the choice of animal to be used for immunization, these include the amount of serum needed, the species used to isolate the antigen, the amount of available antigen and whether or not monoclonal antibodies are needed.[1, 3]

A broad range of vertebrate species can be employed for the antisera production, five most commonly used laboratory animals are rabbits, mice, rats, hamsters and guinea pigs. A single bleed yields 25 mL of serum from rabbits, 100-200 µL from a mouse and 1-2 mL from a rat, hamster or guinea pig. If large volumes of sera are required larger animals are needed and so pigs, horses, sheep and donkeys are all used commercially.[1] For routine production of

polyclonal antibodies rabbits represent a good choice since they are easy to keep and handle and can be safely and repeatedly bled and the antibodies they produce are well characterized and easily purified.[2]

3.1.7.7 Number of animals

The number of animals to be immunized is also of critical importance. Even genetically identical animals generate different antibodies from a single preparation of antigen and when outbred animals such as rabbits are used, these differences are magnified, therefore if sufficient amount of antigen is available, several animals should be immunized.[1]

3.1.7.8 Routes of injection of an antigen

The route of injection is determined keeping three practical aspects in mind, one is the volume to be delivered, second aspect incorporates the buffers and components injected with the immunogen and lastly it is important to consider the speed with which the immunogen must be released into circulation.

The different methods of injections include subcutaneous, intramuscular, intradermal and intravenous injections. Subcutaneous injections are widely used for the immunization of laboratory animals. Intramuscular injections are employed to produce a slow release of an antigen. Intradermal injections are generally used for immunization of larger animals and are known to generate a very slow release rate of the immunogen. Intravenous injections are used for the secondary or any later boost and are rarely used for primary injection. All injection sites are not equally effective at targeting antigens for phagocytosis. The idealistic sites have high numbers of antigen presenting cells (APCs) and low rates of antigen degradation.

Oily adjuvant emulsions have commonly been injected subcutaneously or intramuscularly. Some authors have also suggested intradermal injection into the foot pads. This route should be avoided since it causes considerable pain to the animal. A multiple site intradermal injection procedure in which the immunogen is injected at between 30-50 sites, usually in the back and neck region, is reported to give a very rapid response but can result in extensive skin ulceration. Studies have shown that similar results can be achieved using subcutaneous injection at only four sites.[2]

3.1.7.9 Boosts

The first detectable signs of an increase in the number of B cells bearing the antigen specific surface antibodies appear 5-6 days after the primary injection of antigen. The antibodies become detectable in the serum around the 7th day after injection and remain at low levels for some days. The response is dramatically different on subsequent injection of the same antigen since after the second injection the number of B cells containing the antigen specific cell surface antibodies increases exponentially.[1]

3.1.7.10 Storing and purifying antibodies

One of the practical advantages of antibody molecules having compact and stable protein domain is that they are resistant to a broad range of mildly denaturing conditions. This makes long term storage of antibodies relatively easy. Antibodies are stored conveniently at -20 °C in the serum that they are collected in.[1]

Purified antibodies are required for a number of techniques. There are a wide variety of methods used to purify antibodies; the correct choice of purification method depends on a number of variables including the intended purpose, the species in which the antibodies were raised, its class and subclass.[1]

3.1.8 Antigen and immunogen related factors

3.1.8.1 Epitope

Epitope is defined as the region of an antigen that interacts with an antibody and is often termed as the antigenic determinant.[1] A wide range of synthetic organic chemicals along with carbohydrates, lipids, nucleic acids and amino acids have been identified as epitopes. An epitope is not an intrinsic property, it is described and ascertained by the binding site of an antibody and its size is also determined by the combining site size. The combining site, visualized as a cleft or pocket in which the epitope is docked, is relatively small as shown by X-ray diffraction studies of the structures of cocrystals of antigens bound to antibodies.[1]

3.1.8.2 Continuous and discontinuous epitopes

It has been elucidated that 5-10 amino acids in an intact protein constitute a continuous epitope as the residues are in continuous peptide linkage in the protein. In the case of

discontinuous epitopes the antibody recognizes the residues in spatial proximity to each other and not in continuous peptide linkage within its primary structure.[2]

3.1.8.3 Structural changes

An interaction between an antibody and antigen can occur with or without large structural changes in the antibody or the antigen. Some studies also indicate that antibodies can induce structural changes in the antigens. Even small changes in the antigen structure profoundly affect the antibody-antigen interaction so much so that the strength of interaction can be reduced 1000 fold by the loss of just a single hydrogen bond.[1]

3.1.9 The antibody-antigen reaction

The basis of all immunochemical techniques finds its roots in the interaction between an antibody and an antigen.[1, 2]

The antibody-antigen complex is in equilibrium with the free components, the reactants obey the Law of Mass Action:

$Ab + Ag \leftrightarrow Ab.Ag$

In the above equation, Ab represents the antibody, Ag represents the antigen and Ab.Ag represents the antibody-antigen complex.

Any Ab.Ag complex is capable of undergoing dissociation. The affinity of the antibody determines the extent to which the Ab.Ag complex dissociates.[1, 2]

3.1.9.1 Nature of bonding of the antibody-antigen complex

The antibody and antigen binding entirely depends on non covalent interactions. The stability of the immune complex is determined by a combination of weak interactions depending on a precise antibody-antigen alignment. Forces like hydrogen bonds, van der Waals forces, coulombic interactions and hydrophobic bonds encompass these non covalent interactions. The overall interaction is a balance of many attractive and repulsive interactions.[1]

3.1.9.2 Cross reaction

The molecular basis of a cross reaction comes from the fact that antibodies recognize relatively small regions of antigens and on occasions similar epitopes can be found on other molecules.[1]

3.1.9.3 Strength of an antibody-antigen interaction

Three major factors govern the overall strength of an antibody-antigen interaction, these include the intrinsic affinity of the antibody for the epitope, the valency of both the antibody and the antigen and the geometric arrangement of the interacting components.

3.1.9.3.1 Affinity

The strength of the bond between the antigen and the antibody, or more precisely, between epitope of the antigen and the corresponding paratope of the antibody, is termed as the affinity of the antibody. It is the measure of the binding strength of an epitope to a paratope.[2] The antibody-antigen binding is non covalent and reversible and follows the basic thermodynamic principles of any reversible bimolecular interaction. Hence, if the molar concentration of the unoccupied antibody binding sites is expressed as [Ab], [Ag] represents the molar concentration of the unoccupied antigen binding sites, and molar concentration of the antibody-antigen complex is expressed as [Ab.Ag], the affinity constant K_A will be expressed as:

$$K_A = [Ab.Ag] / [Ab] \,.\, [Ag] \qquad (1)$$

In practical terms, affinity describes the amount of the antibody-antigen complex that will be found at equilibrium.[1]

3.1.9.3.2 High and low affinity interactions

The rate of diffusion determines the time taken to achieve equilibrium. High-affinity antibodies will bind larger amounts of antigen in a shorter period of time compared to low-affinity antibodies. This implies that high-affinity interactions are essentially complete well before low-affinity interactions. High-affinity antibodies generally perform better in all immunochemical techniques due to the stability of the complex. With suitably high affinities,

addition of excess antibody essentially binds the entire available antigen, but with low affinity antibodies a significant fraction of the antigen remains free.[1]

3.1.9.3.3 Factors affecting the affinity of the antibody

The affinity constant for the antibody-antigen interactions, like all equilibrium reactions, is affected by temperature, pH, and solvent. Any change in these factors effects the affinity constant by either driving the reaction towards complete binding or by releasing the bound antigen, thus increasing or decreasing the number of antibody-antigen complexes at equilibrium.

It is possible to exactly determine the affinity of monoclonal antibodies but this does not hold true for polyclonal antibodies. This is because monoclonal antibodies are homogenous whereas antibodies of different affinities constitute the polyclonal sera, making exact determination impossible.[1, 3]

3.1.9.3.4 Multivalent complexes and intermolecular bridges

Both antibodies and antigens can also be multivalent, as the antibody molecule contains two paratopes, or if the antigens have multiple copies of the same epitope or if they contain multiple epitopes recognized by different antibodies. Multivalent interactions stabilize the immune complexes by rendering the reactions practically irreversible.[1] An interaction between an antigen with more than one epitope and polyclonal antibodies leads to the formation of complexes stabilized by intermolecular bridges. One antigen binds to one antibody, if the antigens are linked through more antibody-epitope bonds a cyclic or lattice structure can be formed. The dissociation rate of a single antibody-epitope bond is same as for a simple interaction but since the antigen is still held by other interactions the overall rate of dissociation is very slow.[1]

3.1.9.3.5 Avidity

For multivalent complexes the term affinity is not used, instead the term employed is avidity which refers to the measure of the overall stability of the antibody-antigen complex.[1] In practical terms, it is the ability of an antibody to bind its antigen.[3] Since avidity describes the complete reaction, this value ultimately determines the success of all immunochemical techniques.[1]

3.2 Immunoassays

The basis of all immunochemical techniques finds its roots in the interaction between an antibody and an antigen. Immunoassay is one such an analytical method. Among the immunochemical techniques, it is one of the most accomplished ones, employing various methods to detect and quantify both antigens and antibodies. The term immunoassay usually refers to a quantitative method, but in a broader perspective it includes characterization methods for analyzing the immunological properties of analytes. With appropriate means they can be made to be remarkably quick and easy, generating information that is otherwise difficult to achieve.[1, 4]

Since the discovery of radioimmunoassay technology by Yalow and Berson in 1959, immunoassay methodology has become exceedingly popular for routine clinical diagnostics.[5] Immunoassays are used for the detection and measurement of a near limitless range of analytes in clinical chemistry, microbiology and many other fields with most analytes of interest being either antigens or antibodies themselves.[6]

3.2.1 Different assay systems and possible variations

Assay systems are divided into two groups, one using labeled and the other using non labeled methodology. In the former, the antibody-antigen reaction is detected using a marker labeled substance and in the later the antibody-antigen complex can be detected directly without markers.[4]

There are four possible variations to carry out an immunoassay, these include an antibody excess, or an antigen excess, an antibody competition, or an antigen competition. Assays employing antibody excess or antigen competitions detect and quantify antigens, while assays that make use of antigen excess or antibody competition detect and quantify antibodies.[1]

3.2.2 Assessment of antibodies for use in immunoassays

When selecting a polyclonal antiserum either from a catalogue or from in-house productions there are certain desired characteristics that need to be considered.[2]

3.2.2.1 Titer and specificity

Titer is the dilution of an antiserum that gives a certain specified reaction in a test system, for example, binding 50% of a certain amount of labeled antigen. The titer represents an important aspect in the case of polyclonal antisera. A higher titer means a more economical use of the antiserum.[2]

High specificity is the essential attraction of immunological techniques. The specificity of an antiserum/antibody is its most important characteristic. An antibody has a unique specificity for a particular epitope, but this epitope can be found in one or more antigens leading to cross reactivity. In a polyclonal antiserum there is a heterogeneous mixture of antibodies with a range of specificities.[2]

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

Engvall and Perlman are considered to be the pioneers of ELISA since they first introduced it in 1971 as the first non-isotopic immunoassay employing an enzyme as a label. Ever since it has been gaining importance as a significant analytical technique and has become the most popular immunoassay used in research laboratories today. The assay can be designed, using relatively little equipment, to measure antigens or antibodies with high sensitivity and specificity.[5, 7]

3.2.3.1 Enzyme labels

Enzyme labels provide amplification of the detection signal. The criteria for an ideal enzyme label include that they are available in large amounts, have low cost, high specificity and low non specific binding, valuable precision of detection without any loss of activity, an easy conjugation to the antibody with minimal interferences caused by assay conditions of pH, ionic strength, detergents, buffers and enzyme cofactors. Enzyme labels are inherently stable in native as well as conjugated forms, for more than a year at 4 °C and even longer if freeze dried.[1, 5, 7]

3.2.3.2 Enzyme labeled antigens, antibodies or secondary reagents

All immunoassays rely on labeled antigens, antibodies or secondary reagents for detection. Enzyme labeled reagents are detected using chromogenic substrates. For assays that are carried out in microtiter wells, a soluble substrate that is converted to a soluble coloured

product is used. Enzyme levels are monitored by colour development in the spectrophotometer.[1]

ELISA makes use of enzyme labeled antibodies for the detection of the antibody-antigen complex. Antibodies can be readily labeled by covalent coupling to enzymes. The labeled antibody binds to the antigen and then is detected by using the label as a signal. A large number of enzymes have been used for this purpose, and the ones commonly used include horseradish peroxidase, alkaline phosphatase and β-galactosidase.[1]

3.2.3.3 Direct and indirect labeling

Enzymes may be attached to the antibodies by direct or indirect methods. The direct methods link enzyme to antibody covalently whereas indirect methods use an intermediate molecule to link the enzyme non covalently. Direct coupling of enzymes to antibodies must preserve the activity of both the enzyme and the antibody. Indirect methods use an enzyme labeled, secondary molecule that binds to the primary antibody in a second step of the assay. Two of the most popular secondary molecules are streptavidin and protein A. Streptavidin is an effective secondary molecule when used with the vitamin biotin which is a small molecular weight molecule with a strong non covalent affinity.[7]

3.2.3.4 Important aspects of ELISAs

Although various different configurations of immunoassays exist, ELISAs often prove to be the one of choice owing to certain attributes and features. The three important characteristics are: the solid phase adsorbent, the enzyme label and ease of operation. The solid phase may be paper, plastic or glass beads, latex emulsions or microtiter plate well with a significant advantage of quick and thorough washing of unbound reagents. The second advantage is that the enzyme label makes it safe, compared to other labels such as radionuclide, hence making it possible to work without any safety issues concerning the waste and with no requirement of a specialized lab. The ease of the ELISA procedure is noteworthy since ELISAs are relatively trouble free to apply hence allowing even amateurs to apply an assay according to their requirements.[5, 7]

3.2.3.5 Types of ELISAs for antigen detection

Numerous ELISAs exist as several variations and modifications are possible among the immunoassays to determine either antigen or antibody level. The classification is based on different criteria; the four classes of immunoassays are discussed below.

3.2.3.5.1 The antibody capture assay and factors affecting its sensitivity

The antibody capture assay, also known as the competitive assay, has its own significance. It is less time consuming since it requires fewer steps and is able to measure both low and high molecular weight antigens. Antibody capture assays can be used to detect and quantify antigens or antibodies; they can also be used to compare the epitopes recognized by different antibodies. For such assays, concerning antigen detection, the protocol is as follows. An unlabeled antigen solution is immobilized or adsorbed directly on to the microtiter well, the solid support, without the use of a primary antibody. This coating is usually carried out at temperatures such as 4 °C or 37 °C. Later, sample and an antibody in a limited amount are added, this leads to a competition between the two antigens, the one that was coated and the one that is added as the sample. Depending on the concentration of the antigen in solution, the antibody then binds to the immobilized antigen. The antibody can either be labeled directly or detection can be carried out by using a labeled secondary reagent that specifically recognizes the antibody. Following incubation with a labeled secondary reagent such as an anti-immunoglobulin antibody the amount of antibody bound to adsorbed antigen or retained on the solid support is measured and related inversely to the antigen concentration in the sample.[1, 7] For detection purposes substrate is added, the enzyme catalyzes the conversion of the substrate into a coloured product. This reaction can be stopped by enzyme denaturation. The optical density measured by a photometer is indirectly proportional to the analyte concentration in the sample implying that the higher the signal the lower the concentration of the antigen. A standard curve can be obtained by plotting the optical density (OD) against the logarithm of the antigen concentration.[8]

A pivotal problem concerning this assay is the requirement of substantial amounts of pure or partially pure antigen since the plate is coated with the antigen. This can be a source of concern when dealing with rare antigens.[7]

The three factors that will affect the sensitivity of a labeled antibody assay are discussed in the following text. The **amount of immobilized or bound antigen** to the solid phase can be controlled easily either by dilution or concentration of the antigen solution. The **avidity of the antibody for the immobilized antigen** is the second factor that affects the sensitivity of an antibody capture assay. The **type and number of labeled moieties** used to label the antibody. Antibodies are either labeled with enzymes or biotin. The sensitivity of detection is adjusted by varying the number of labeled molecules bound to the antibody.[1]

3.2.3.5.2 The antigen capture assay

In this case, the primary antibody is adsorbed to the solid support and sample antigen and a labeled antigen are added. In comparison to other assays, the antigen capture assay measures low molecular weight antigens more effectively by this incorporation of the labeled antigen. In this case a constant amount of the labeled antigen is added to the unlabeled sample antigen. Both antigens then compete for binding with the primary antibody on the plate. Washing removes any unbound proteins; quantification is carried out by measuring the amount of the labeled bound antigen. A higher sample antigen concentration means less labeled antigen is bound to the antibody and hence the final signal is smaller.[1, 7]

3.2.3.5.3 Indirect antibody capture immunoassay

The indirect antibody capture assay is used for detecting the presence of an antibody rather than antigen in test sample. This assay uses commercially available labeled secondary antibody along with fairly large quantities of pure or partially pure antigen to coat the plate. In this case an antigen solution is added for coating and then an antibody sample is added. If antigen specific antibody is present in the sample, the antibody binds to the well and is detected by a labeled isotype-specific antibody. Thus the captured test antibody is detected indirectly with a second labeled antibody.[7]

3.2.3.5.4 Antibody sandwich immunoassay and factors affecting its sensitivity

The antibody sandwich assay is thought to be the most sensitive and the most versatile one for detecting the concentration of high molecular weight antigens like proteins by ELISA without any prerequisites of antigen purity and only suitable antibodies being essential. Since binding of two antibodies to the antigen is required, only multivalent antigens with either different or repeating epitopes can be detected. This is not a disadvantage for proteins since

they are almost always multivalent.[7] Either two monoclonal antibodies that recognize discrete sites or a batch of affinity purified polyclonal antibodies can be used.[1]

For carrying out such assays the primary antibody is purified and bound to the solid phase. An antigen in the sample solution is then allowed to bind to this antibody forming the antibody-antigen complex. Washing removes any unbound compounds and the labeled secondary antibody, recognizing a different epitope, is allowed to bind to the antigen. Since the antigen is in between two antibodies this ELISA type is called sandwich ELISA. The analyte is quantified after washing by measuring the amount of bound labeled secondary antibody. In the next step a chromogenic substrate is added which is transformed to a coloured product by the enzyme. The OD value is directly proportional to the concentration of the analyte in the sample. The major advantage of this technique is the high specificity and that no antigen purification prior to the use is necessary. However, this assay requires substantial amounts of both primary antibody and labeled secondary antibody.[1, 7]

The sensitivity of two antibody assays is dependent on four factors. The first one being the number of molecules of the primary antibody bound to the solid phase. This can be adjusted very easily by varying the concentration of the antibody solution. The second and third factors affecting the sensitivity deal with the avidity of the primary and secondary antibody for the antigen, respectively. The fourth factor deals with the specific activity of the labeled secondary antibody, that is the number and type of the labeled moieties on the secondary antibody.[1]

For the detection and quantification of antigens, two antibody sandwich assays have proved to be the best choice. However, in the absence of two monoclonal or affinity purified polyclonal antibodies, competitive assays prove to be the next most useful assays for antigen quantification.

3.2.3.6 Sensitivity and reproducibility of ELISAs

The sensitivity and reproducibility of an immunoassay is determined by acknowledging various parameters. First and foremost a high affinity antibody capable of binding to an accessible epitope(s) is critical for a sensitive assay. Secondly, the adsorption of antibody or antigen to the solid phase should not in any way reduce the antigen and antibody binding. As

a result of adsorption, the molecules may be oriented in an ineffective manner or slight changes in the protein structure may lower or modify antibody binding. Fortunately this does not appear to be a common problem. Third, consistent incubation times, identical buffers and reagents and redundant standard curves are necessary for every plate to accomplish reproducible results.

The antibody sandwich assays are capable of detecting protein concentrations as low as 0.1 ng/mL (pmol) and are more sensitive than capture assays. The antibody capture assays, typically measuring 1-10 pmol of antigens are, however, preferred for low molecular weight antigens.[7]

3.3 Chemical sensors

The term sensor comes from the Latin word *'sentire'*, which implies *'to perceive'*. As defined by IUPAC, chemical sensor is a device that converts chemical information into an analytically useful signal. Thus the objective of a chemical sensor is to translate chemical information into a measurable quantity; preferably, a change in voltage. Chemical information varies from a specific component concentration to total composition analysis. [9-11]

3.3.1 Advantages of chemical sensors

Sensors are simple, low in cost, rugged and easy to operate miniature systems and thus can be applied in various fields including biomedicinal chemistry, warfare safety, environmental and industrial chemistry. Owing to their simplicity, high maintenance is not required and they can be operated by non trained personnel. Miniaturization renders the possibility to integrate several different sensors in one device, making multi-analyte measurements possible and eliminating cross reactivity.[12, 13]

3.3.2 Components of a chemical sensor

A chemical sensor consists of a sensitive layer, a physical transducer, an electronic setup for measurement and a data acquisition unit. The details of each are discussed in the following sections.[9]

3.3.2.1 Sensitive layer

The sensitive layer is a chemical recognition layer monitoring the chemical constituents within the environment of the sensor selectively. The layer is either natural, comprising of bio-macromolecules such as enzymes or antibodies, or it can be synthetic that comprises of a polymer.[9] The layer composition critically influences effectiveness and applicability of the sensor as it controls the selectivity, sensitivity, response time, and lifetime of the sensor.[14]

There are three major types of sensitive layers; the ion selective layers detect ionic concentration changes, whereas the molecular recognition layers rely on the molecular size interactions. Lastly the bio-sensitive layers consist of biologically sensitive species, enzymes, receptors, nucleic acids and antibodies.[14]

3.3.2.2 Transducers

Chemical sensors use various transduction principles; the major types include optical, electrochemical, thermal and mass sensitive transducers. For mass sensitive transducers, surface acoustic wave devices (SAW) and quartz crystal microbalances (QCM) are the most common ones. Since mass sensitive transducers have been used for the present research, they will be elaborated in detail in forthcoming sections.[14]

3.3.2.3 Measuring electronics and data acquisition

Some form of measurement electronics and software is required to convert the signal produced by the transducer into a readable form, so that the data obtained gives analytical information.[14]

3.3.3 Principle of a chemical sensor

Figure 3 elaborates pictorially the working principle of the sensor. The recognition layer when exposed to the analyte interacts with it, this results in a change in one of the physical properties of the layer, mass, optical absorbance, reflectance, polarity, impedance, voltage, fluorescence behavior are to name a few.

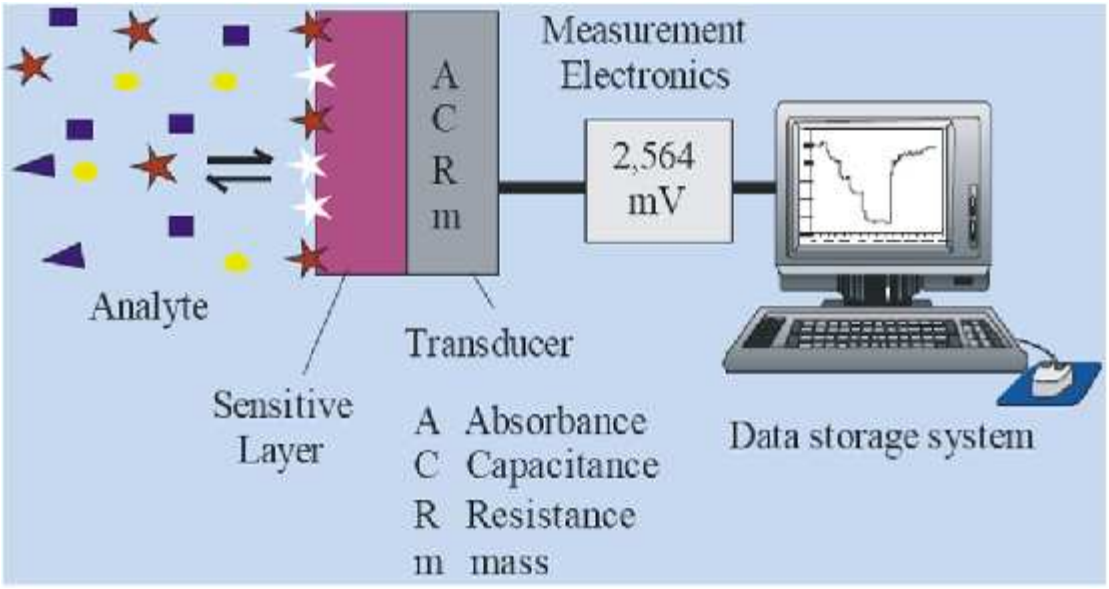

Figure 3: Working principle of the sensor[9]

The transducer system detects and converts these changes to an electrical or optical signal. The electronics and software then detect, amplify and process this signal. Finally, the data acquisition unit processes and stores the acquired data.[9, 14-16]

3.3.4 Characteristics of a sensor

In order to ensure the usage of chemical sensors they should posses certain basic properties. The following text discusses such characteristics, which a good chemical mass sensor, especially a quartz microbalance, must possess.[9]

3.3.4.1 Sensitivity

Sensitivity of a sensor is the relationship between signal output and the analyte concentration thus it recognizes the mass changes of the sensitive layer on exposure to the analyte. Sensitivity depends on the type, thickness and structure of the sensitive layer and on the fundamental frequency and damping of a quartz crystal. Various parameters like operating temperature, viscosity and temperature of the analyte solution, type of the cell and measurement electronics also affect the sensitivity. The mass sensitivity of a QCM device is given by frequency change per unit mass change.[9, 14, 17]

3.3.4.2 Noise level

An ideal sensor should have low intrinsic noise level with high sensitivity as this allows the sensor to respond and to detect very small changes in the concentration of the analyte.[14]

3.3.4.3 Selectivity

Selectivity towards the species being analyzed is vital; the selectivity of a mass-sensitive device refers to the degree to which it is free from interference by any other species present in the sample matrix. Typically, selectivity is induced both by a selective and/or sensitive layer and the physical and/or chemical properties of the analytes.[9, 14]

3.3.4.4 Response time

Response time is the time required by the sensor to reach a constant signal or to get the frequency to a constant value. Fast response times are essential for online monitoring of processes, a chemical reaction for instance. Response time is affected by the type of interaction between the layer and the analyte and the operating temperature; it is shorter at higher temperatures.[9, 14]

3.3.5 Drift

A slow or non-random change of the response of a sensor under unaltered experimental conditions is called drift. It can be observed due to continuous loss or gain of layer material or due to some electrical reasons. For good sensor results ideally there should be no drift and under inevitable conditions it should be as low as possible.[9, 14]

3.3.6 Reproducibility, repeatability, reversibility and stability

Reproducibility of the sensor response is signified by the same response obtained with experiments conducted under the same conditions at different times. Repeatability of the sensor response is determined in experiments that are repeated under the same experimental conditions but at different times and different ambient conditions. Reversibility is the return of the measured frequency to its original value on analyte removal. Stability is a characteristic that takes into account the reproducibility of measurements and is directly associated with the stability of the chemical layer deposited onto the quartz. A sensor can only be a stable one if it has high repeatability and reproducibility.[9, 14]

3.3.7 Temperature effects

The temperature dependence of measurements is crucial, therefore it is necessary to regulate the temperature at the time of measurement. A small change in temperature can cause a change in the resonance frequency of quartz in several Hz as in the case of liquids, water or aqueous solutions; the change in resonance frequency is ~ 40 Hz for 1 °C rise in temperature. This is due to the fact that temperature may affect sensor response, response time and viscosity of the liquids.[18]

3.3.8 Matrix effects

The presence of any substance in the sample other than analyte that affects the measured signal is known as the interfering substance. These interfering substances interact with the sensor layer in a similar way as the analytes and induce a response similar to that generated by the analyte, thus their presence results in an apparent increase in the concentration or activity of the analytes. At high concentrations, even highly discriminated substances may induce interference.[9]

3.3.9 Classification of sensors

The chemical sensors are divided into three basic categories depending on the type of transducers which are different depending on their type of signal generation principles. These include electrochemical sensors, optical sensors and acoustic wave or mass sensors. The acoustic wave or mass sensors include the bulk acoustics and surface acoustics. Chemical sensors that measure change in mass on the surface of a chemically sensitive layer can be employed to a large variety of analytes. Typical examples of this class of sensors are bulk acoustic wave (BAW) and surface acoustic wave (SAW) devices. [17, 19, 20]

3.3.9.1 QCM as a mass sensitive device and its principle and applications

Quartz crystal microbalance, QCM, is a typical example of a bulk acoustic wave device which primarily relies on the change in mass. Commercially, QCMs are available for frequencies up to 50 MHz; above that limit, the plates become too thin for mechanical stability. In sensor applications, the quartz crystals operating in the frequency range of 5-10 MHz are commonly used. QCM based sensors employ a quartz crystal oscillator with a metallic film placed on it for electrical contact. The QCM is basically a mass sensing device

with the ability to measure very small mass changes on a quartz crystal resonator in real-time. Besides being highly sensitive a major advantage of the QCM technique used for liquid systems is its ability to allow a label-free detection of molecules.[14]

Coating the quartz crystals with a chemical recognition layer renders selectivity to the sensor. The coated QCM, when placed into an oscillating circuit, resonates with its fundamental frequency. This fundamental frequency depends on the thickness, structure, shape and mass of the crystal. The interaction between an analyte and the surface of the sensitive layer results in a shift of the resonance frequency, which can be measured with high sensitivity. The relationship of the change in resonance frequency of a QCM can be related to the mass of the deposited analyte and is given by the Sauerbrey equation.

$$\Delta f = -{f_o}^2 \frac{2}{A_{cr}(\rho_m C_q)^{1/2}} \Delta m \qquad (2)$$

The Sauerbrey relationship is given for the measurements in gas phase and relates the change in frequency, (Δf), to the change in mass, (Δm), making use of the density of the chemical film (ρ_m), the shear modulus of the quartz crystal (C_q), the fundamental resonance frequency of the crystal (f_o) and the area of the crystal, (A_{cr}). As indicated by the equation an increase in mass results in a decrease in the frequency. For liquid phase measurements, the properties of liquids are to be considered. Therefore the equation is represented as:

$$\Delta f = -{f_o}^2 \frac{2}{A_{cr}(\rho_m C_q)^{1/2}} \sqrt{\frac{\rho_l \eta_l}{4\pi f_o}} \qquad (3)$$

The equation describes the frequency change when the quartz crystal is placed in a liquid environment. The equation gives the relationship between the change in frequency (Δf) to the density (ρ_l) and viscosity (η_l) of the surrounding liquid as well as to the density (ρ_m) and shear modulus (C_q) of the quartz crystal. The theoretically expected frequency shift from gas to aqueous phase for a 10 MHz crystal is about 2 to 4 kHz. However, the observed frequency shift is often 1.5 to 3 times higher. This variance is attributed to several factors including the differences between the surface and bulk values of viscosity and density, hydrophilic or hydrophobic nature of the film and its surface roughness, intermolecular forces, electric double layer structure, etc.

There are various fields where QCM sensors can be applied for analysis; electrochemistry of interfacial processes at electrode surfaces, biotechnology, functionalized surfaces, thin film formation, surfactant research, drug research, liquid plating & etching and in situ monitoring of lubricant and petroleum properties are to name a few. Also a large number of analytical applications have already been published in the area of gas sensing and trace ion determination.[21, 22]

3.3.10 Immuno sensors

Immuno sensors are biosensors that make use of the antibodies as molecular recognition element for specific analytes that is antigens, to form a stable antibody-antigen complex and provide concentration dependent signals.[23-25] Performance of a biosensor depends on the stability of the immobilization, therefore stable immobilization of biomolecules is crucial to obtain a sensitive biosensor.[26, 27] Working of an immuno sensor is a two phase process; the first phase is to sense the formation of the antibody-antigen complex. The second one is then a signal transfer process that responds to the change in one of the properties caused by the antibody-antigen complex formation.[25]

The immuno sensors have certain advantages over other methods, these include high selectivity, miniaturizing, a label free (without any enzymatic label) detection of the binding reaction, being cost effective, fast and robust.[28, 29] Due to these features, immuno sensors tend to overcome the shortcomings of other available methods.

3.4 DNA - a universal biological analyte

Deoxyribonucleic acid (DNA) technology has remarkably affected various sectors like healthcare, pharmaceutical production, diagnostics, agriculture, food and forensic analysis. The use of nucleic acids, particularly DNA, as an analyte offers tremendous advantages including an increased scope of application, low detection limits, high speed and high sensitivity. DNA is the genetic material of the majority of forms of life and an ideal universal analyte for biological methodologies. Nearly every cell of an organism has an identical copy of the genome and theoretically a single copy of the gene can be detected using the polymerase chain reaction.[30]

3.4.1 DNA extraction

The isolation of genomic DNA is a fundamental requirement for analytical procedures based on DNA analysis; therefore, a well characterized and robust DNA extraction technique that reproducibly isolates DNA and is suitable for the sample matrix is essential.[31]

3.4.1.1 Factors determining the suitability of the extraction method

The suitability of isolated DNA as an analyte for a given technique is generally determined by the amount or concentration, purity and integrity of the DNA. The amount and concentration of the isolated DNA must be sufficient enough for subsequent analyses. The extracted DNA must be free from any contaminants that may inhibit analysis. Also, a high molecular weight ranging from 50 to 200 kbp can be of significance for certain analysis. Each of these factors can be influenced by the extraction technique employed.[31]

3.4.1.2 Various steps of DNA extraction

DNA extraction encompasses various steps from sample preparation to the determination of the concentration and purity of the obtained DNA. Sample preparation incorporating homogenization, centrifugal separation or minimizing the effects of surface contaminants prior to cell lysis may prove to be beneficial. The next step, cell lysis, is critical as it frees the DNA and can be done by microwave heating, chemical, mechanical or enzymatic means. The stabilization and protection of the released DNA is a significant step. For this purpose an extraction buffer that prevents DNA degradation by cellular nucleases liberated during lysis, is usually present. Next, the separation of nucleic acids from cellular and matrix debris and other biological macromolecules is achieved, e.g. by phenol - chloroform and chloroform extractions. DNA purification by removing RNA through appropriate nucleases is the next crucial step. Other impurities can be removed by ion exchange columns or the addition of chelating agents. Lastly, to concentrate DNA to a working molarity, alcohol precipitation or commercial columns are used.[31]

3.4.1.3 Choosing the most appropriate DNA extraction procedure

Before endeavoring upon an extraction method, it is essential to consider certain aspects of the sample from which the DNA has to be extracted. The history of the sample is important since it gives information regarding any possible contaminations and so the sample

preparation steps employed must remove contaminants. Also, the source of the sample, if it was maintained in a stable environment, is important to determine whether a method optimized for high molecular weight or degraded DNA should be employed. The composition of the sample has its own significance. If the sample is heterogeneous with respect to the analyte, it must be homogenized in the initial steps. Some information on the cellular structure of the sample should be there so as to ensure appropriate lysis.

3.4.1.4 Quantification of total DNA by spectroscopy

To determine the yield obtained from a DNA extraction process, DNA must be quantified. The simplest and most commonly used technique is spectrophotometric determination, that is, either UV absorption or fluorescent emission. In the UV region, all nucleic acids absorb strongly with maximum absorbance at a wavelength of 260 nm. This physical property is used for concentration determination of nucleic acids in solutions. For determining the purity, a simple method is to determine the A_{260}:A_{280} ratio; this ratio should be 1.8-2.0 in an uncontaminated sample of DNA.[32]

3.4.2 PCR – Polymerase chain reaction

Kleppe and colleagues in 1971 elucidated the theoretical concept of generating numerous copies of a specific DNA molecule using DNA polymerase and oligonucleotide primers in a cyclic process. Kary Mullis in 1983 introduced the procedure for the polymerase chain reaction and won the Nobel Prize in 1993 for it. Automated oligonucleotide synthesizers were available when the polymerase chain reaction (PCR) process was demonstrated by Saiki and colleagues in 1985.[33, 34]

Real-time PCR is a laboratory genetic amplification technique that renders high levels of sensitivity and specificity to bioanalysis with a huge impact on various facets of biological research. In PCR, DNA polymerase is used to copy a segment of template DNA, as the reaction proceeds, its concentration increases and the fluorescent signal from the polymerase chain reaction is continuously collected over a range of cycles. Real-time PCR assays can be completed very rapidly since no manipulations are required post amplification.[33-36]

3.4.2.1 Basic principles underlying the real-time polymerase chain reaction

Basically, the amplification is defined by four phases; baseline, exponential, linear and plateau. Figure 4 illustrates some of these phases.

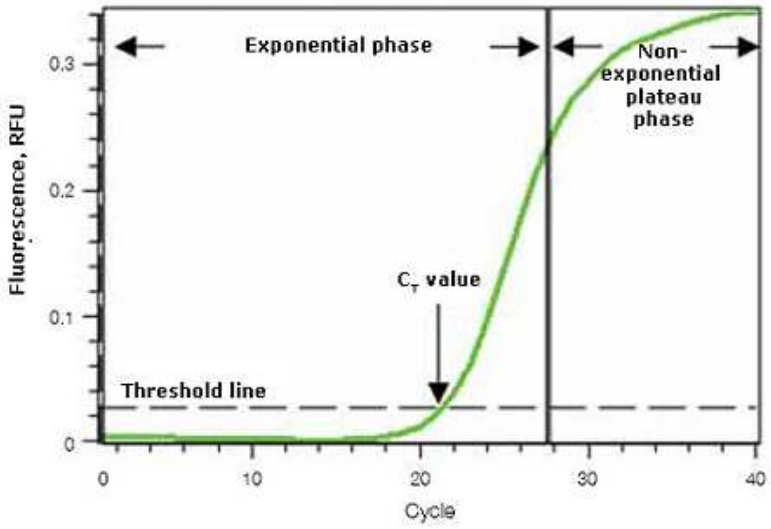

Figure 4: Different phases of PCR[52]

The amplification during the baseline phase is well below the detection level of the real-time PCR instrument with no detectable signal but the exponential amplification of the template is occurring. The earliest detectable signal from PCR is detected in the exponential phase where the amplification proceeds at its maximal exponential rate. The template concentration and the quality of the real-time assay determine the length of this phase. If an assay is 100% efficient, 2 products result per cycle from each template. As the amplification efficiency begins to taper off, linear phase starts, which is also a straight line as indicated in figure 4 but the amplification falls from a ratio of 2 products from 1 template to 1.95 from 1. The template replication efficiency continues to decline until the fourth phase, the plateau, is reached where the amount of DNA produced in an amplification reaction reaches a maximum level and the amplification rapidly ceases for the remaining cycles of the experiment. The number of PCR cycles before plateau depends on the amount of target at the start of the reaction but the level of plateau is independent of the initial target concentration. Consequently, the DNA yield in reactions amplified to plateau cannot be used for quantification purposes.[33, 35]

3.4.2.2 Factors affecting amplification and reproducibility

The exponential nature of the amplification process implies that any subtle differences in amplification efficiency can lead to large differences in product yield and results. Variations may arise as a consequence of pipetting differences, using different batches of reagents, or temperature variations at different positions in the thermal cycler. Variations may also arise in different runs on the same thermal cycler. Besides these, a false positive resulting from the presence of contaminating DNA poses additional menace. It is therefore essential to not only calibrate the thermal cycler but also to use proper positive and negative controls for reliability of results.[35]

3.4.2.2.1 Reaction components

The reaction components which are of significant value are the template (DNA target), the deoxynucleotide triphosphates and magnesium chloride. The amount of target DNA added is important, if it is too less it may generate a negative signal while too much may result in the production of non specific products. The concentration of the deoxynucleotide triphosphates, dNTPs, must be optimized to maximize specificity. DNA polymerase used for amplification is magnesium dependent therefore magnesium serves as an enzyme cofactor. The dNTPs bind magnesium with a stoichiometry of 1:1 so the concentration of magnesium must be set accordingly.[35]

3.4.2.2.2 Primer design and primer optimization

Designing appropriate primers is an essential requirement for real-time PCR, while designing these oligonucleotide primers certain aspects must be taken into account. It is important to know the desired product as the placement of primers on the template establishes the composition of the product. The primer sequence must be designed such that they anneal the correct strands. **Primer specificity** is crucial since the greatest challenge in developing a successful real-time PCR method is the prevention of non specific amplification. Mispriming can be prevented by comparing sequence similarity between the primers and all other template sequences in the design space. Several primers can be designed and individually checked for cross matches with basic local alignment search tool (BLAST). **Primer length** is significant; if too short, it is difficult to design gene specific primers and choosing an optimal annealing temperature is hard. Very long oligos besides being expensive to synthesize are

likely to form secondary structures that decrease the PCR efficiency and promote primer dimer formation. PCR primers are typically 16-28 nucleotides long. The melting temperature, T_m, determines the optimal PCR **annealing temperature**. An ideal PCR reaction has forward and reverse primers with similar T_m values.

There are software programs with various guidelines that can help with planning oligonucleotides, however, following them strictly does not guarantee success. Since primer synthesis is relatively cheap it is only cost effective to try several primers to achieve the desired result rather than attempting to optimize what may turn out to be a suboptimal primer pair.[35-37] **Optimizing primer concentration** is of vital importance since each primer set works best at a different concentration. An optimal primer concentration is the one where the specific amplification relative to primer dimers is maximal, in a positive and negative control experiment.[38, 39]

3.4.2.2.3 Polymerases

The first polymerase reaction was demonstrated by using the Klenow fragment of *E. coli* DNA polymerase. Since the DNA polymerase enzyme was not heat stable and fresh polymerase had to be added after each denaturation step, the original process was burdensome. The true potential of PCR was realized when a thermo stable DNA polymerase from *Thermus aquaticus* was used for amplification; it is the heart of modern PCR. Although *Thermus aquaticus* (*Taq*) remains the most widely used polymerase, a number of other enzymes are now in use for various applications.[33, 35]

3.4.2.2.4 Thermal cyclers

A broad range of automatic thermal cyclers are available commercially. Any difference in the machines or the individual machine performance has profound effects on the end result. While taking the effect of a thermal cycler on PCR it is important to consider what occurs during each cycle. There is generally a delusion that three discrete temperatures exist in PCR; DNA denaturation occurring at one, primer annealing at another and primer extension occurring at the third. More realistically, it is a dynamic process where denaturation progresses to annealing and then to extension before the cycle is repeated. Denaturation is likely to be complete as the set temperature is approached, annealing starts as the set

temperature is approached, becoming progressively faster at the optimal temperature, continues past the set annealing temperature and extension follows primer annealing.[35]

The three steps of denaturation, annealing and elongation are elaborated in figure 5.

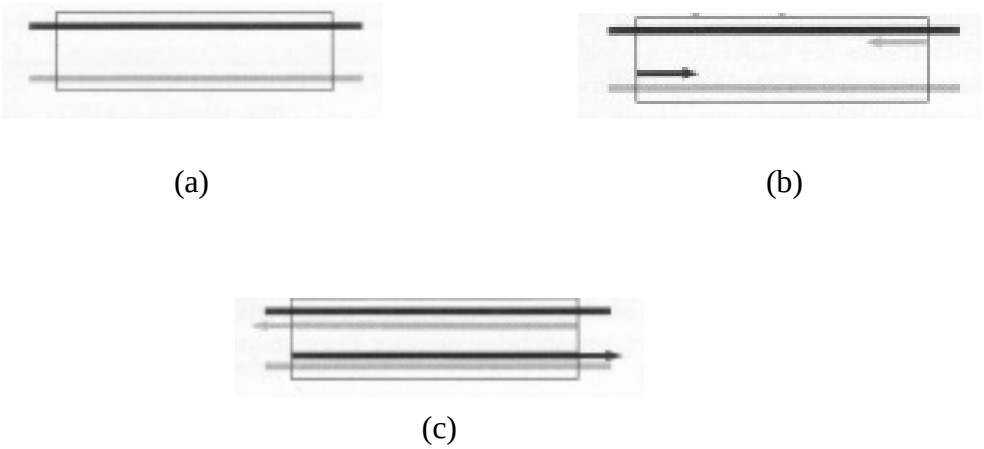

Figure 5: Denaturation, annealing and elongation stages[40]

(a): Denaturation of double-stranded DNA in two single strands, (b): annealing of primers on the single strands, (c): elongation of the DNA templates by Taq DNA polymerase

During denaturation, the hydrogen bonds are broken and the double stranded template DNA is denatured at 92 – 95 °C and two single strands are obtained. (a). At annealing temperatures between 45 and 65 °C, primers, two specific oligonucleotides complementary to the target DNA hybridize to their complementary sequence of 3´ or 5´end of the single stranded DNA. (b). Elongation is carried out at 72 °C, the temperature optimum for the Taq DNA polymerase which binds to the 3´end of the primer and synthesizes a new DNA strand complementary to the template. The required dNTPs for this synthesis are in the PCR reaction mix. (c).[40]

3.4.2.3 Detection and detection chemistries

The basic methodologies utilize fluorescent dyes and the fluorescence signal governs the detection of real-time PCR; a proportional increase in the fluorescent signal in subsequent cycles is directly proportional to the increase in the amplified product. The assay delta is of

crucial importance in any fluorescent assay, it is the difference between the initial and final signal intensities and greater difference is essential.

According to the role in the assay, fluorescent molecules used in real-time PCR are divided into three classes. Firstly, a donor dye i.e. reporter, gives a fluorescent signal monitored during the course of the experiment. Second is an acceptor or quencher molecule, responsible for the initial quenching of the signal from the reporter. Lastly, a reference dye, common to all reactions, with no interaction with assay components, functions to normalize the well to well signal in the software. Quencher molecules can either be fluorescent dyes or any molecules that absorb light within appropriate wavelength range. Theoretically any fluorescent dye can be a reporter; most commonly used is 6-FAM (6-carboxy flourescein), SYBR Green I is also used widely.[33]

3.4.2.3.1 SYBR Green based detection

The incorporation of a free dye into the newly formed double stranded DNA product represents the simplest detection system. DNA binding dyes have been extensively used for the direct analysis and quantification of nucleic acids. Commonly used dye for this purpose is SYBR Green I, when stimulated by appropriate wavelength, its background fluorescence in solution as a free dye is very low. There is, however, a dramatic increase in the fluorescence output when it is bound to double stranded DNA and the fluorescence is proportional to the amount of double stranded DNA. The signal to noise ratio of this assay system is excellent.[33]

The SYBR dyes are popular for real-time detection and are readily available commercially. The popularity of SYBR Green I assays is three fold; low cost of the dye, ease of assay development that requires only one pair of primer and possibility of using the same detection mechanism for each assay. The only downside being that every double stranded molecule generates a signal, be it an inappropriate PCR product or primer dimer, therefore careful primer design is important.[33]

In real-time PCR the readouts are given as the number of PCR cycles necessary to achieve a certain level of fluorescence, i.e. threshold cycle, C_t. Initially the fluorescence signal emitted by SYBR Green I bound to the PCR product is too weak to register above the background, however fluorescence doubles in each cycle in the exponential phase. The intensity of the

fluorescent signal usually begins to plateau 30 to 35 cycles later, indicating that the PCR has reached saturation. Primer design needs to be more stringent for SYBR Green based assays than for TaqMan assays and it is important to determine the sequence that needs to be targeted by the PCR.[38, 39, 41]

3.4.2.3.2 TaqMan based detection

Another category of signaling systems involves the use of a third, fluorescently labeled oligonucleotide located between the primers called the **probe.** Such assays have the added specificity of the intervening third oligo. Signals from primer dimers are not detected which proves to be a further advantage of a probe based assays. The only PCR amplicon that is detected is the one to which both primers and probe bind simultaneously.[37, 39]

Like the primers, the real-time PCR probes are designed using software, various aspects that are taken into account while designing the probe include the probe melting temperature or the annealing temperature, it must nearly be 10 °C higher than the forward or reverse primer. This is to ensure that the primers bind before the probe participates in the reaction. Also, the probe should not self anneal and form secondary structures. The probe should, without overlapping with the primer sequence, be as close to the forward and reverse primers as possible.[35, 39]

TaqMan probes or hydrolysis probes, one of the five probe based assay systems in use today, are linear oligonucleotides that have been labeled with a reporter on the 5´end and a quencher on the 3´end. The reporter signal is quenched if reporter and quencher dyes are in close proximity in solution. In the elongation step Taq DNA polymerase binds and rapidly extends the new strand following primer binding.[33]

3.4.2.4 Data analysis and reporting

The C_t value, also known as the crossing point, C_p, is defined as the cycle when sample fluorescence exceeds a chosen threshold above background fluorescence; positive C_t results from genuine amplification. The C_t is determined by the chosen baseline setting and the threshold setting which can be either default values of the software or selected manually. The negative controls play a vital role in setting the threshold; therefore, no template controls

(NTCs) must be included on every plate in every experiment and help distinguish between a sample and a contaminant.[42]

3.4.2.4.1 Cycle efficiency and amplicon size

PCR is usually explained as a reaction in which the amount of template is doubled in each cycle, leading to geometric growth from 1 template to 2, then 4, 8, 16, 32 and finally 1024 after 10 cycles; hence making it an exponential amplification process. This is a little fictitious because the efficiency of the PCR is not 100% in each cycle. In practice, the efficiency of the amplification approaches 100% for only a limited part of the process. The amplicon size, an important factor for accurate expression of quantification, affects the PCR efficiency. Very long amplicon decreases the PCR efficiency, therefore the amplicon should be smaller than 250 bp, between 100-250 bp is a typical size range.[36, 37]

3.4.2.4.2 False positives in PCR

PCR being a powerful genetic amplification tool, theoretically detects a single DNA target by producing approximately 10^{12} copies of a selected sequence in a few hours and thus the technique may suffer from its own success. All PCR amplification products can be re-amplified giving rise to false positive results if not excluded from subsequent amplifications. PCR controls hold great significance; no template (negative) controls should always be included in real-time runs along with appropriate positive control. Methods relying on probe hybridization to produce a fluorescent signal are less liable to produce false results compared to the other methods where intercalating dyes are used.[34, 35, 39]

3.4.2.4.3 Melt curve analysis

The melt curve analysis, also known as heat dissociation analysis of the PCR product, adds significantly to the utility of fluorescent DNA binding dyes since the data can be used to determine a number of important variables. It renders the possibility to characterize the PCR amplicon in situ on the machine and determines if the amplified signal is for a single product or not or if there were any primer dimers formed, thus making the dimer issue obsolete.[34, 38 ,41]

3.4.2.4.4 Specificity and sensitivity of the reaction

The specificity of the reaction is dictated by the uniqueness of the priming sequence within the template DNA used in the assay, and control under which the primers are allowed to interact with target and to non-target sequences. The sensitivity of the reaction results from consecutive amplification cycles, controlled by a thermal cycler, where products of one cycle act as targets in the next cycle, theoretically allowing the amount of selected target to be doubled at each cycle.[35]

3.4.2.4.5 Optimization of real-time PCR assays

Every factor associated with PCR has the potential to affect the amplification efficiency. For a robust and specific amplification protocol, various factors have to be optimized. Optimization of reagents is essential for the development of robust assays to obtain reliable results. A robust assay is the one in which the crossing threshold (C_t) is not affected by slight variations. Factors like the primer concentration, probe concentration, cycling conditions and buffer composition need to be optimized since any alteration in these affects the specificity, sensitivity, efficiency and reproducibility of the assay.[35, 39]

3.5 Immuno-PCR (IPCR)

A combination of an immuno assay and a PCR is termed as immuno-PCR. Sano et al. developed an immuno-PCR method, extending the scope of PCR for ultra sensitive detection of proteins. It is an immunoassay similar to ELISA that, instead of a colorimetric enzyme reaction, uses PCR for detection.[43] It is a highly promising technique for the ultrasensitive analysis of proteins and other antigens as it combines the sensitivity and the signal amplification power of PCR with the versatility and antigen specificity of ELISA by exchanging the signal generating antibody enzyme conjugate used in ELISA with an antibody-DNA conjugate.[44-46] The sensitivity of immuno-PCR is much higher than that of ELISA; as a consequence the limit of detection, LOD, of a given ELISA is generally enhanced 100-10000 fold by the use of PCR as a signal amplification system.[47, 48]

Initially the amount of PCR product was assessed by either gel electrophoresis as explained by Sano et al. who constructed a steptavidin-protein A chimera that was bound to a biotinylated linear plasmid. The protein A-streptavidin-DNA-conjugate bound the detection

antibody which was bound to the analyte in the microtiter plate. The DNA was amplified using PCR and the PCR product was detected by gel electrophoresis.[43] Using real-time PCR, the amount of DNA could be quantified with high sensitivity and accuracy over a wide concentration range.[49]

Owing to the extreme sensitivity of the PCR amplification step, immuno-PCR is very sensitive to contamination and false positive signals which result from the non specific binding of reagents. Therefore, the requirements for IPCRs are much more stringent than for conventional ELISA. Thus synthesis of antibody-DNA conjugate and the readout method of the amplified nucleic acid fragments are of vital importance. These two steps distinguish the ELISA from immuno-PCR and are the key for the successful conversion of a given ELISA into a highly sensitive immuno-PCR assay.[48, 50]

3.5.1 Assemblages for real-time PCR

Immuno-PCR can be assembled in several ways depending on the binding of the capture antibody to the surface of the reaction vessel and of the DNA label to the detection antibody.[43] In a protocol for immuno-PCR reported by Lind et al. antibodies specific for the protein target were immobilized to the reaction vessel. On adding the sample, targeted proteins bound to the immobilized antibody molecules. Addition of the detection antibody, coupled to a DNA molecule, leads to a bond between the antibody and the immobilized protein target. After careful washing to remove all unbound reagents, the DNA is amplified. During the exponential phase of the PCR, the amount of product formed reflects the amount of target protein that was bound to antibodies.[49] Further developments include various modifications such as the stepwise coupling of biotinylated antibodies and biotinylated DNA using the biotin binding protein, streptavidin, as the linking unit. [45]

3.5.2 Various aspects of immuno-PCR

Various aspects to be considered during an immuno-PCR are discussed in the following text.

3.5.2.1 Attaching capture antibody

A simple way to attach the capture antibody to the vessel surface, where no special linking groups are needed, is by physical adsorption. A solution containing the antibody is added to the wells; overnight incubation allows the antibodies to adsorb to the surface. Another

possibility is through a streptavidin-biotin link. For this the capture antibody is biotinylated, the surface is coated with streptavidin. The capture antibody can either be attached to the surface before being exposed to the antigen or it can be incubated in a test solution to bind the antigen followed by immobilization to the surface. The assay time with this approach is much shorter but it is also more expensive.[43]

3.5.2.2 DNA label and labeling detection antibody with DNA

The DNA label must be carefully designed as it serves as the template for PCR. It is important to ensure that the DNA label and the primer pair produce negligible amounts of non specific primer dimer formation. DNA label can be attached to the detection antibody in two ways, through streptavidin-biotin linkage or by covalent coupling using a cross linking agent. The streptavidin-biotin link is usually achieved stepwise, incubating one component at a time during the assay. This is simple but requires additional incubation and washing steps. The covalent antibody / DNA conjugate is prepared and purified in advance.[43]

3.5.2.3 Reaction containers and instruments

Immunoassays are performed in plates optimized for protein binding. For real-time immuno-PCR, containers that fit real-time PCR instruments are needed. Regular ELISA plates cannot be used as they do not fit the PCR instruments having flat bottoms.[43]

3.5.2.4 Blocking agents

In immuno-PCR the sensitivity is affected due to background signal resulting from non specific binding reagents, blocking agents are used to avoid this. These are often inert protein blends, such as bovine serum albumin (BSA) and milk powder or detergents such as Tween 20. The blocking agents cover any empty surface preventing direct binding of the reaction components to the vessel.[43]

3.5.2.5 Controls

Running controls parallel to the test samples determines the performance of the real-time immuno-PCR assay. Any background signal from non specific binding of assay components is assessed by a background control (BC), containing the same amount of antibodies and DNA as positive samples but no antigen, it should have a high C_t. The second control is the

no template control (NTC); containing only the real-time PCR master mix. It indicates any master mix contaminations and any primer dimer formation is indicated by its C_t value.[43]

3.6 Gel electrophoresis

To study properties like size and molecular weight of proteins and DNA, electrophoresis proves to be a simple, quick and highly sensitive tool. The separation of proteins and DNA by electrophoresis is based on the fact that upon application of an electric field, usually provided by the immersed electrodes, charged molecules tend to migrate through a matrix. This support matrix, where the sample is run is either agarose or polyacrylamide gel. Polyacrylamide gel is widely employed to separate proteins while agarose is mainly used to separate larger macromolecules such as nucleic acids. By gel electrophoresis, the apparent sizes of linear fragments of DNA in comparison to standards are determined. Also the migration of two bands can be compared with each other without the standards.[36, 51]

3.6.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis has become the principle tool in analytical chemistry, biochemistry and molecular biology since it can be utilized to analyse the size, amount, purity and isoelectric point of polypeptides and proteins.

One-dimensional polyacrylamide gel electrophoresis is simple with various modifications, amongst which the most commonly used system is the sodium dodecyl sulphate (SDS)-polyacrylamide discontinuous electrophoresis where proteins are separated by their size.

In the SDS-PAGE system, the protein mixture is denatured by heating at 100 °C in the presence of excess SDS and a thiol reagent, employed to break the disulphide bonds. Under these conditions, all proteins are dissociated into their individual polypeptide subunits. These polypeptides bind the same amount of SDS in a constant weight ratio and form SDS: polypeptide complexes with identical charge densities independent of the amino acid composition and sequence of protein. The SDS-protein complex forms a rod with its length more or less proportional to the molecular weight of the protein. Thus, proteins are separated in polyacrylamide gels strictly according to their size. This procedure denatures proteins and hence cannot be used to analyze native proteins and proteins whose biological activity needs to be retained for subsequent functional testing.[51]

3.6.1.1 Gel porosity

The amount of total acrylamide used per unit volume and the degree of cross-linkage primarily determines the gel porosity. The latter is determined by the relative percentage of bisacrylamide used. On increasing the proportion of cross-linker, the pore size decreases. The average pore size reaches a minimum when the amount of bisacrylamide represents about 5% of total acrylamide.[51]

3.6.1.2 Some considerations

Certain things that need to be taken into account are discussed in this section. In a discontinuous SDS-PAGE system, the samples are not loaded directly onto the resolving gel with small pore size but onto a gel, called the stacking gel, with a larger pore size that is polymerized on top of the resolving gel. The purity of the reagents is another critical aspect since contaminants in acrylamide, bisacrylamide, initiators, buffers and necessary additives like SDS may inhibit or accelerate polymerization. Also, ammonium persulphate is highly hygroscopic and when dissolved in water it begins to break down immediately. Therefore ammonium persulphate solutions must always be freshly prepared. Oxygen traps free radicals thus inhibiting the polymerization of acrylamide. Therefore oxygen dissolved in a gel mixture must be removed before polymerization by degassing under vacuum.[51]

3.6.1.3 Casting gels

Gels are cast immediately after mixing the various components. However, mixing all components except ammonium persulphate allows the mixture to be stored for 4-6 weeks at 4 °C since it will not polymerize. To initiate polymerization, ammonium persulphate is added and gels are cast.[7]

3.6.2 Agarose gel electrophoresis

Nucleic acids are separated on their ability to move through the gel during electrophoresis; their size and shape affect mobility through the gel. Electrophoresis of nucleic acids is usually performed in gels composed of agarose or polyacrylamide. Agarose, a polysaccharide, is a natural product of kelp, and it is available in many grades of purity, melting temperature, and mechanical strength. For most applications, the agarose used is

"high melting point", implying that it is solubilized in a boiling aqueous solution and solidifies below 50 °C.

During electrophoresis, DNA moves towards the positive terminal or the anode as the phosphodiester back bone of the DNA is negatively charged, DNA is a polyanion and the number of charges it carries is proportional to its length. As the DNA moves through the gel matrix which acts as a sieve, it fits through small and narrow openings, this in turn slows down the DNA migration.[36]

3.6.2.1 DNA migration

For electrophoresis, the DNA samples are loaded in wells, at the origin of the gel, that are cast for the purpose of holding the sample before the voltage is applied. The DNA moves in straight lines across the gel towards anode. The individual bands on the gel have the same shape as the well and each band simply represents DNA molecules that happen to migrate together because of their size and shape.[36]

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4 RESULTS AND DISCUSSION

The present research deals with the development of analytical methods for the detection of potentially allergenic sesame, poppy and lupine. Four methods were developed; an ELISA and an immuno sensor for the detection of sesame, a real-time PCR method for the detection of poppy seeds and an immuno-PCR assay for lupine detection. This chapter deals with the results obtained and inferences made from all four experimental setups developed. The details of each method are discussed in the following text.

4.1 Development, optimization and validation of a competitive enzyme linked immuno sorbent assay (ELISA) for sesame

The first section describes the development and optimization of a competitive ELISA allowing the determination of sesame proteins in foods. For developing an ELISA for the detection of sesame proteins, the experimental setup involved various steps as mentioned in the experimental section, 5.1.

4.1.1 Sesame extract

A sesame protein extract was prepared to serve two purposes, one to produce antibodies, when injected as an immunogen, and secondly for obtaining a protein extract to be used for standard solutions during various steps of ELISA development and optimization. The sesame extract was prepared in a multistep process; white, peeled sesame seeds were initially homogenized to obtain a paste, which was then defatted using soxhlet extraction with n-hexane. After drying overnight at room temperature, it was mixed with PBS buffer and stirred. This mixture was then subjected to centrifugation and the supernatant was used as the protein extract, the details are described in section 5.1.1. The protein concentration was determined using the Bradford assay as explained in section 5.1.2. Figure 6 is the calibration curve obtained for BSA standards from which the concentration of the unknown sesame sample was determined. According to it, the extract yielded gave a protein concentration of 7.3 mg/mL.

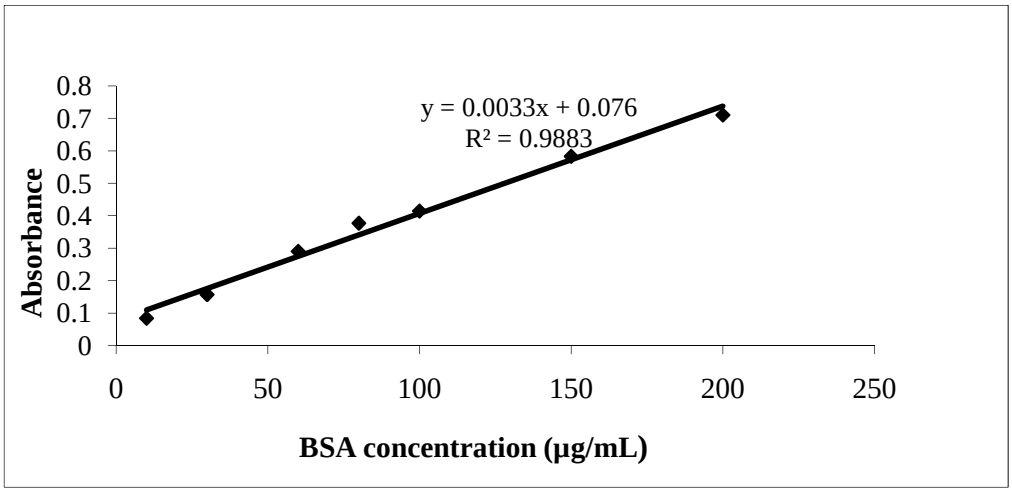

Figure 6: Calibration curve for BSA standards

4.1.2 SDS-PAGE

A SDS-PAGE was carried out for the obtained sesame extract so as to determine the molecular weight of the proteins present in the extract. The experimental details are given in section 5.1.3 and figure 7 represents the results obtained for this experiment.

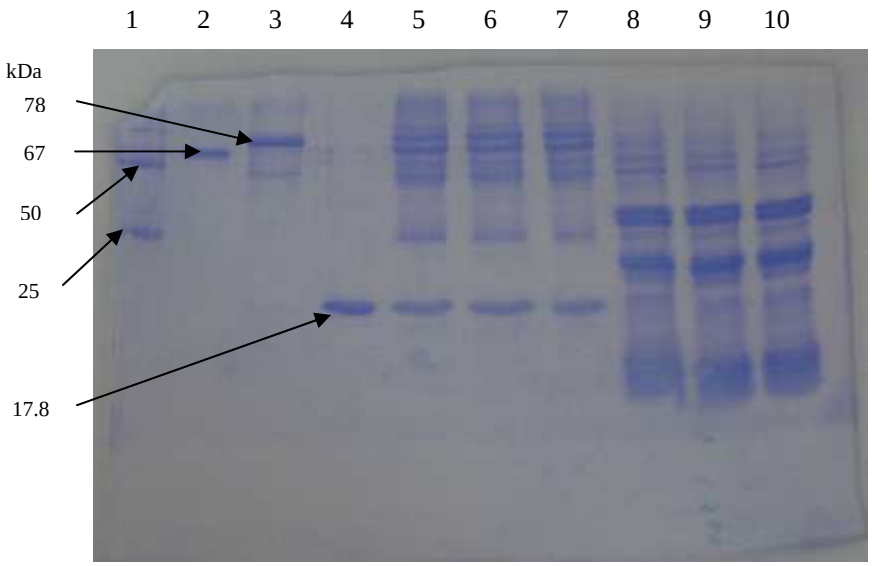

Figure 7: SDS-PAGE of the sesame extract

1: Immunoglobulin G, 2: BSA, 3: Conalbumin, 4: Myoglobin 5, 6 & 7 Mix containing immunoglobulin G, conalbumin, ovalbumin, BSA and myoglobin, 8, 9 & 10: Sesame extract

The bands on the gel indicate that the sesame extract contained proteins of approximately 22, 39 and 48 kDa.

4.1.3 Production, isolation and titer determination of anti-sesame antibodies

Two hens were immunized once each month with a fraction of the defatted sesame extract in a concentration of 1 mg/mL in PBS, the details can be found in section 5.1.4. The eggs obtained from these hens were stored at 4 °C. Titer determination was carried out with the yolks of eggs laid seven days after each immunization. For titer determination, the microtiter plates were coated with sesame extract and dilutions of the egg yolks were incubated for 30 min. IgY bound to sesame protein was detected with the commercially available rabbit antibody raised against IgY, labeled with horseradish peroxidase. The details are given in section 5.1.6. The titer was determined regularly, and the results in figure 8 illustrate the absorbance obtained from egg yolks prior to immunization or pre-immunization as well as from all five post immunization yolks.

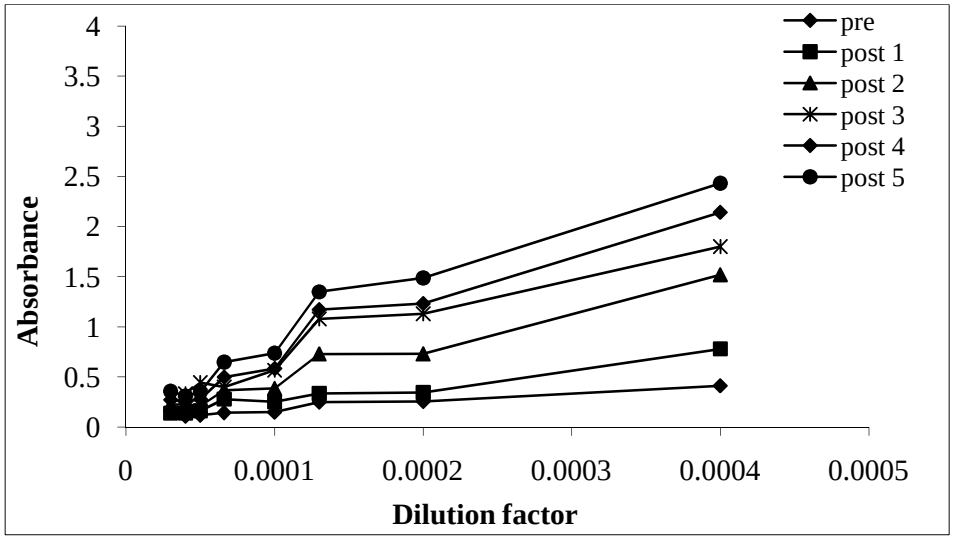

Figure 8: Results of the titer determination

Regular titer determination indicated that the egg yolks of both hens gave a greater titer after each immunization in comparison to the previous one. However, one of the hens stopped laying eggs after the third immunization and hence ELISA was developed with the antibodies produced by the hen that continued laying eggs.

The highest titer was obtained from the egg yolks produced after the fifth immunization. From the eggs obtained a week after the immunization, the yolks were separated from the egg whites; an equal volume of PBS buffer was added, the mixture was shaken and then centrifuged. The supernatant was filtered and used for isolating antibodies by the ammonium sulphate/caprylic acid precipitation method; the details are given in section 5.1.5. The isolated antibodies were then used for ELISA. The concentration of the isolated antibodies was determined by the Bradford assay using immunoglobulin G for standard solutions and the concentration obtained for the isolated antibodies was 29 mg/mL.

4.1.4 Development and optimization of the ELISA

In the development of the ELISA, the following parameters were optimized: concentration of the coating antigen, coating temperature, blocking time, concentration of the primary antibody and incubation time of the antigen and the primary antibody. The details of optimization experiments are given in section 5.1.10. Figure 9 represents the results for the optimization of the primary antibody concentration.

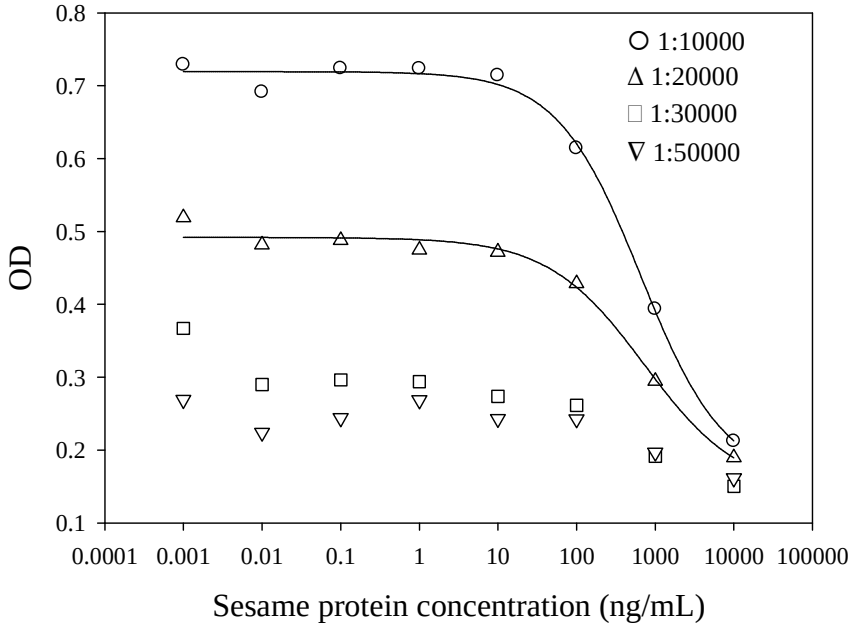

Figure 9: Influence of the primary antibody (anti-sesame IgY) concentration on the calibration curve

Initially, the concentration of the primary antibody was optimized by diluting the primary antibody solution from 1:10000 to 1:50000, (the concentration of the undiluted antibody

solution was 29 mg/mL). Dilution factors of 1:10000 and 1:20000 resulted in slopes of 0.8179 mL/ng and 0.7110 mL/ng, respectively. Figure 9 indicates that a dilution of 1:10000 resulted in the calibration curve with the highest slope; therefore, this dilution was applied in all further experiments.

Incubation time of the primary antibody was varied from 15 min to 1 h, the optimum time was found to be 45 min.

To optimize the coating antigen concentration and the coating temperature, two microtiter plates were coated with four different coating antigen concentrations, one plate at 36 °C and the other one at 4 °C. The influence of the coating antigen concentration on the calibration curve is shown in Figure 10. The four dilutions used for the coating antigen were 1:6000, 1: 12000, 1:18000 and 1:24000. The corresponding concentrations for these dilutions were 1.22 µg/mL for 1:6000, 0.61 µg/mL for 1:12000, 0.41 µg/mL for 1:18000 and 0.31 µg/mL for 1:24000.

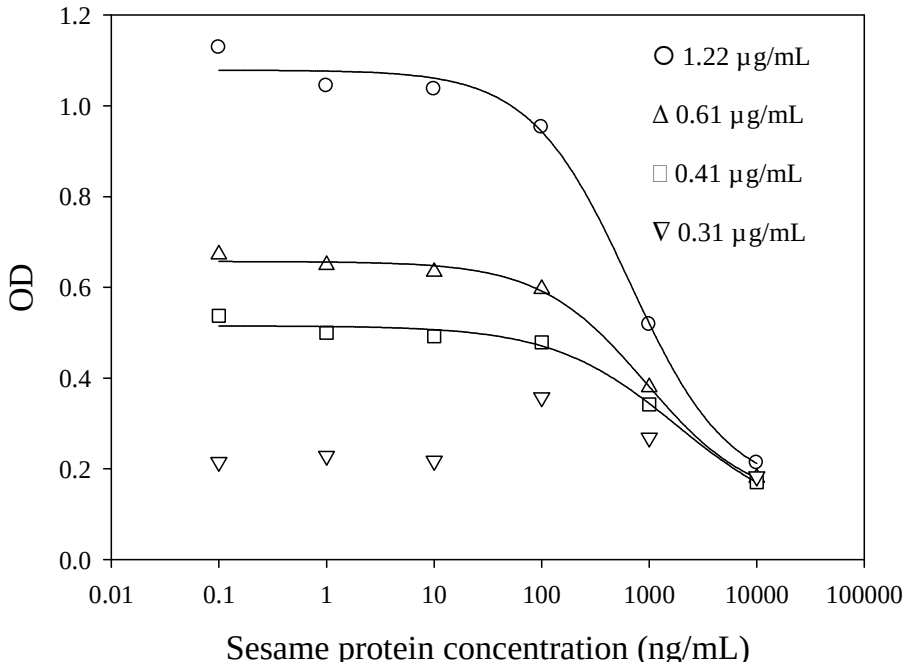

Figure 10: Influence of the coating antigen concentration on the calibration curve

The coating antigen concentrations of 1.22 µg/mL, 0.61 µg/mL and 0.41 µg/mL yielded calibration curves with slopes of 0.9424 mL/ng, 0.8848 mL/ng and 0.7561 mL/ng, respectively, and a coating antigen concentration of 1.22 µg/mL yielded the steepest calibration curve. In contrast to the coating antigen concentration, the coating temperature did not have any influence on the calibration curve. In all further experiments coating was carried out at 4 °C.

To optimize the blocking time, a microtiter plate was divided into four parts and the blocking time varied from 15 min to 1 h. The results are illustrated in figure 11. Since the obtained slopes of the calibration curves were rather similar, in all further experiments a blocking period of 15 min was applied.

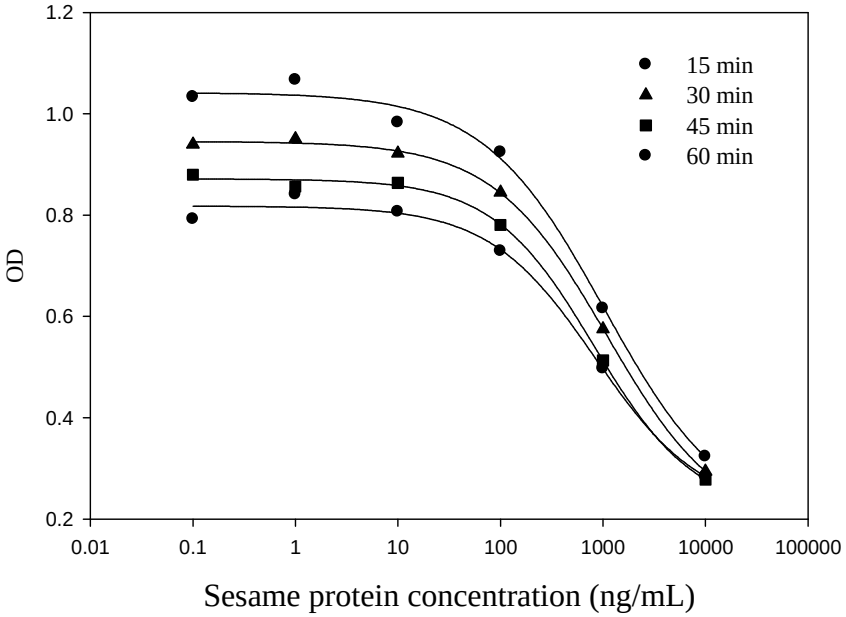

Figure 11: Influence of the blocking time on the calibration curve

The optimized conditions that were used for future ELISA experiments included coating at 4 °C with 1.22 µg/mL of the coating antigen solution, blocking with 2% (w/v) casein solution for 15 min and incubation for 45 min with a 1:10000 dilution of the primary antibody.

4.1.5 Cross reactivity of the ELISA

The specificity of the ELISA is an important aspect in terms of its applicability to real life samples. Therefore several tests were conducted to determine if the developed method showed any cross reactivity with other food stuffs. In order to determine the cross reactivity of the ELISA, the protein fraction of food ingredients commonly found in sesame containing food stuffs was extracted. The food ingredients and the protein concentrations of the extracts are listed in Table 1. The extracts were diluted with PBS to obtain protein concentrations between 10 ng/mL and 1 mg/mL and subjected to analysis by the ELISA. Figure 12 is a representative figure of the results obtained when there was no cross reactivity.

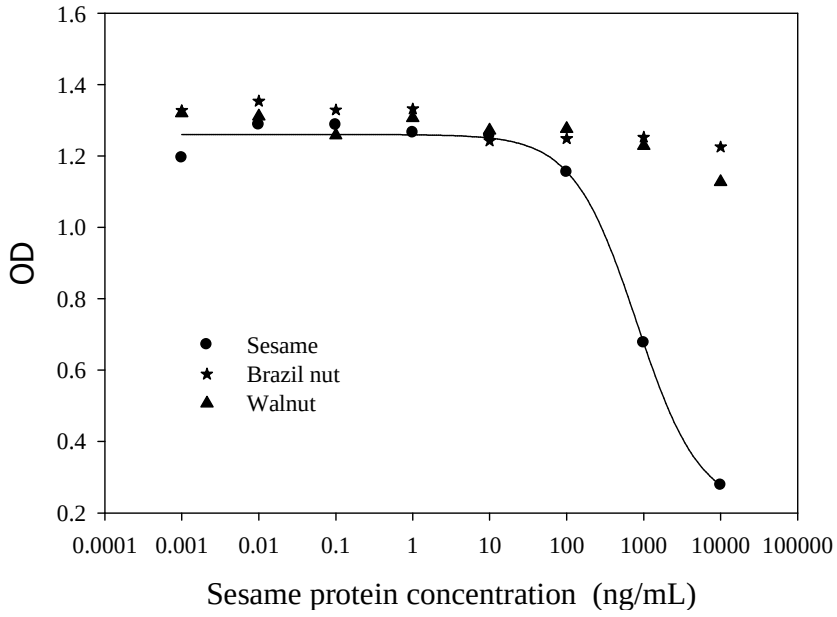

Figure 12: Cross reactivity test for Brazil nut and walnut

Table 1 summarizes the results for cross reactivity tests and indicates that the ELISA did not show any cross reactivity with 12 from the 13 food ingredients tested. Only for chocolate a low cross reactivity of 0.7% was observed.

Table 1: Cross reactivity of selected foods and food ingredients in the sesame ELISA

Food/ingredient	Protein concentration of the extract (mg/mL)	Cross reactivity (%)
Peanut	12.3	n.d.
Hazelnut	13.4	n.d.
Walnut	2.5	n.d.
Brazil nut	48.5	n.d.
Almond	51.9	n.d.
Sunflower seed	16.6	n.d.
Poppy seed	18.0	n.d.
Rice	2.0	n.d.
Wheat	13.1	n.d.
Rye	17.0	n.d.
Oat	14.4	n.d.
Chocolate	9.2	0.7
Honey	0.05	n.d.

n.d. below the limit of detection (LOD)

4.1.6 Matrix influence

In order to test for any matrix influences, the ELISA was carried out with sesame protein standard solutions which had been prepared by three different methods. In one case, the standard solutions were prepared by diluting the sesame extract with PBS. In the next case, the sesame extract was diluted with the extract of blank crackers (diluted 1:20 with PBS) to obtain the highest concentrated standard solution and then serially diluted with PBS to obtain standard solutions of lower concentrations. And in the last case, all standard solutions were prepared by diluting the sesame extract with the extract of blank crackers (diluted 1:20 with PBS). The standard curves obtained with the different sesame protein standard solutions are shown in figure 13.

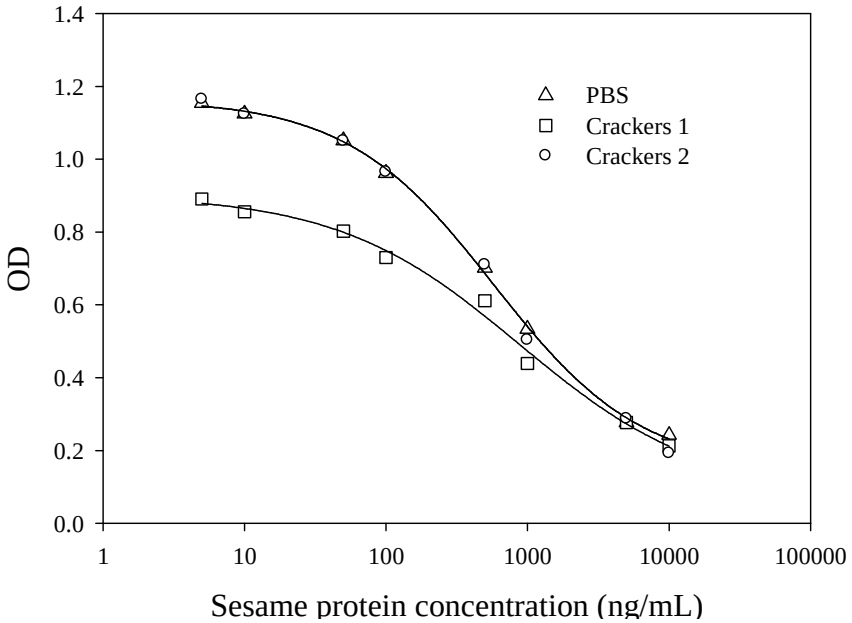

Figure 13: Influence of the matrix on the calibration curve I

PBS: sesame protein standards prepared with PBS; Crackers 1: sesame protein standards prepared with a 1:20 PBS diluted cracker extract; Crackers 2: highest sesame protein standars prepared with cracker matrix and dilutions made with PBS

The curve labeled as “PBS” represents the case where sesame protein standards were prepared in PBS. The curve labeled as “crackers 1” represents the case where sesame protein standards were prepared with the extract of blank crackers, which had been 1:20 diluted with PBS. The last case labeled as “crackers 2” is where the highest concentrated standard solution was prepared by diluting the sesame extract with the extract of blank crackers, which had been 1:20 diluted with PBS, further dilutions were prepared by dilution with PBS.
Preparing the standard solutions by diluting the sesame extract with the extract of blank crackers (1:20 diluted with PBS) yielded a calibration curve which differed from the other two calibration curves, indicating a matrix effect.
In order to check the influence of other food matrices a series of experiments was carried out the results of which are represented by figure 14.

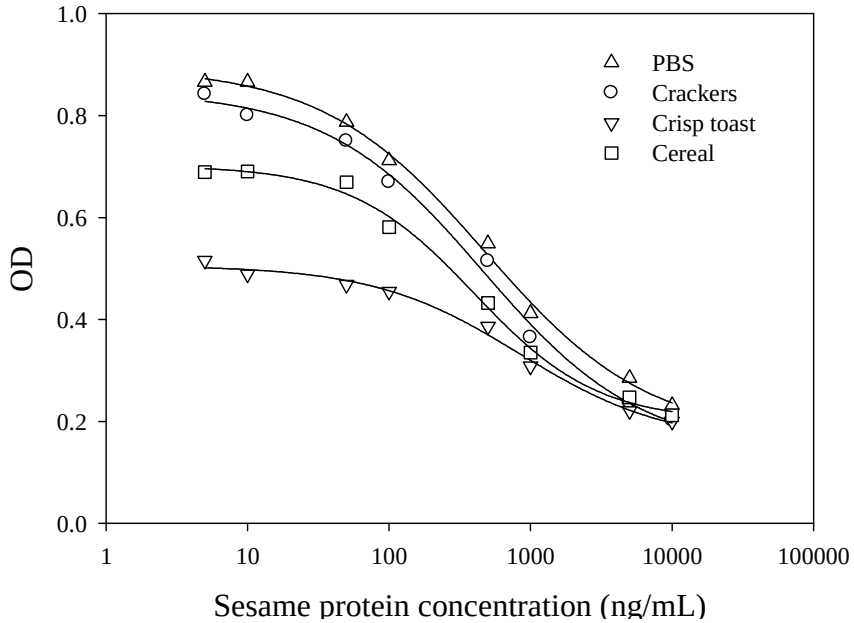

Figure 14: Influence of the matrix on the calibration curve II

In this case, sesame extract was diluted with extracts of various blank food matrices. Figure 14 represents these results.

Sesame protein standard solutions were prepared either in PBS, labeled as PBS, the extract of blank crackers, the extract of blank cereals, or the extract of blank crisp toast. All food matrices had previously been diluted 1:20 with PBS. The figure shows that the food matrices influenced the calibration curve to a different degree. Among the matrices tested, crisp toast showed the biggest influence.

In order to take these matrix effects into account a matrix similar to the samples to be analyzed had to be chosen for future experiments. The blank cracker extract was used in the analysis of all kinds of crackers and cookies, the crisp toast for all kinds of crisp toasts and the cereal extract when analyzing cereals, mueslis and muesli bar snacks.

4.1.7 Recovery studies - accuracy and precision

The accuracy and precision of the ELISA were determined in recovery studies performed by spiking blank food matrices with sesame protein. The blank food matrices were the ones in which according to previous ELISA analysis, sesame could not be detected.

In one series of experiments, the protein fraction of commercially available cereal and snack was extracted, followed by adding sesame protein extract to achieve sesame protein concentrations from 25 µg/g to 200 µg/g. Mean recovery of sesame protein in cereal and snack ranged from 99% to 114% and 82% to 99%, respectively, as indicated in table 2.

Table 2: Recovery of sesame protein in commercially available blank food stuffs spiked after extraction

Sample	**Spiking concentration (µg/g)**	**Recovery (%)**		**Mean recovery (%)**
		Day 1	**Day 2**	
Cereal	25	123	106	114
	50	99	100	99
	100	112	98	105
	200	121	91	106
Snack	25	83	81	82
	50	98	85	91
	100	87	98	92
	200	106	92	99

Table 3 summarizes the recoveries obtained by the analysis of different blank food matrices which had been spiked with sesame protein prior to extraction. The extracts of the spiked food stuffs were analyzed on either three or four subsequent days. In general, the recovery of sesame protein was in the range from 85% to 120%, independent of the spiking level, indicating the high accuracy of the ELISA. However, in the case of the multi grain crisp toast, too high recoveries (117%-160%) and in the case of the whole grain bread, too low recoveries (70%-85%) were obtained. Relative standard deviation ranged from 3% to 33%, indicating a high interday repeatability of the ELISA.

Table 3: Recovery of sesame protein in commercially available blank food stuffs spiked prior to extraction

Sample	Spiking concentration (µg/g)	Recovery (%)				Mean recovery (%)	Interday relative standard deviation (%)
		Day 1	Day 2	Day 3	Day 4		
Crisp toast 1	25	87	105	96	91	95	8
	50	102	99	108	104	103	3
	100	91	97	106	110	101	8
	200	98	94	96	81	92	8
Crisp toast 2	25	78	91	89	-	86	8
	50	88	119	97	-	101	16
	100	93	102	100	-	98	5
	200	101	139	105	-	115	18
Multi grain crisp toast	25	186	180	146	128	160	17
	50	127	141	137	134	134	4
	100	98	148	134	145	131	17
	200	77	93	151	150	117	32
Snack	25	85	115	105	93	99	13
	50	85	110	97	112	101	12
	100	108	123	135	138	126	10
	200	72	72	76	85	76	8
Roll	25	80	115	86	98	94	16
	50	96	118	77	115	101	19
	100	95	116	78	117	101	19
	200	88	102	75	109	93	16
Whole grain bread	25	77	57	76	-	70	16
	50	78	70	99	-	82	18
	100	67	76	114	-	85	29
	200	51	68	99	-	73	33

(-): Not determined

4.1.8 Limit of detection (LOD) and limit of quantification (LOQ)

In order to determine the limit of detection (LOD, S/N=3) and the limit of quantification (LOQ, S/N=10) of the ELISA, the extracts of blank food matrices (diluted 1:20 with PBS) were filled in six wells of a microtiter plate and subjected to ELISA analysis. The LOD of the ELISA was calculated by subtracting three times the standard deviation of the obtained absorbance from the mean absorbance and calculating the corresponding concentration by using the equation of the calibration curve established with sesame protein standard solutions. The LOQ was calculated in the same way but by subtracting ten times the standard deviation of the absorbance.

In the case of diluting the sample extract 1:20, in crisp bread, cracker, cereals and snacks the LOD and LOQ were found to be 5 and 30 µg sesame protein/g food. In fresh breads and buns, the LOD and LOQ were found to be 11 and 49 µg sesame protein/g food. The concentration of sesame was determined taking the extraction method and the dilution factor into consideration. According to Poms et al.[2] the LOD of an analytical method suitable for the detection of allergenic foods should be between 1 and 100 ppm. The ELISA developed in the present study meets these requirements.

4.1.9 Applicability of the ELISA to commercial food samples

To demonstrate the applicability of the developed ELISA, 28 commercially available samples, with different declarations, such as “may contain sesame”, “contains sesame” or with no sesame declaration were analyzed. The results are summarized in Table 4. In 12 of the 13 sesame containing food samples, sesame could be detected. Sesame could not be detected in sesame oil. Only in 3 samples sesame protein could be quantified, for the other samples the OD values measured were outside the quantification region of the calibration curve. In 8 samples the sesame protein concentration was so high, that the resulting OD values did not differ from the NSB value although the sample extracts were diluted up to 1:500. In sunflower crisp toast, the sesame protein concentration was below the LOQ.

With the ELISA, sesame was not detected in food stuffs which did not have any information about containing sesame. Sesame was detected in one out of nine samples which as a precaution were labeled with “might contain sesame”.

Table 4: Analysis of commercial food samples

Sample	Declaration	Result (sesame protein concentration (µg/g))
Sesame snack 1	(+)	+ (5928)
Sesame snack 2	(+)	+ (2575)
Sesame snack 3	(+)	+ (3404)
Sesame balls 1	(+)	+[a]
Sesame balls 2	(+)	+[a]
Crisp flakes	(+)	+[a]
Sesame flakes	(+)	+[a]
Sesame cookies	(+)	+[a]
Sesame peanut cookies	(+)	+[a]
Sunflower crisp toast	(+)	< LOQ
Peanut cookies (packed with sesame cookies)	(+)	+[a]
Cashew nut cookies (packed with sesame cookies)	(+)	+[a]
Sesame oil	(+)	-
Whole grain crisp toast 1	(+/-)	-
Butter biscuits	(+/-)	-
Crackers	(+/-)	-
Muesli bar with lemon	(+/-)	-
Muesli bar	(+/-)	< LOQ
Nut muesli	(+/-)	-
Whole grain crisp toast with poppy and sunflower seeds	(+/-)	-
Muesli	(+/-)	-
Muesli bar with grapes	(+/-)	-
Cereal 1	(-)	-
Cereal 2	(-)	-
Flakes with fruits	(-)	-

Whole grain crisp toast 2	(-)	-
Crisp toast	(-)	-
Chocolate biscuits	(-)	-

Declaration: (+) sesame listed, (-) sesame not listed, (±) may contain sesame. Result: +[a] sesame was detected but could not be quantified (OD value ~ NSB), - below the LOD

4.1.10 Analysis of roasted sesame

To investigate the applicability of the ELISA to detect roasted sesame, white peeled sesame seeds were roasted for 10 min at four different temperatures: 100 °C, 150 °C, 200 °C or 250 °C. The roasted sesame was then subjected to extraction and the protein concentration in the extract was determined using the Bradford assay. The extracts were then diluted 1:20 with PBS and analyzed by the ELISA. Table 5 summarizes the protein concentration obtained by the Bradford assay and the sesame protein concentration determined by the ELISA.

Table 5: Analysis of roasted sesame

Roasting temperature (°C)	**Protein concentration of the extract (mg/mL)**	**Sesame protein concentration determined by the ELISA (µg/g)**
100	12.3	88.5
150	11.1	93.7
200	1.2	< LOQ
250	0.1	-

- Below the LOD

It can be seen that the protein concentration of the extracts drastically decreased with increasing roasting temperature. The protein concentration of the extracts obtained from sesame seeds roasted at 200 °C and 250 °C were only 1.2 and 0.1 mg/mL, respectively. Due to this low protein concentration, sesame roasted at 250 °C could not be detected with the ELISA.

4.2 Development and validation of a QCM immuno sensor for the detection of potentially allergenic sesame proteins

The aim of the second part of the thesis was to develop a quartz crystal microbalance immuno sensor to detect sesame proteins, the experimental details are provided in section 5.2. For the development, anti-sesame IgY antibodies were immobilized on the quartz surface as the sensitive layer which on contact with sesame in the environment of the cell forms the antibody-antigen complex to give a mass sensitive signal.

4.2.1 Measurement cell

AT-cut quartz crystals with a fundamental resonance frequency of 10 MHz were used and particular electrode geometry was generated via silk-screen printing of the gold paste with the help of a designed sieve. This was done on both sides of the quartz but the sizes of electrodes generated were different to reduce damping. The prepared quartz was mounted in the measurement cell which ensured a stable environment for carrying out measurements and was equipped with an inlet and outlet for the solution.

4.2.2 Antibody immobilization and formation of the antibody-antigen complex

For injecting a solution in the measurement cell, the solution inlet was used and the solution outlet was used to remove the solution from the measurement cell using a pipette. During the measurement only one face of the QCM came in contact with the sample solution and so the damping of the quartz was half to what the damping would be if quartz would be in contact with the liquid on both sides. After injecting the solution it took a few minutes to get the frequency signal stable.

The immobilization of the antibody was carried out by injecting the antibody solution in a concentration of 2 mg/mL into the flow cell through the solution inlet. Later an antigen solution comprising of a sesame protein extract in deionized water in a concentration of 1.5 mg/mL was injected, after removing the antibody solution, resulting in the formation of the antibody-antigen complex. Figure 15 illustrates these results.

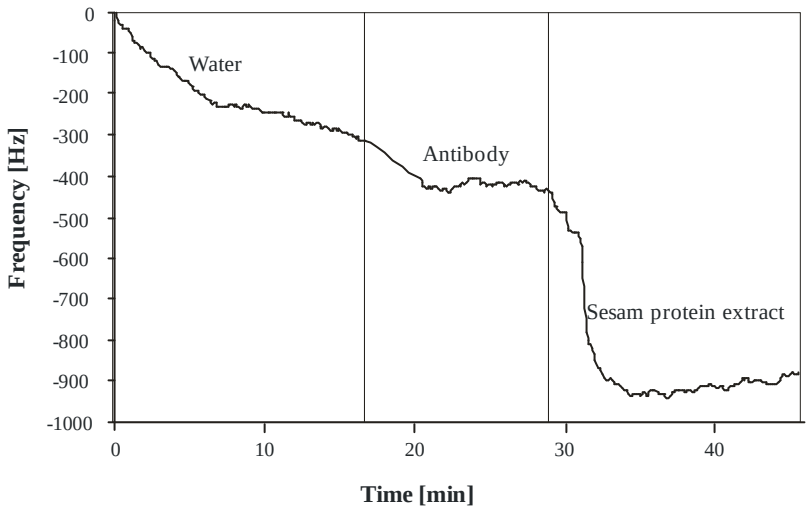

Figure 15: Sensor response to anti-sesame IgY antibody solution and the sesame extract (Antibody concentration: 2 mg/mL; sesame protein extract concentration: 1.5 mg/mL)

The change in signal after adding the antibody solution indicates that the antibodies were immobilized on the electrode surface. A change in frequency, a signal response after the addition of the sesame extract results from a mass change and indicates the formation of the anti-sesame IgY- sesame protein complex. The same experiment was also tried with injecting PBS as a first step and making dilutions with PBS instead of deionized water but since the result was more or less the same, water was used to minimize any interference with the measurements.

4.2.3 Dissociation of the antibody-antigen complex

After the formation of the antibody-antigen complex, the next significant step was to figure out a way to dissociate the bond in such a way that the antibody layer immobilized on the quartz is not disturbed. This was important as this was the only way in which the sensor could be proven to be reusable. The dissociation step was termed as elution and a 2 M guanidine hydrochloride solution was used for this purpose, the details of which are in section 5.2.6.2. In an experiment a 1 mg/mL sesame protein solution was injected, the signal response was measured and then elution was carried out using guanidine hydrochloride. Later sesame protein in the same concentration was added again to determine if the

immobilized antibody layer was intact after elution or not. The results for this experiment are illustrated by figure 16.

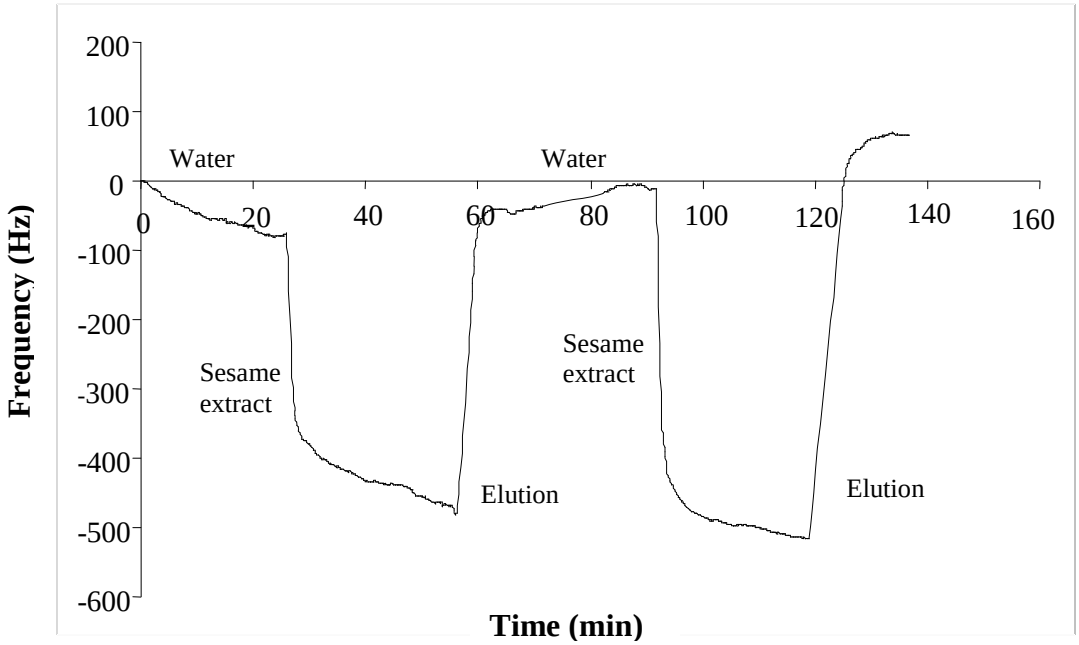

Figure 16: Sensor response to sesame protein extract (1 mg/mL) before and after elution with 2 M guanidine hydrochloride

It was clearly evident from the experiment that the elution with guanidine hydrochloride does not damage the immobilized antibody layer since the signal response is almost the same in the two instances. Therefore guanidine hydrochloride was then used in all experiments to dissociate the antibody-antigen complex. After the dissociation of the antibody-antigen complex by guanidine hydrochloride, the antibodies were renatured by injecting PBS in the measurement cell and allowing it to incubate for 5-7 min.

Next, it was determined if it was possible for the sensor to work for a lower concentration if a higher one was injected before. For this the sensor response was measured using three different concentrations starting with 1 mg/mL, then injecting sesame protein in a concentration of 250 µg/mL and lastly in 500 µg/mL. The results shown in figure 17 indicate that the sensor gave a greater signal response when a higher concentration of sesame protein

was injected. Upon elution the bond is broken and when a lower concentration is added the signal response corresponds to the concentration of sesame protein added.

This indicates that upon elution with guanidine hydrochloride only the antibody-antigen complex is dissociated, the sensor is able to detect the analyte upon further sample injections and the signal response is dependent on the amount of sesame protein injected.

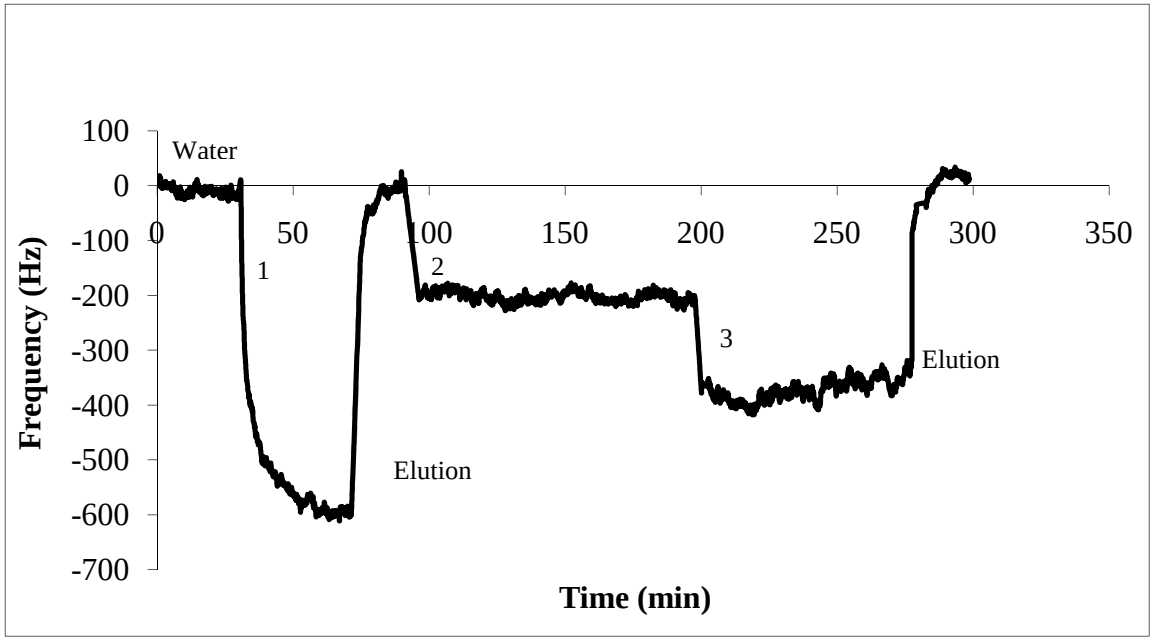

Figure 17: Influence of the sesame protein concentration on the sensor response

Sesame protein extracts: 1: 1 mg/mL, 2: 250 µg/mL, and 3: 500 µg/mL

Similar experiments were conducted again to demonstrate the suitability of the elution medium. A suitable elution medium is the one that particularly elutes the antigen without irreversibly denaturing the antibodies. Other elution media were also used; these included acidic media of pH of 5 and 6. The results obtained in those cases indicated that the antibody layer was denatured. To ensure that guanidine hydrochloride was an appropriate elution medium and that the sensor is reusable and works even if a higher concentration is added before adding a lower one a experiment was conducted in which a sesame protein extract was added in a concentration of 500 µg/mL, followed by injecting 2 mg/mL and then 500 µg/mL. The results are illustrated in figure 18.

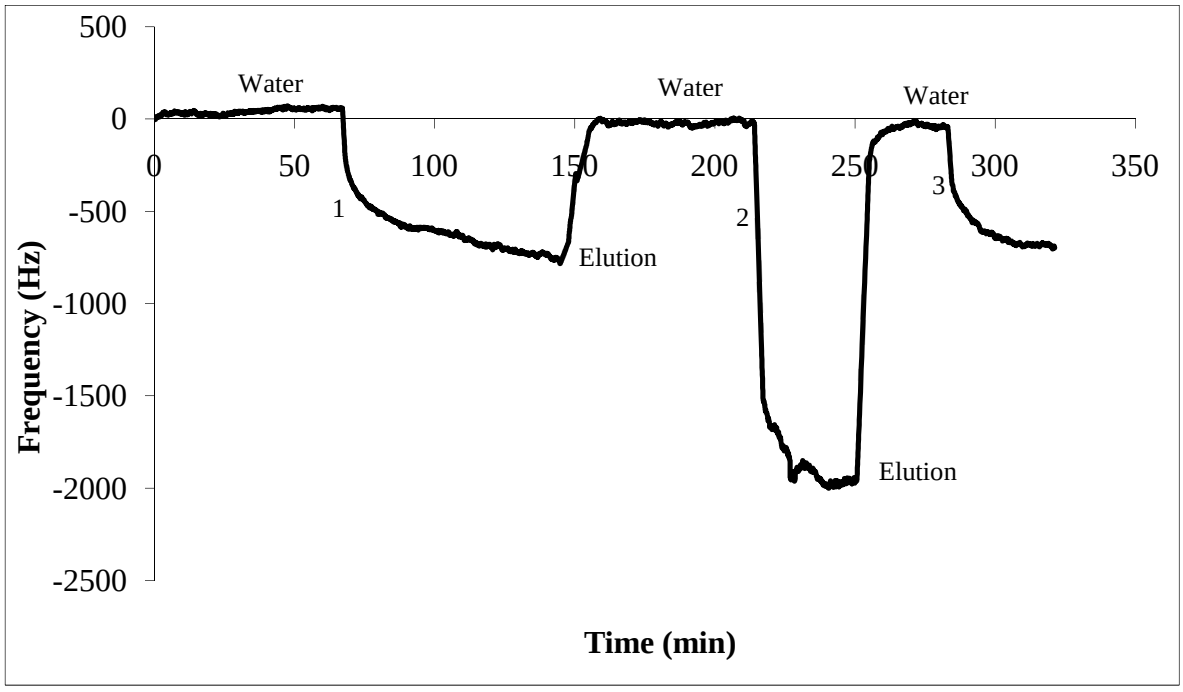

Figure 18: Sensor response illustrating the suitability of 2 M guanidine hydrochloride as the elution medium

Sesame protein extracts: 1: 500 µg/mL, 2: 2 mg/mL, 3: 500 µg/mL

It can be inferred from the figure that the signal response for the concentration of 500 µg/mL is the same even when it was injected after the concentration of 2 mg/mL and also that the signal response for 2 mg/mL is about four times higher than for 500 µg/mL.

4.2.4 Washing step

While dealing with complex food matrices, a washing step was incorporated after the sample injection but prior to the measurement to remove any non specifically bound matrix compounds. In the washing step, the injected sample extract was removed after an incubation of 5 min and deionized water was injected and the measurement was carried out. For an experiment incorporating the washing step, sesame extract in a concentration of 1 mg/mL was added, followed by injecting a concentration of 500 µg/mL and in the end, again a concentration of 500 µg/mL but in a complex food matrix comprising of whole grain bread.

After the injection of sesame protein in water and antibody-antigen complex formation, elution step was carried out, the antibodies were renatured and sesame protein extract in a concentration of 500 µg/mL was added. Again the elution was followed by renaturing of the

antibodies and finally sesame protein in whole grain bread was added, prior to carrying out the measurement, the washing step was incorporated. The results are depicted by figure 19.

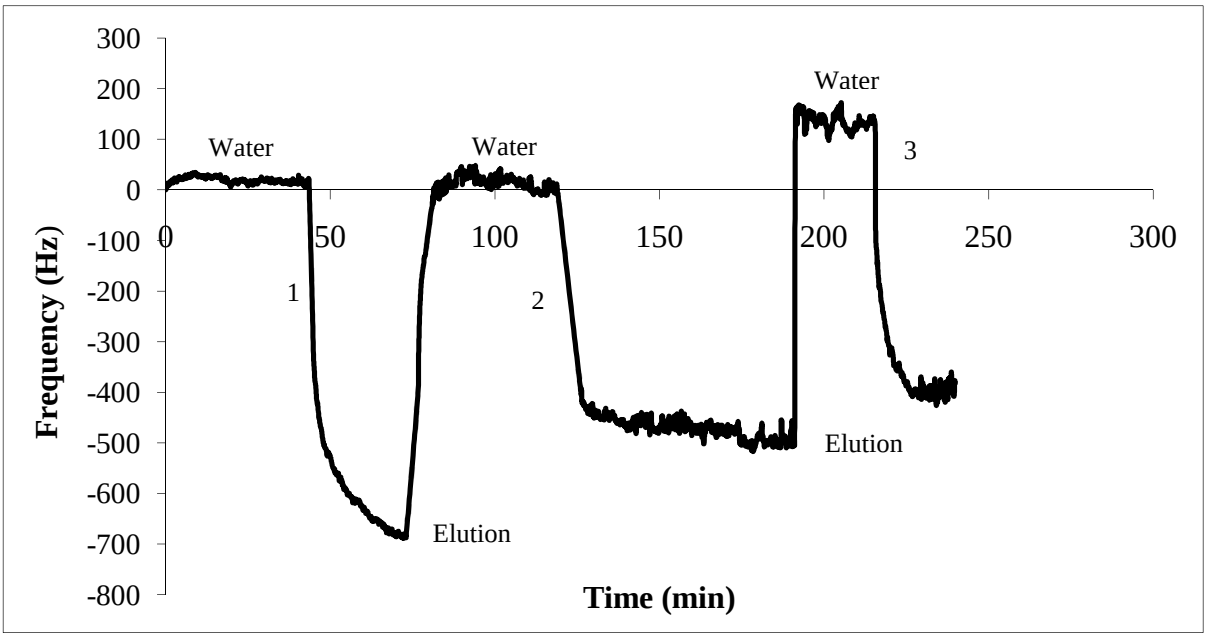

Figure 19: Measurements in a complex food matrix

Sesame protein extracts: 1: 1 mg/mL, 2: 500 µg/mL, 3: 500 µg/mL in whole grain bread matrix

The results depicted by figure 19 clearly indicate that there was a signal response upon injecting sesame extract in water indicating the formation of the antibody-antigen complex. The response is much higher for 1 mg/mL than the response for 500 µg/mL which is similar to the response of 500 µg/mL sesame protein in the whole grain bread matrix.

4.2.5 Cross reactivity tests

To determine the specificity of the developed sensor towards sesame, cross reactivity tests were carried out with 14 different food samples including various nuts, legumes and honey and chocolate. The food extracts used for this purpose were the same as those prepared for the ELISA setup. The details of extraction are given in section 5.1.7 and their concentration determination by Bradford assay is given in table 1. The results of cross reactivity tests are depicted in figures 20 to 24.

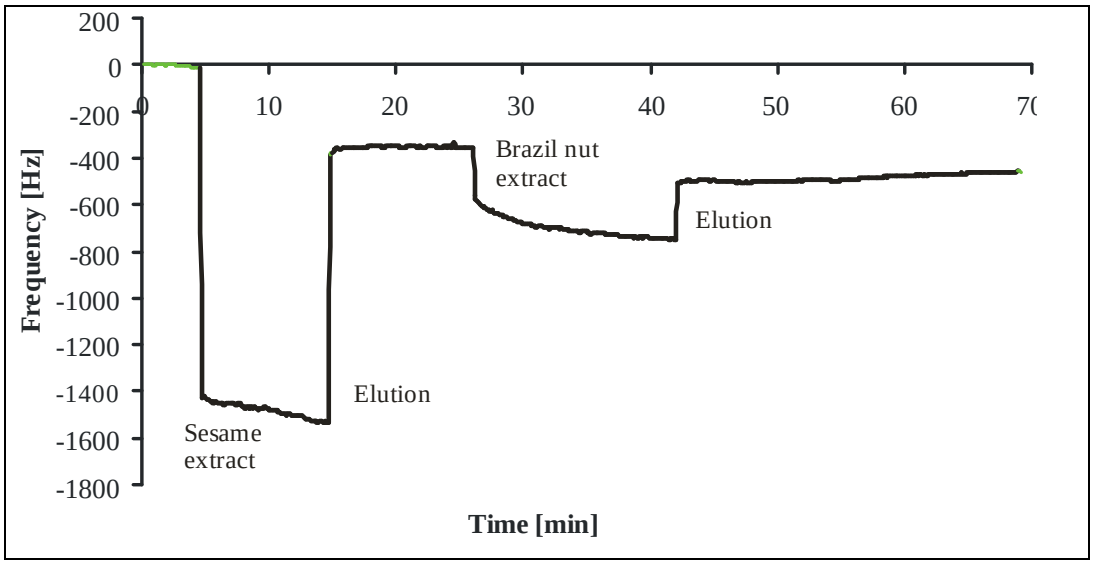

Figure 20: Cross reactivity test 1; sensor response to sesame and Brazil nut extract

Sesame protein extract: 3.5 mg/mL, Brazil nut protein extract: 3.5 mg/mL

Figure 20 represents the cross reactivity test done for Brazil nut. Sesame extract in a concentration of 3.5 mg/mL was injected and the sensor response was measured. The signal response indicates a change in mass which is a result of the formation of the antibody-antigen complex. The signal goes up after the elution step and goes down again slightly when 3.5 mg/mL of Brazil nut extract was added. A very high concentration of Brazil nut extract shows a relatively small signal response.

Next, chocolate and walnut extracts were injected and the cross reactivity was determined; figure 21 shows results for cross reactivity tests done for chocolate and walnut.

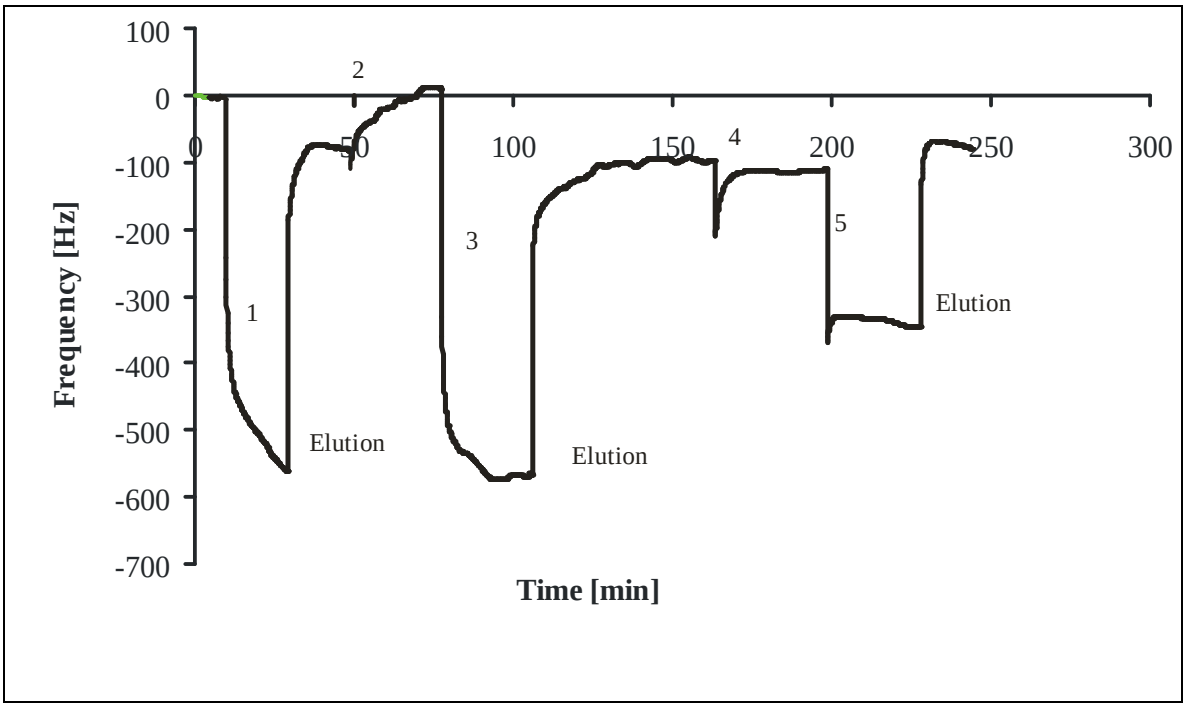

Figure 21: Cross reactivity test 2; sensor response to sesame, chocolate and walnut extracts

Sesame protein extract: (1), chocolate protein extract: (2), chocolate protein extract spiked with 0.88 mg/mL sesame protein: (3), walnut protein extract: (4), walnut extract spiked with 0.44 mg/mL sesame protein: (5)

Sesame extract when injected in a concentration of 0.88 mg/mL gave a sensor response, whereas there was no sensor response when measurements were carried out after injecting the chocolate and walnut extracts.

The sensor, however, gave a response when chocolate and walnut extracts spiked with sesame protein were injected in the measurement cell. It is also evident from the figure that the magnitude of the signal response also depends on the amount of sesame used to spike the extract. The signal response was almost half in the case of walnut spiked with 0.44 mg/mL sesame protein to that of chocolate extract spiked with 0.88 mg sesame protein per mL.

The next experiment was to test the cross reactivity of peanut, the results of which are represented by figure 22. Although there is a high noise in this particular case, even then it is evident that there is no response of the sensor towards the peanut extract alone. However, peanut spiked with sesame protein in a concentration of 1 mg/mL showed a signal response.

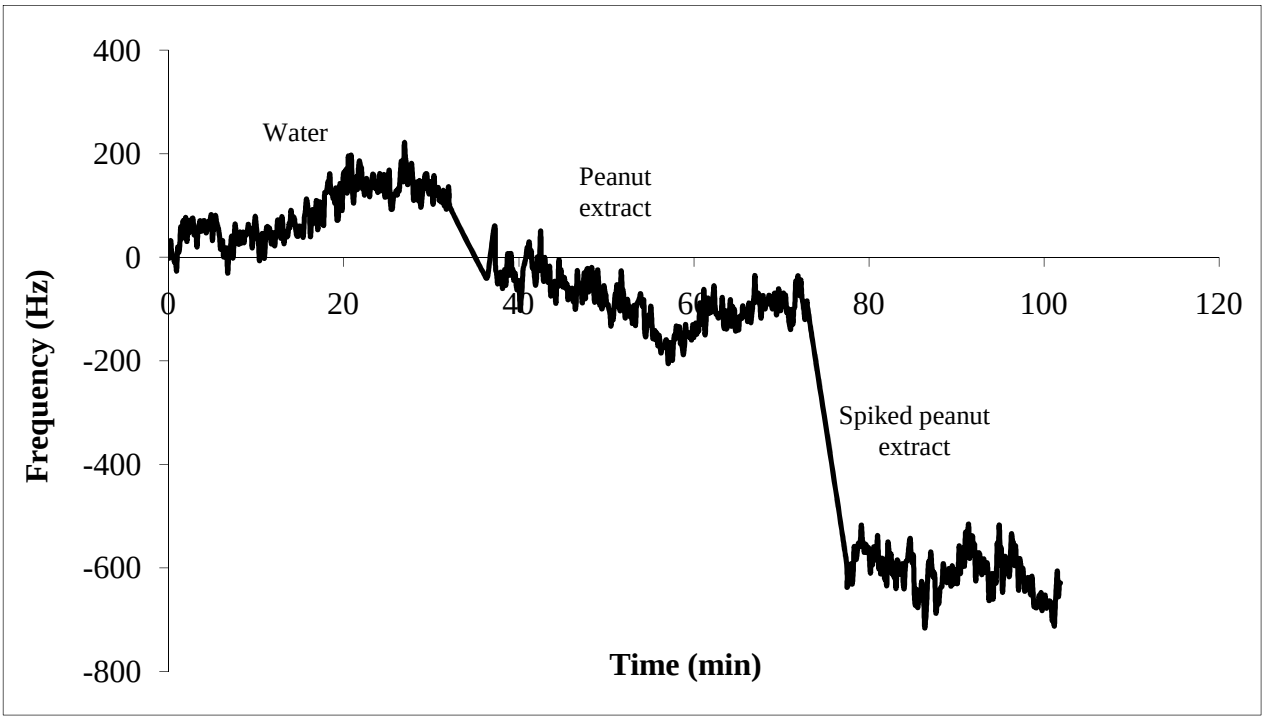

Figure 22: Cross reactivity test 3; sensor response to peanut and peanut extract spiked with 1 mg/mL sesame protein

Later, almond, wheat, sunflower seeds and honey were tested for cross reactivity. The results are shown in figure 23. From the curve it is obvious that there is no response at all for wheat, sunflower and honey. For almond extract, however, the sensor showed a small change in the frequency. That is why almond extract was analyzed again to be sure of its cross reactivity. In addition, all samples were spiked with sesame extract and analyzed to ensure that there are no false negative signals and that the sensor is in a working condition. For all spiked extracts the sensor gave a response.

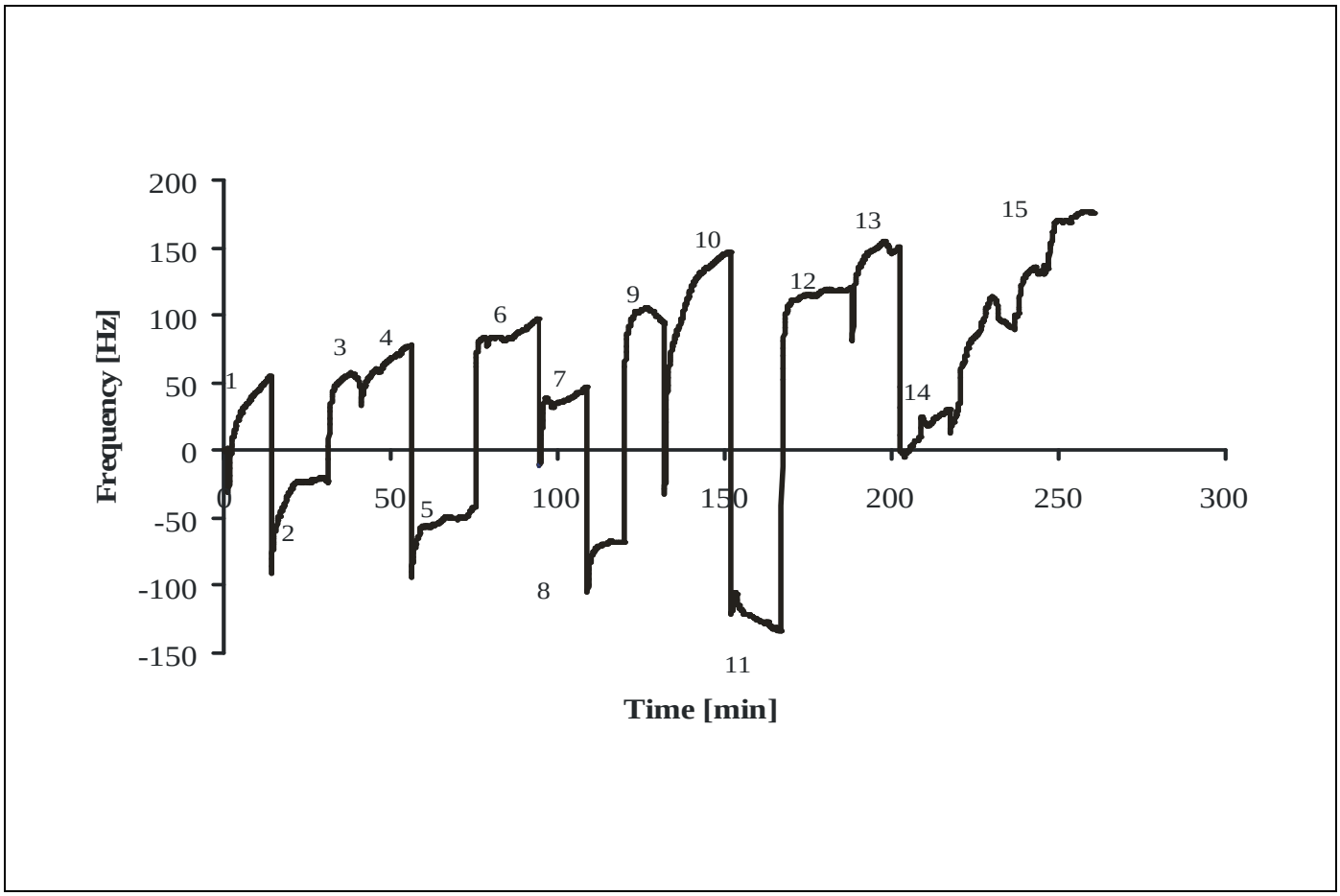

Figure 23: Cross reactivity test 4; sensor response to sesame, wheat, almond, sunflower seeds and honey extracts

Water: (1), (3), (6), (9), (12), (15); sesame extract 300 µg/mL (2); wheat extract (4); spiked wheat extract (5); almond extract (7); spiked almond extract (8); sunflower seed extract (10); spiked sunflower seed extract (11); honey extract, 50 µg/mL (13); spiked honey extract (14). All samples except honey were added in a concentration of 1 mg/mL and all samples were spiked with 300 µg/mL of sesame protein.

Later, six more samples were tested. These included extracts of poppy seeds, hazelnut, rice, oat, rye, soya bean and almond was also tested again. The results illustrated by figure 24 indicate that the sensor gave a sensor response for sesame and sample extracts spiked with sesame proteins. For all others including almond there was no response, indicating that none of these tested samples showed any cross reactivity.

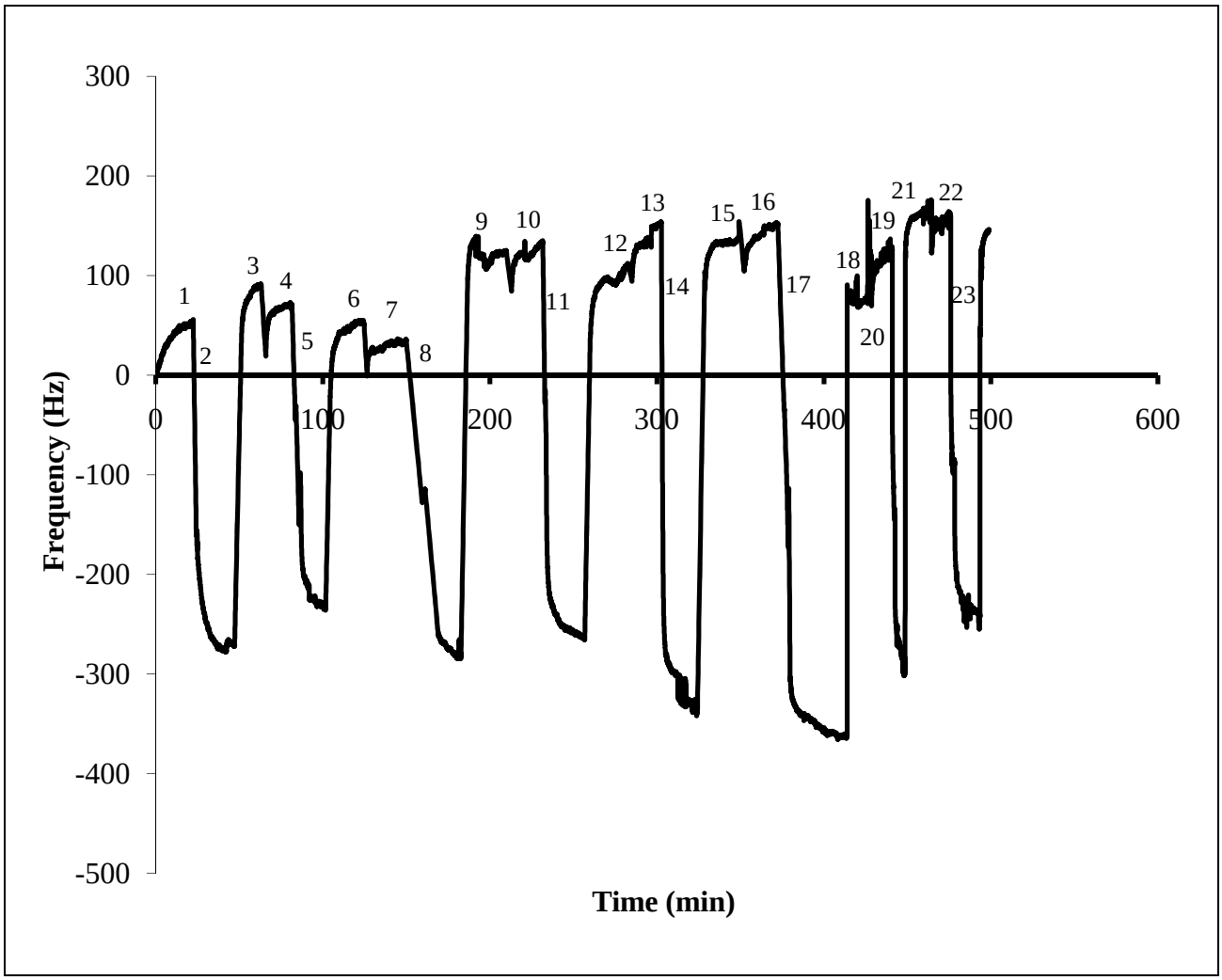

Figure 24: Cross reactivity test 5; sensor response to sesame, poppy seeds, hazelnut, rice, oat, rye, soya bean and almond extracts

Water: (1), (3), (6), (9), (12), (15), (18) & (21); sesame extract 400 µg/mL (2); poppy seed extract (4); spiked poppy seed extract (5); almond extract (7); spiked almond extract (8); hazelnut extract (10); spiked hazelnut extract (11); rice extract (13); spiked rice extract (14); oat extract (16); spiked oat extract (17); rye extract (19); spiked rye extract (20); soya been extract (22); spiked soya been extract (23). All samples were added in a concentration of 1 mg/mL and all samples were spiked with 400 µg/mL of sesame protein.

4.2.6 Analysis of roasted sesame

Next, it was investigated if the developed sensor allowed the detection of roasted sesame seeds. The roasting of sesame seeds is described in detail in section 5.1.7 and the protein extraction in 5.1.7. Figure 25 represents the results obtained when extracts of roasted sesame samples were injected in the measurement cell. The order of the injected samples was sesame extract, extract of sesame roasted at 100 °C, after that extract of sesame roasted at 150 °C, and then the extract of sesame roasted at 200 °C and at the end the extract of sesame roasted at 250 °C was injected.

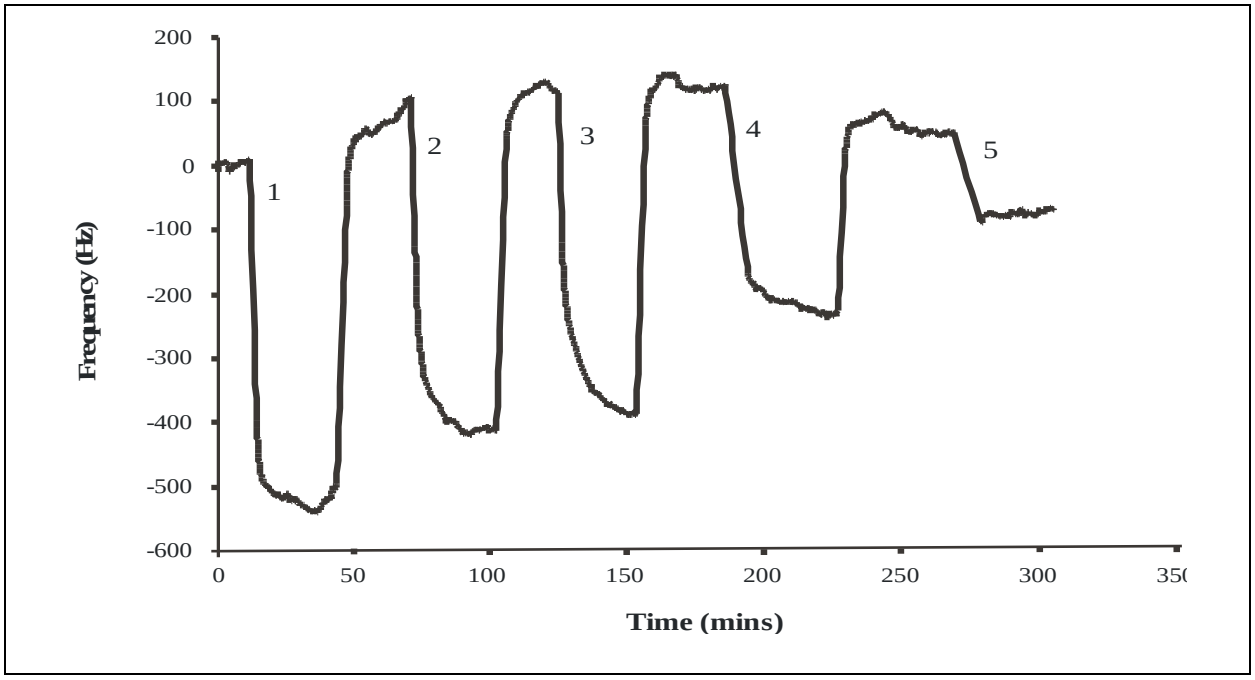

Figure 25: Sensor response to sesame protein extract obtained from sesame seeds roasted at different temperatures

Unroasted sesame: (1), sesame roasted at 100 °C: (2), sesame roasted at 150 °C: (3), sesame roasted at 200 °C: (4), sesame roasted at 250 °C: (5)

From the curve it can be deduced that with the increase in the roasting temperature the magnitude of the signal decreases accordingly. This is in accordance with the results obtained by Bradford assay for protein concentration determination of these roasted sesame samples

represented by table 5. A significant decrease in the signal magnitude is observed for the sesame roasted at 200 and 250 °C.

4.2.7 Validation of the sensor

The developed immuno sensor was validated by determining the reproducibility and repeatability of the sensor and its limit of detection.

4.2.7.1 Inter day repeatability

To check if the sensor would give same responses on different days, sesame extracts in a concentration of 1 mg/mL were injected twice on one day and twice on the other day. The results illustrated by figure 26 show that the signal response was the same in all four instances. This depicts the inter day repeatability of the sensor.

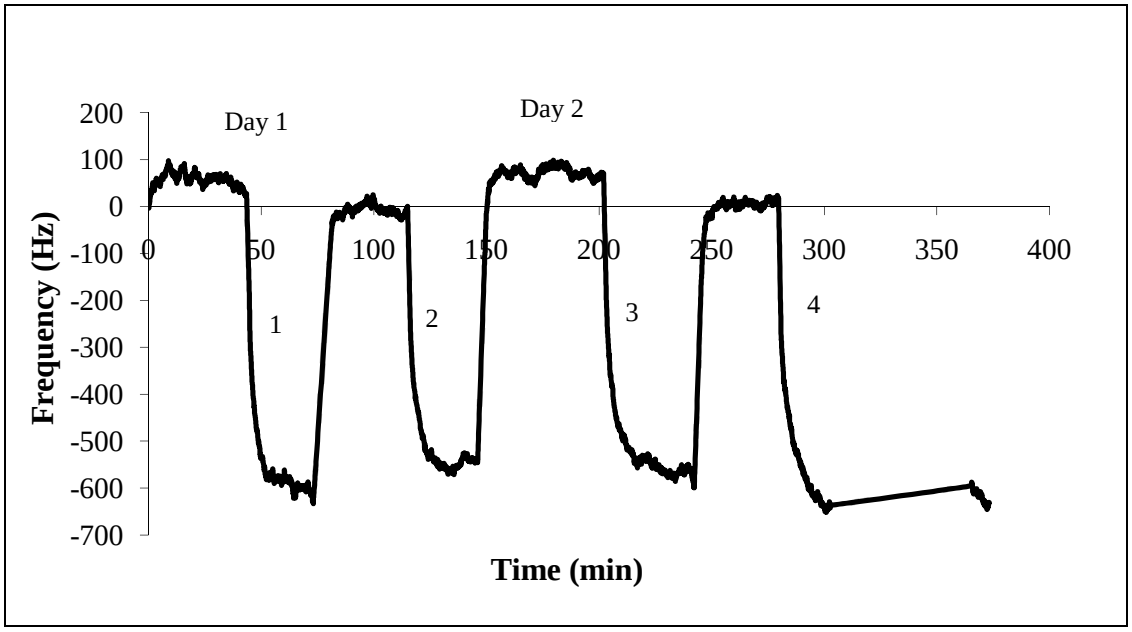

Figure 26: Inter day repeatability of the sensor

Sesame protein extract concentration: 1 mg/mL

4.2.7.2 Batch to batch reproducibility (inter assay reproducibility)

In the next experiment it was determined whether the same kind of sensor can be produced again or not. For that purpose sesame extract in the concentration of 500 µg/mL was added

and the sensor response was measured. The antibody-antigen complex was dissociated and in this case the antibody layer was also removed by incubating the antibody layer with guanidine hydrochloride for 40 min. PBS was injected in the measurement cell and it was stored at 4 °C over night. The next day a new sensor was prepared by immobilizing a new antibody layer and again a sesame protein extract was injected in a concentration of 500 µg/mL. This was done for 3 days and the results shown in figure 27 indicate that the response is reproducible.

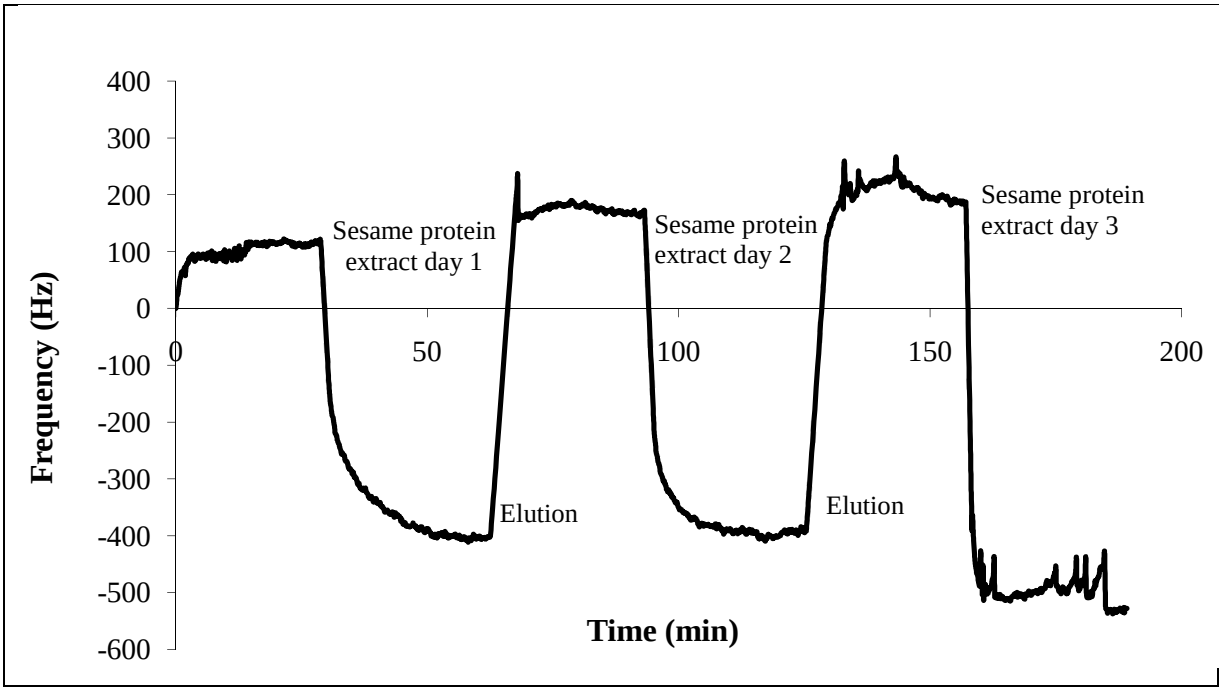

Figure 27: Batch to batch reproducibility of the sensor

Sesame protein extract concentration: 500 µg/mL

4.2.8 Limit of detection (LOD)

To determine the limit of detection (LOD) of the sensor, two experiments were carried out. In the first one, first of all the antibody layer was immobilized, washed away and immobilized again and then different sesame concentrations in water were injected in the measurement cell and the sensor response was measured. For this experiment, starting from a higher sesame concentration several lower concentrations were added one after the other and

the results are depicted in figure 28 and 29. For a sensor response to be detected the signal to noise ratio should be 3. Sesame could be detected in a concentration as low as 0.007 mg/mL which corresponds to 35 µg of sesame protein per gram food, taking into account the sample extraction protocol.

Since the antibody layer was immobilized twice this experiment also shows the batch to batch reproducibility of the method.

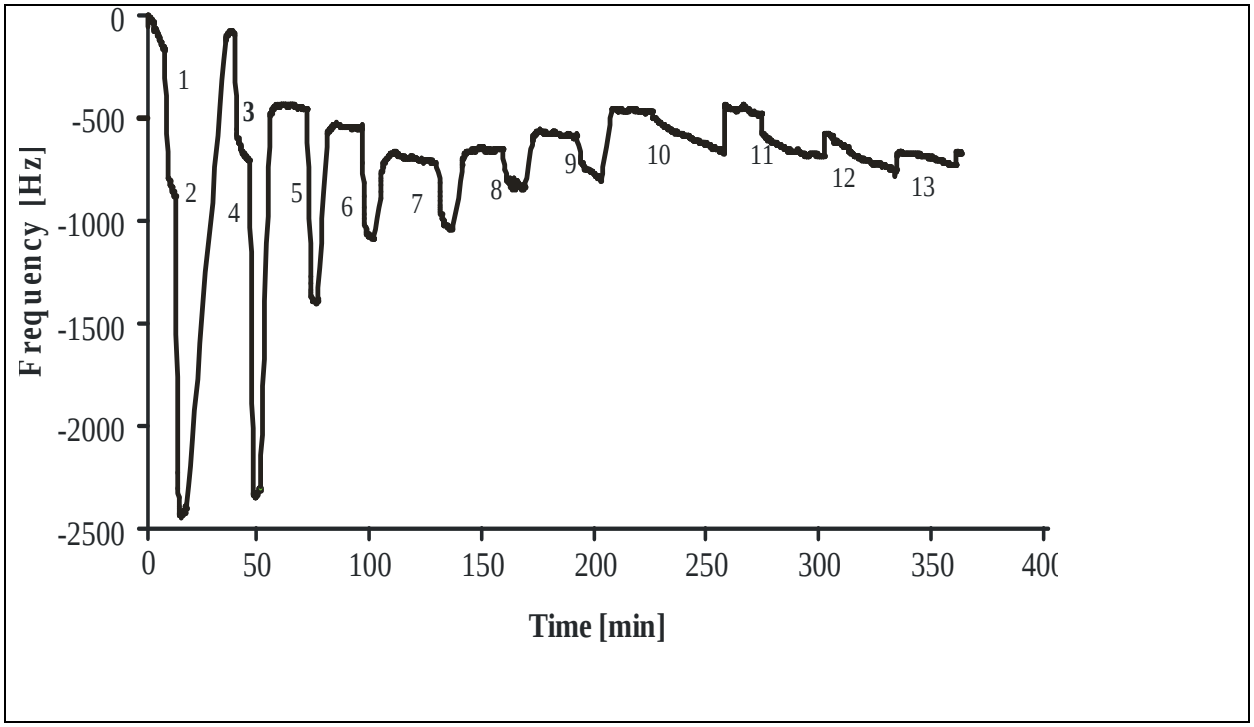

Figure 28: Sensor response to various sesame protein concentrations I

(1) & (3): Antibody immobilization, (2) & (4)-(13): sesame protein extract, (2): 3.5 mg/mL, (4): 3.5 mg/mL, (5): 1.75 mg/mL, (6): 0.875 mg/mL, (7): 0.438 mg/mL, (8): 0.219 mg/mL, (9): 0.11 mg/mL, (10): 0.055 mg/mL, (11): 0.027 mg/mL, (12): 0.014 mg/mL, (13): 0.007 mg/mL

Sesame protein concentration (mg/mL)	Effect (Hz)
3.5	1625
1.75	928
0.875	537
0.438	331
0.219	189
0.11	176
0.055	150
0.027	100
0.014	60
0.007	20

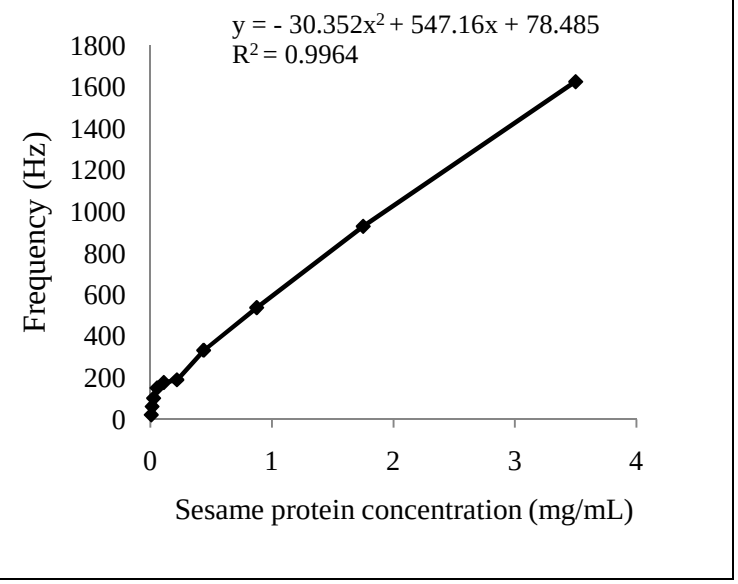

Figure 29: Sensor response to various sesame protein concentrations II

In order to determine the LOD in a food matrix in the next experiments different sesame concentrations in whole grain bread were analysed and the results are depicted by figure 30 and 31.

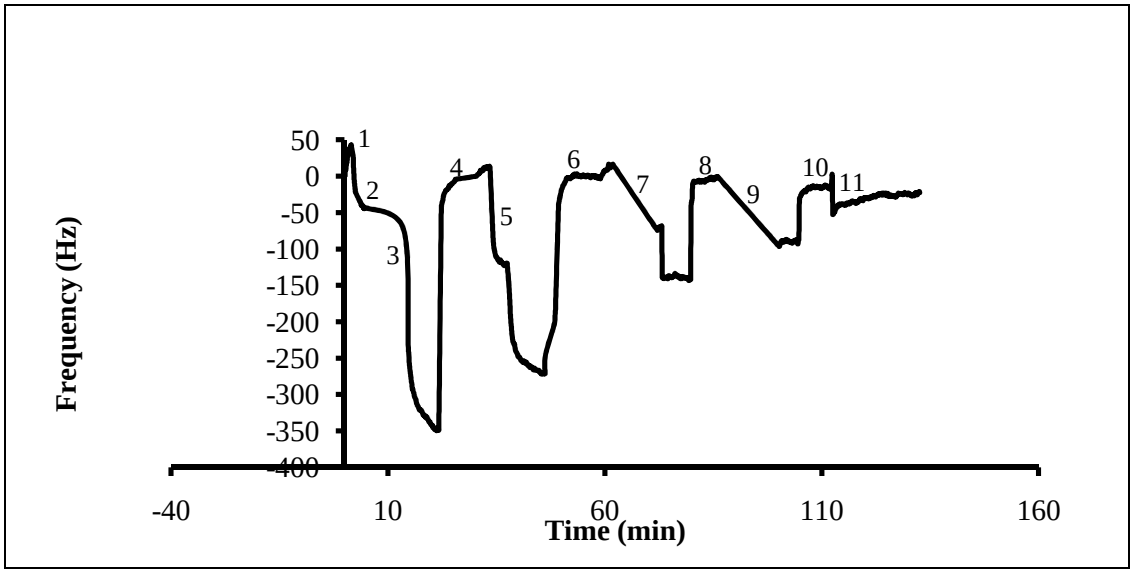

Figure 30: Sensor response to various sesame protein concentrations in a complex matrix (whole grain bread) I

Water: (1), whole grain bread extract: (2), whole grain bread spiked with 0.438 mg/mL sesame protein: (3), water: (4), whole grain bread spiked with 0.219 mg/mL sesame protein: (5), water: (6), whole grain bread spiked with 0.055 mg/mL sesame protein: (7), water: (8), whole grain bread spiked with 0.014 mg/mL sesame protein: (9), water: (10), whole grain bread spiked with 0.007 mg/mL sesame protein: (11)

Sesame protein concentration (mg/mL)	Effect (Hz)
0.438	300
0.219	150
0.055	70
0.014	38
0.007	15

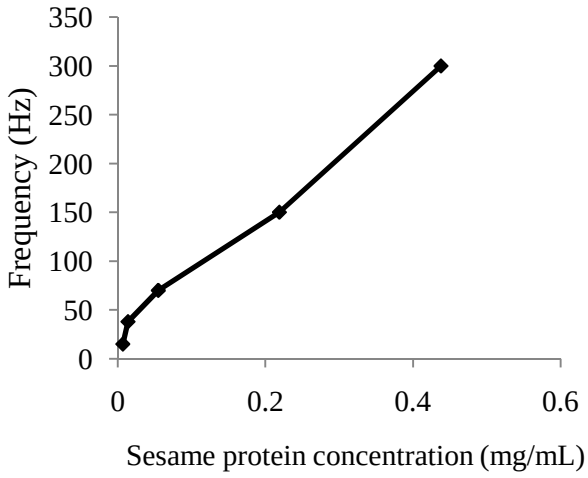

Figure 31: Sensor response to various sesame protein concentrations in a complex matrix (whole grain bread) II

In this case as well it was possible to detect sesame protein down to a concentration of 0.007 mg/mL which corresponds to 35 µg of sesame protein per gram food.

4.2.9 Analysis of commercial food samples

The applicability of the developed sensor was determined by analysing various commercially available food samples. For this purpose the samples chosen had three kinds of declaration on them; samples containing sesame and samples with no mention of sesame were tested along with samples labeled with “may contain sesame”. The results are illustrated in figures 32and 33 and also summarized in table 6.

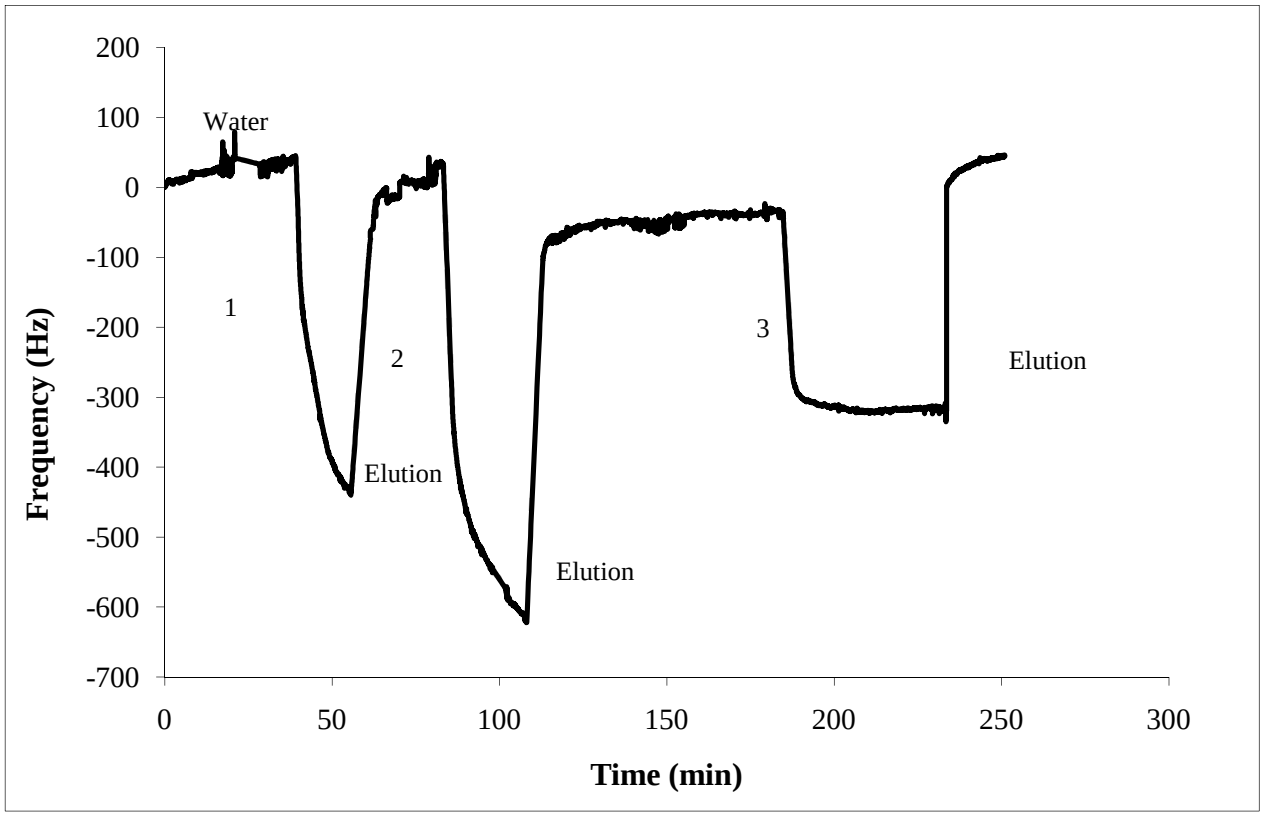

Figure 32: Sensor response to sesame containing commercial food samples

Extract of: sesame balls (1), sesame and peanut cookies (2), sesame and cashew nut cookies (3)

The figure represents the results for sesame containing food samples which included sesame balls, sesame and peanut cookies and sesame and cashew nut cookies. The sensor shows a response for all three.

For whole grain cookies, chocolate cookies and a muesli sample the results depicted in figure 33 indicate that the sensor response was below the LOD for all these samples.

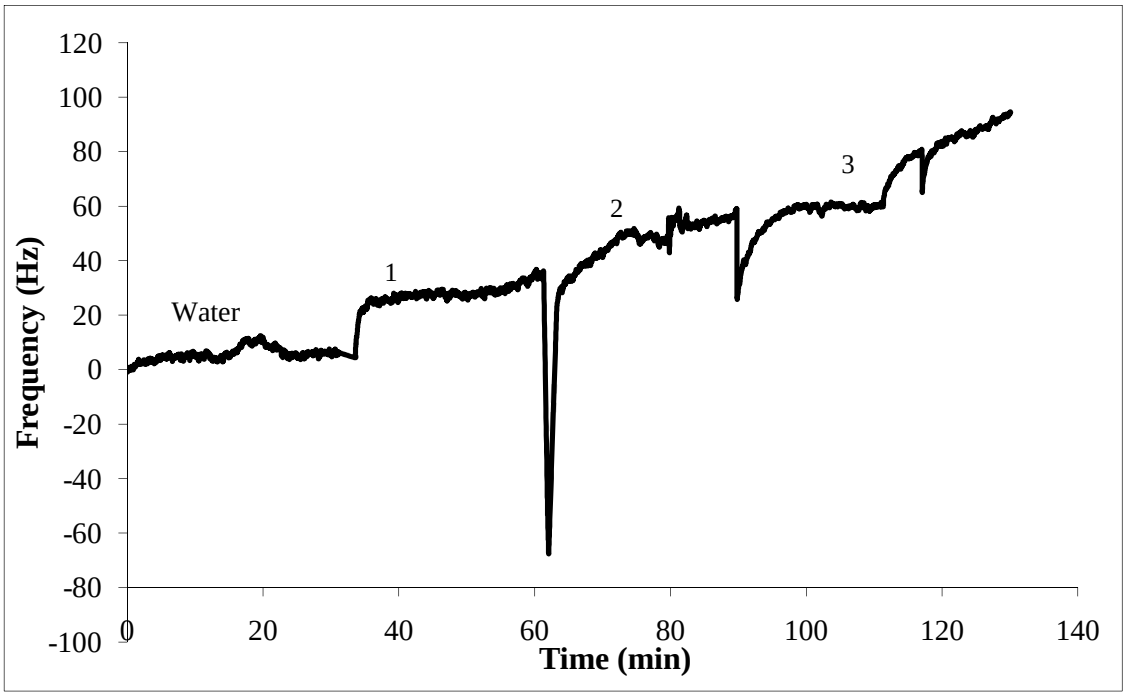

Figure 33: Sensor response to commercial food samples free of sesame

Extracts of: whole grain cookies (1), chocolate cookies (2), muesli (3)

The results for the analysis of commercial samples are summarized in table 6.

Table 6: Commercial samples: declaration and results

Sample	Declaration	Result
Sesame balls	(+)	(+)
Sesame and peanut cookies	(+)	(+)
Sesame and cashew nut cookies	(+)	(+)
Whole grain cookies	(+/-)	(-)
Chocolate cookies	(-)	(-)
Muesli	(-)	(-)

Result: (-) : < LOD

4.3 Development and validation of a real-time polymerase chain reaction (PCR) method for poppy (*Papaver somniferum*)

A real-time polymerase chain reaction method was developed for the detection of poppy DNA, the experimental details of which are given in section 5.3. Initially, DNA was extracted from poppy powder and poppy seeds. Secondly, three pairs of primers were designed and the cross reactivity tests were conducted. Then, for the primer pairs that did not show any cross reactivity with other food stuffs, probe was designed and the method was validated. Two kinds of assays were conducted, a SYBR green assay and a TaqMan assay. The following sections discuss the results obtained at various stages of method development and its validation.

4.3.1 DNA extraction of poppy

The DNA extraction was carried out from poppy seeds and also from poppy powder available commercially. Table 7 represents the results. DNA was extracted with the CTAB method as explained in section 5.3.2 and the concentration and purity of the extracted sample was determined as explained in section 5.3.2.1, the only difference being that shorter incubation times were used. After adding the CTAB extraction buffer the incubation time was 30 min and the addition of proteinase K was followed by an incubation of 1 hour in the oven at 65 °C. Each poppy sample was extracted thrice.

Table 7: Concentration and purity of the DNA extracts for poppy I

Sample	**Concentration** (ng/µL)	**Purity** (A_{260}/A_{280})
Poppy powder	149.7	1.97
	45.0	1.68
	42.5	1.77
Poppy seeds	39.5	2.02
	135.5	1.95
	153.7	1.94

To increase the yield and improve the purity the extraction method was modified. Out of all options tried the CTAB method with prolonged incubation times as explained in section 5.3.2 proved to be the best option. Table 8 represents the results obtained.

Table 8: Concentration and purity of the DNA extracts for poppy II

Sample	**Concentration** (ng/µL)	**Purity** (A_{260}/A_{280})
Poppy powder	187.00	2.12
	191.50	2.05
Poppy seeds	144.00	2.12
	204.00	2.10

A PCR experiment with these poppy DNA samples loaded as positive controls was carried out. The DNA obtained from poppy seeds was used instead of poppy powder since it gave lower C_t value.

4.3.2 Primer and probe design

Specific primers and probe were designed as explained in section 5.3.4. Table 9 illustrates the sequences for primer pairs and probes designed, their amplicon size and melting temperatures.

Table 9: Primer pair and TaqMan used

Primer / Probe	**Sequence 5´→ 3´**	**Tm (°C)**	**Amplicon size (bp)**	**Tm (°C)**
Primer pair 1: Accession number: AY217335				
Forward primer	TAGATTGGTTACTTCTGCATTGGC	58.3	118	71.9
Reverse primer	AGCTTTAGATATGGCTTTCACTGC	58.4		

TaqMan probe	TGGTTGATGTCGGCGGTACGG			

Primer pair 2: Accession number: AF108434

Forward primer	TCTGTTGCCCAGGTTAGTATGAG	58.5		
Reverse primer	CCTTCATCCTCCCTTCATTGAAC	58.5	85	71.7
TaqMan probe	AGCAAGGCGCGAGTCTAGTGGTGA			

Primer pair 3: Accession number: AF191772

Forward primer	CCCGAAAGAGTGTCAGATTATGG	57.6		
Reverse primer	GTGAAAACTTCAATGGATCAGTCC	57.3	83	71.5
TaqMan probe	AACGCTTGGGGCATTGGTCGGGAT			

4.3.3 Real-time PCR - SYBR Green assay

SYBR Green assay was carried out as explained in section 5.3.6, the experiments conducted with this assay included the primer pair concentration and annealing temperature optimization and the cross reactivity tests.

4.3.3.1 Primer pair concentration and annealing temperature optimization

Three pairs of reverse and forward primers that were designed were then optimized as explained in section 5.3.6.1. The results for primer pair 1 are depicted in figure 34.

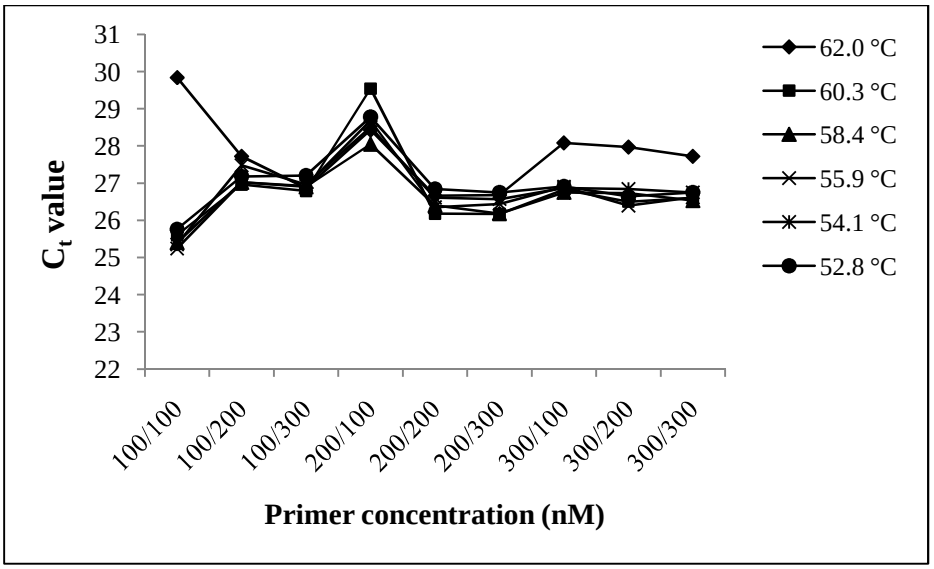

Figure 34: Primer pair concentration and Tm optimization results for primer pair 1

As the results indicate the minimum C_t value, 26.17 is obtained for a primer pair concentration of 200/300 nM and a temperature, T_m of 60.3 °C. Therefore for primer pair 1 a concentration of 200/300 nM and an annealing temperature of 60.3 °C was used for all future experiments including those of cross reactivity tests. Figure 35 represents the results obtained for primer pair 2 and figure 36 for primer pair 3.

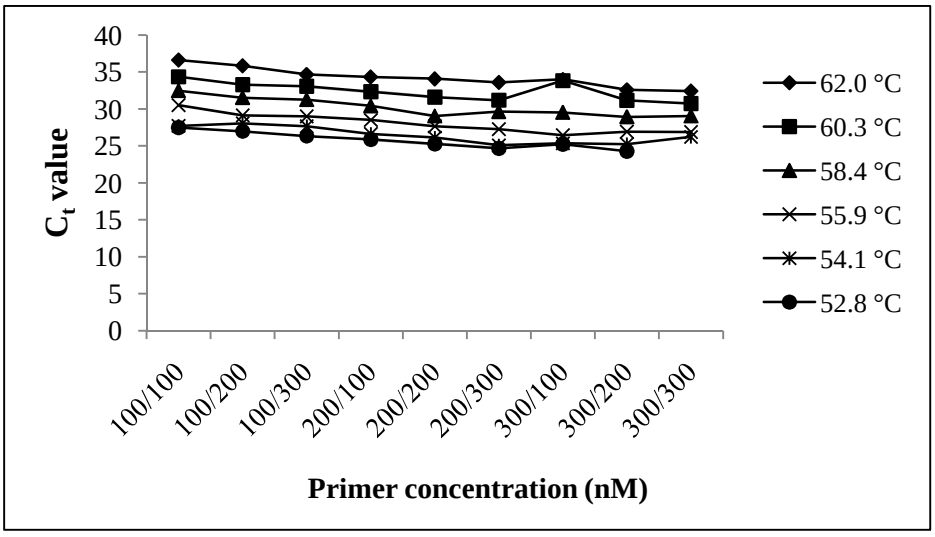

Figure 35: Primer pair concentration and Tm optimization results for primer pair 2

As the results indicate the minimum C_t value 24.29 is obtained for a primer pair concentration of 300/200 nM and a temperature, T_m of 52.8 °C. Therefore for all the next experiments dealing with primer pair 2 the conditions used were 300/200 nM of the primer concentration and an annealing temperature of 52.8 °C.

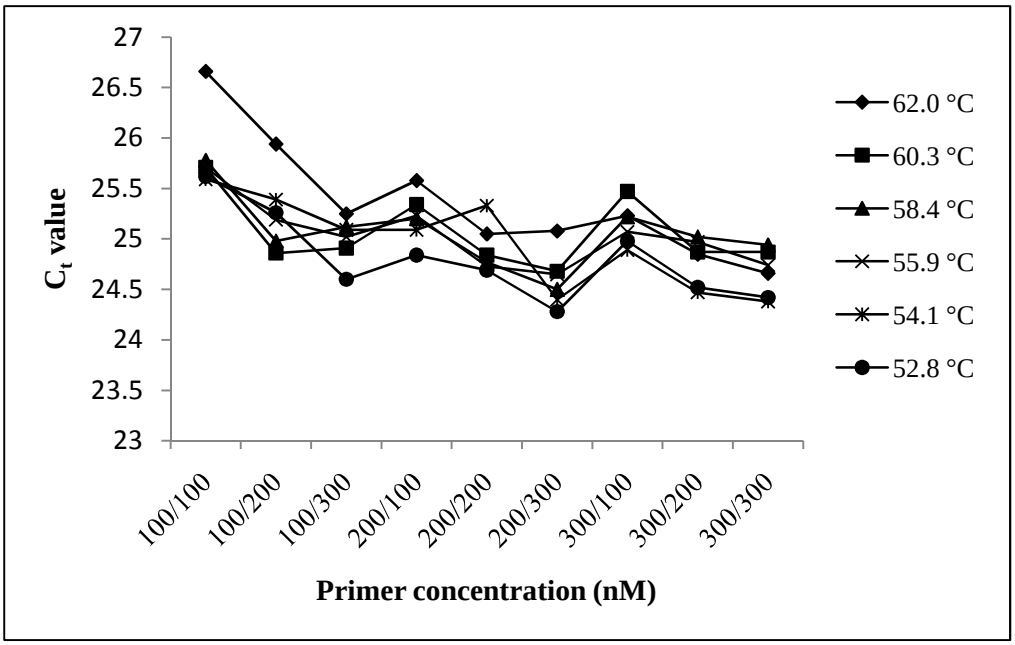

Figure 36: Primer pair concentration and Tm optimization results for primer pair 3

In the case of primer pair 3 the lowest C_t value obtained is 24.28 which is at a primer pair concentration 200/300 nM and at 52.8 °C. Therefore these conditions were considered to be optimum for this primer pair.

4.3.3.2 Cross reactivity tests

The second important experiments conducted by SYBR Green assay are the cross reactivity tests. These tests were conducted as explained in section 5.3.6.2 and table 10 illustrates the results obtained for all 28 food stuffs checked with primer pair 1 and 3. Primer pair 2 was not used to carry out these experiments as the primers got contaminated during the previous experiments.

Table 10: Results for cross reactivity tests with primer pair 1 & 3

Sample number	Sample	Primer pair 1		Primer pair 3	
		PCR analysis	Gel electrophoresis	PCR analysis	Gel electrophoresis
1	Sesame	-		-	
2	Black sesame	-		-	
3	Oat	-		-	
4	Wheat	-		-	
5	Lentils	+	-	-	
6	Corn	-		+	-
7	Macadamia	-	-	+	-
8	Pecan	-		+	-
9	Hazelnut	-		-	
10	Peanut	+	-	-	
11	Pistachio	+	-	+	-
12	Chocolate	-		-	
13	Soya	-		-	
14	Cashew	+	-	+	-
15	Sunflower	-		-	
16	Chick pea	+	-	+	-
17	Rice	+	-	+	-
18	Almond	-		-	
19	Walnut	+	-	-	
20	Rye	-		-	
21	Brazil nut	-		-	
22	Peas			-	
23	Barley			-	
24	Pine nut			+	-
25	Pumpkin seed	-		+	-
26	Coffee			+	-
27	Lupine			-	
28	White beans			-	

PCR analysis: (+): C_t value < 40, (-): no increase in the fluorescence signal in the first 40 cycles. Gel electrophoresis: (-): band but of a product of a different size than that of the amplicon.

Negative results were obtained for certain nuts and legumes whereas positive results were obtained for a few food stuffs. Melt curve analysis was carried out in this case and the melt curve ruled out the chances of cross reactivities since the melting temperatures obtained were different compared to the amplicon of poppy. Assuming that the positive result in the PCR run was a result of primer dimer formation and not a cross reactivity and to rule out the possibility of any cross reactivity gel electrophoresis was carried out as explained in section 5.3.6.2.1 for samples that in the melt curve analysis showed similar melting temperature values to that of the positive control.

4.3.3.3 Analysis by PCR and agarose gel electrophoresis

The analysis by agarose gel electrophoresis enabled to determine whether a positive result was an actual cross reactivity or was due to primer dimer formation.

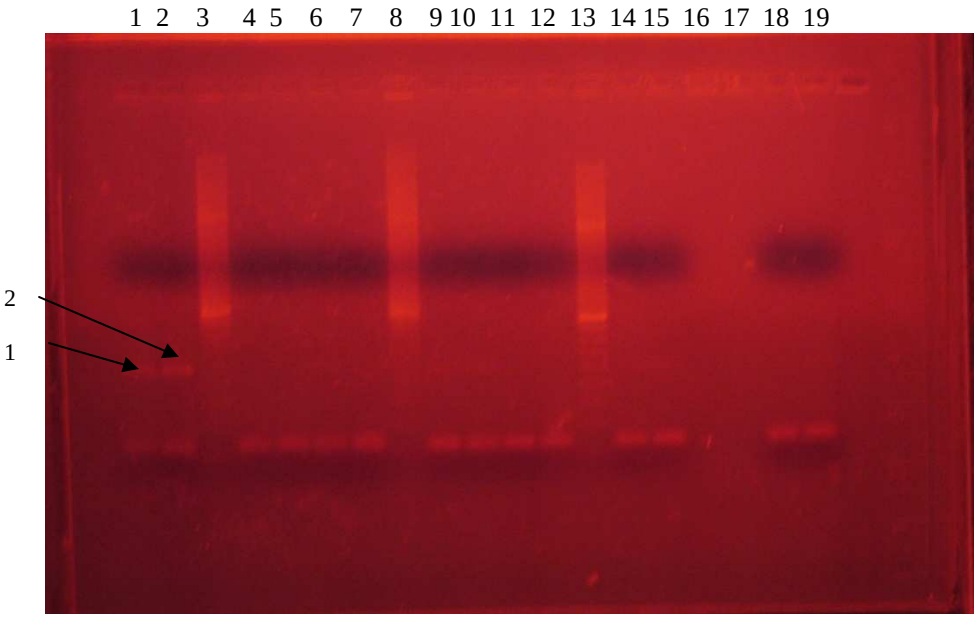

Figure 37: Cross reactivity tests of primer pair 1 by agarose gel electrophoresis

1 & 2: poppy seed, 3, 8 & 13: molecular marker, 4 & 5: chick pea, 6 & 7: pecan, 9 & 10: macadamia nut, 11 & 12: pistachio, 14 & 15: corn, 16 & 17: negative control, 18 & 19: walnut.

For further analysis by PCR, primer pair 1 was chosen since PCR analysis for primer pair 1 showed that seven food stuffs gave a C_t value of less than 40 whereas for primer pair 3, ten such results were obtained. Primer pair 2 was not used in further experiments due to primer contamination.

4.3.4 TaqMan assay

The TaqMan assay was carried out for all further experiments like efficiency measurement, analysis of commercial food samples and of spiked food samples. First, the probe concentration had to be optimized.

4.3.4.1 Probe concentration optimization

The concentration of the probe was optimized using the experimental setup explained in the section 5.3.7.1. The results are illustrated in figure 38.

Probe concentration (nM)	C_t value	C_t value	Mean C_t value
25	26.96	26.56	26.76
75	26.34	27.15	26.74
100	25.94	26.51	26.22
150	26.1	26.06	26.08

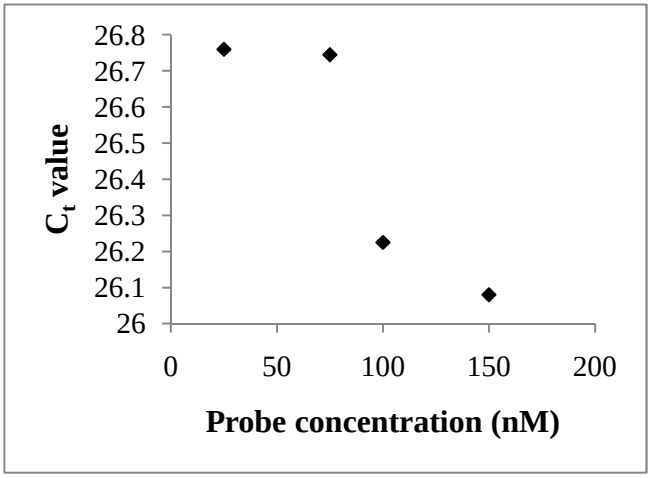

Figure 38: Probe concentration optimization

The optimum concentration of the probe was found to be 150 nM.

4.3.4.2 Optimization of primer concentration and annealing temperature

Next, the optimum probe concentration of 150 nM was used and the primer pair concentration and annealing temperature were optimized using the TaqMan assay. Primer

pair 1 was used in this experiment and since an optimization has already been carried out for these two variables, only four temperatures were analyzed again. Figure 39 depicts the results obtained.

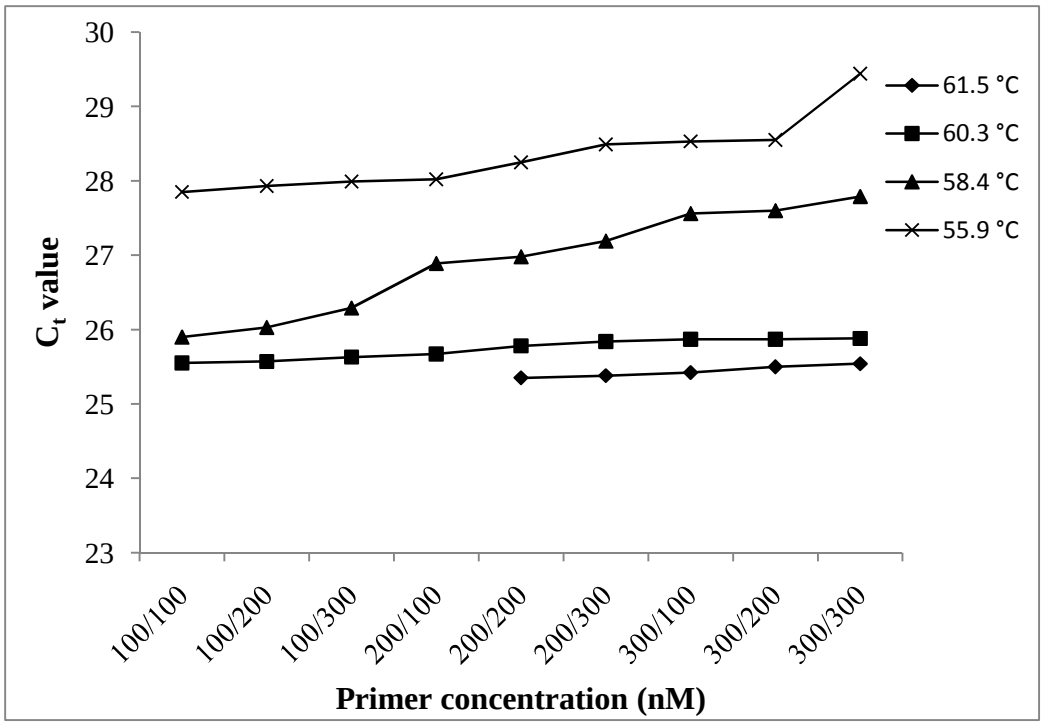

Figure 39: Results of primer pair concentration and Tm optimization using TaqMan assay

As the results indicate the lowest C_t value of 25.35 was obtained for a primer pair concentration of 200/200 nM at an annealing temperature of 61.5 °C. Therefore these conditions were used for all future experiments.

4.3.5 Validation of the developed real-time PCR method

4.3.5.1 Efficiency and LOD

Serially diluting a positive control comprising of poppy DNA extract with a concentration of 20 ng/µL, five standards were prepared as explained in section 5.3.8. The dilutions used were 1:10, 1:100, 1:1000, 1:10000 and 1:100000 which correspond to 2 ng/µL, 0.2 ng/µL, 0.02

ng/µL, 0.002 ng/µL and 0.0002 ng/µL of poppy DNA, respectively. The results are illustrated by figure 40.

The efficiency was calculated from the slope of the standard curve using the following equation:

$$E = 10^{-1/slope} \qquad (4)$$

$$E(\%) = (E - 1) \times 100 \qquad (5)$$

An efficiency of 100% indicates a 2-fold increase in the amount of DNA with each cycle. In practice, the amplification efficiency should be in the range from 90 to 105%.[3]

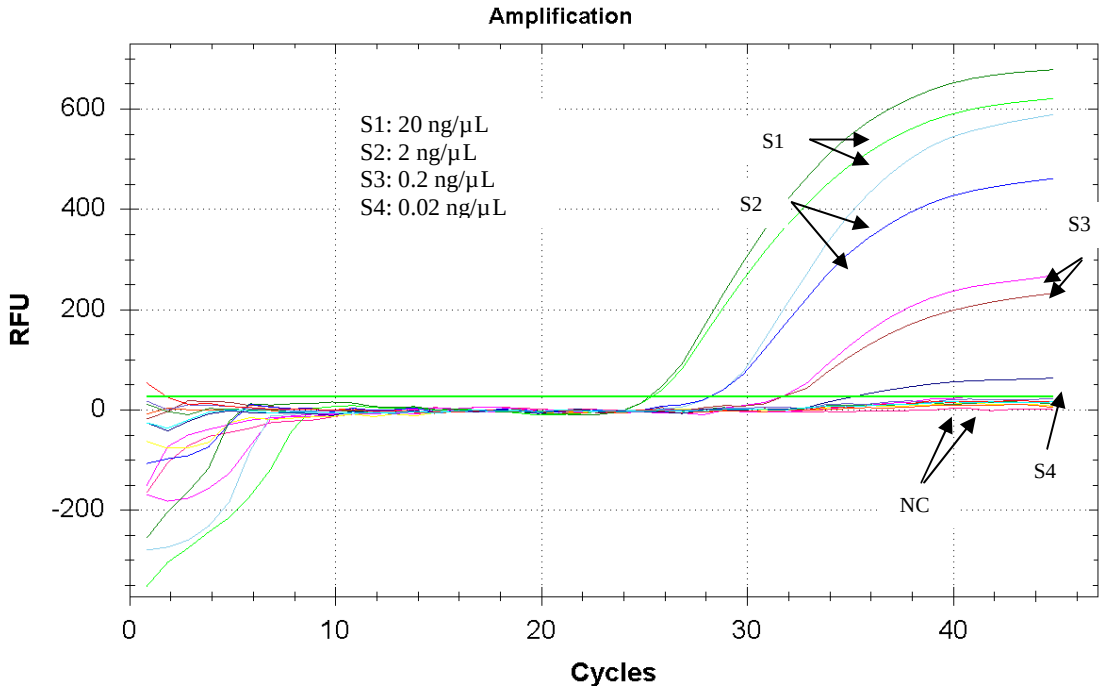

Figure 40: Efficiency determination I

The figure indicates that there is an increase in the C_t value as the dilution increases or as the concentration decreases. The C_t values for S1 are 25.35 and 25.22, for S2 the values are 28.18 and 28.17, for S3 the values are 31.64 and 31.70 and for S4 as only one sample shows amplification, the value is 35.16.

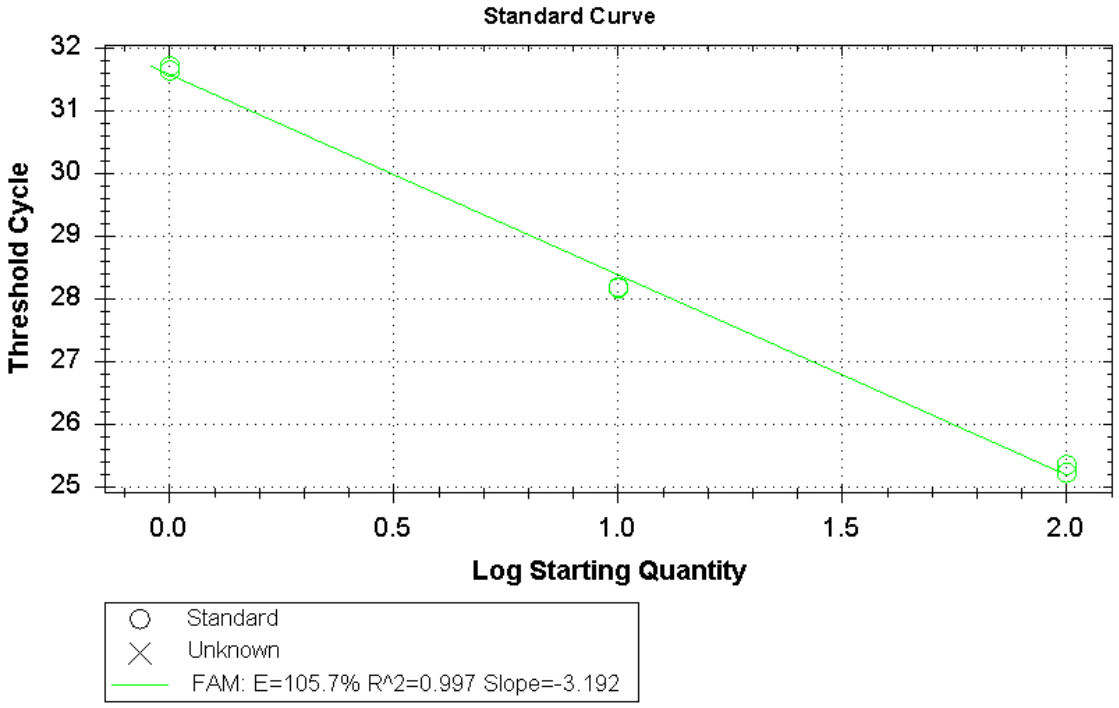

Figure 41: Standard curve for efficiency determination I

As figure 41 clearly indicates that although the efficiency is 105.7% the minimum concentration that could be detected was of 1:100 which corresponded to a concentration of 0.2 ng/µL of the poppy DNA. No increase in the fluorescence signal was observed for lower concentrations.

A series of experiments were carried out in the similar way except that a higher initial concentration was loaded on the plate and the dilutions were made from a poppy DNA extract of 45 ng/µL. In this case however, S1, S2 and S3 were loaded only once whereas duplicates were loaded for S4 and S5. The loading of positive and negative controls helped rule out the possibility of any false results. The results are shown in figures 42 and 43.

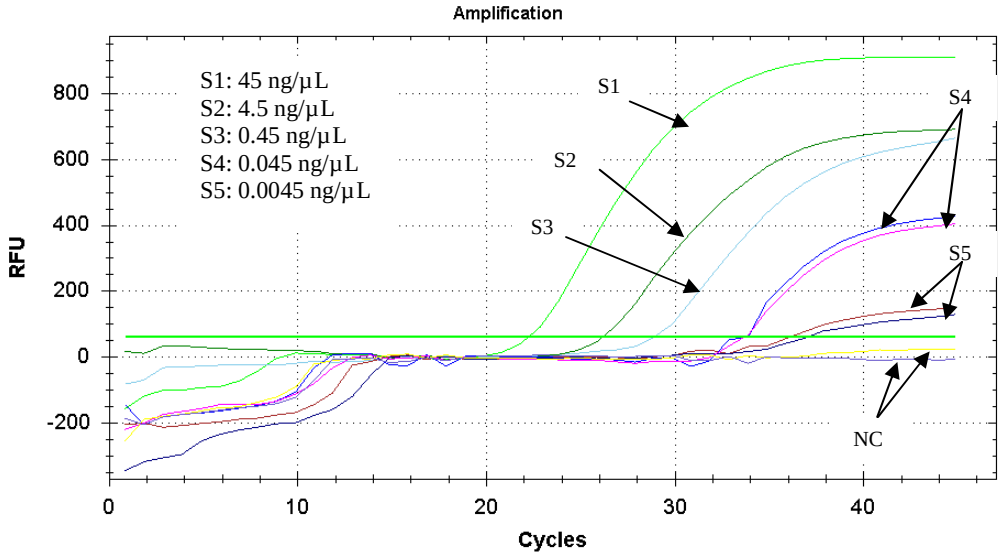

Figure 42: Efficiency determination II

As indicated by the figure the C_t value for S1 is 22.24, for S2 it is 26.18, for S3 it is 28.90, for S4 the values are 33.88 and 33.72 and for S5 the values are 36.17 and 37.10.

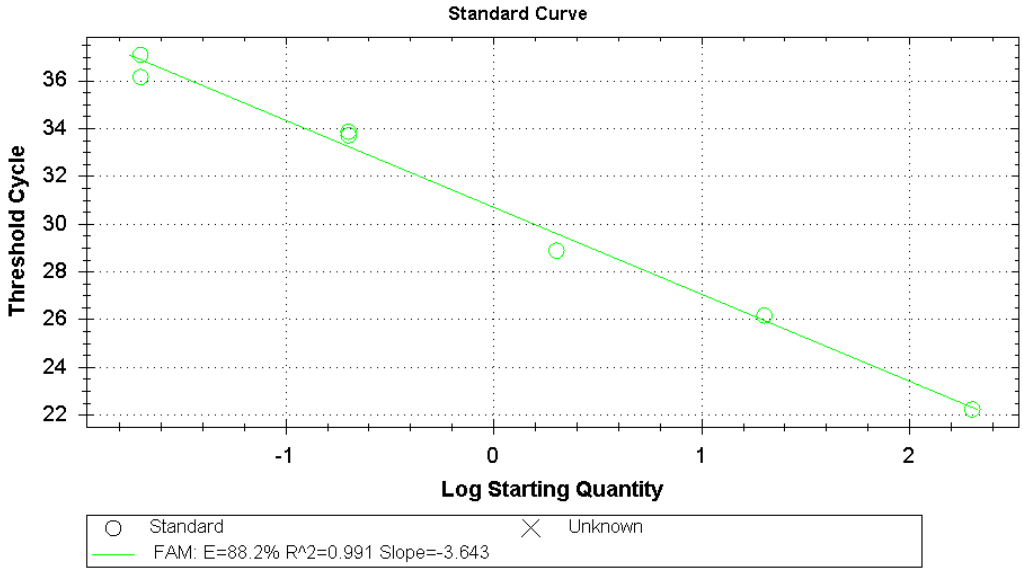

Figure 43: Standard curve for efficiency determination II

In this case the fluorescent signal was obtained until the 1:10000 dilution, i.e. 0.0045 ng/mL and the 1:100000 (0.00045 ng/mL) dilution did not show any amplification. There is also

high correlation between the C_t values and the log of the initial DNA concentrations. The slope indicates that the efficiency is 88.2%.

Thus, changing to a higher initial DNA concentration enabled detection of lower concentration.

4.3.5.2. Spiking experiments

As described in detail in the experimental section 5.3.9 spiking of two blank matrices, crisp toast and grissini, was carried out. These two matrices were chosen for spiking since the PCR analysis showed that these samples were free of poppy. The spiked samples were then used to extract DNA and the concentration and purity was determined by spectroscopic determination. For each spike level the DNA was extracted thrice and the best one in terms of concentration and purity was chosen for carrying out further PCR experiments.

Table 11 represents the results of DNA extraction of the samples obtained from spiking of commercially available crisp toast free of poppy.

Table 11: DNA extraction of spiked commercial food samples (crisp toast)

Spike level (%)	**DNA concentration** (ng/µL)	**Purity** (A_{260}/A_{280})
1	211.2	1.94
0.5	206.5	1.90
0.1	230.5	1.97
0.05	180.8	1.99
0.01	265.8	1.91
0.005	251.3	1.88
0.001	314.8	1.99

The accuracy of the developed PCR method was determined by analyzing the DNA extracted from these spiked samples. Figure 44 represents results obtained from spiking crisp toast. In this case too, a positive (PC) and a negative control (NC) were loaded on the PCR plate along with the rest of the spiked samples.

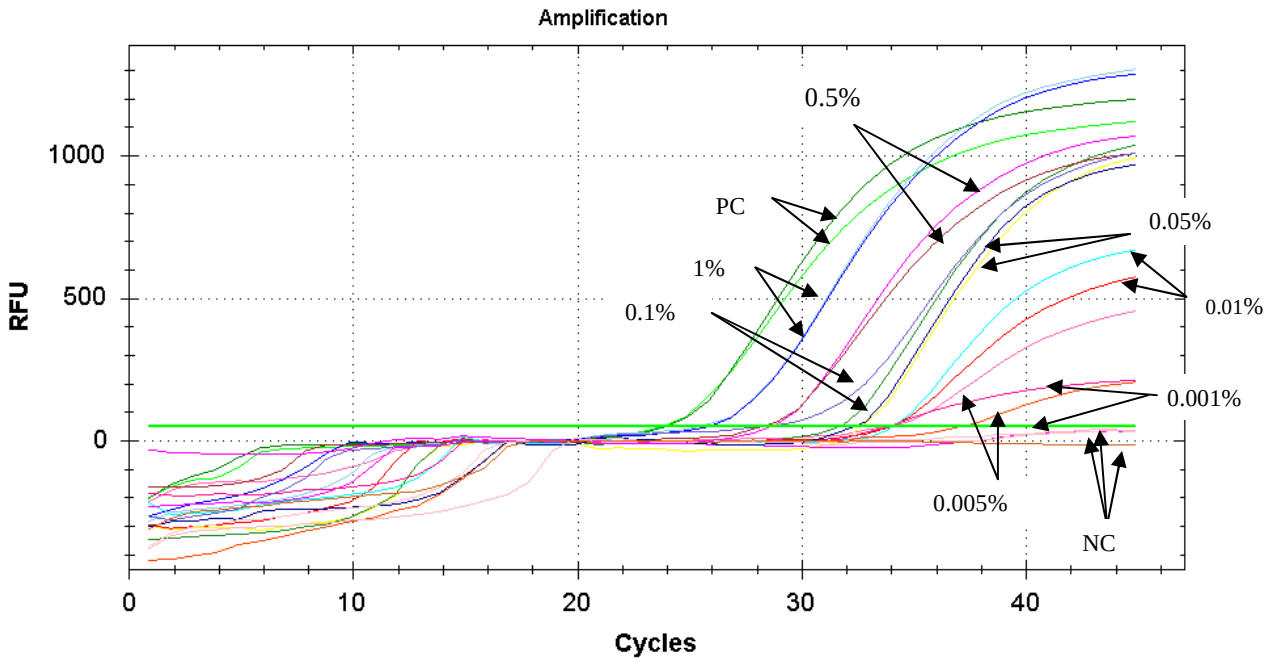

Figure 44: PCR analysis of spiked crisp toast samples

The figure indicates that the negative controls show no amplification and hence the possibility of a false positive signal can be ruled out. Also there is a periodical shift in the C_t value with a decrease in the poppy concentration of the spiked sample. The C_t values of different spiked levels are depicted in table 12.

Table 12: C_t values obtained for spiked crisp toast samples

Spike level (%)	C_t value		Mean C_t value
1	27.06	27.12	27.09
0.5	29.55	29.46	29.50
0.1	33.15	31.04	32.09
0.05	33.29	32.41	32.85
0.01	34.81	34.97	34.89
0.005	38.83	35.40	37.11
0.001	35.73	-	-

(-): No increase in the signal until the 40th cycle.

A standard curve was obtained by plotting the obtained mean C_t values against the logarithm of the different spiking levels. Figure 45 represents these results. The slope of the standard was -3.922 which corresponds to an efficiency of 79.9%

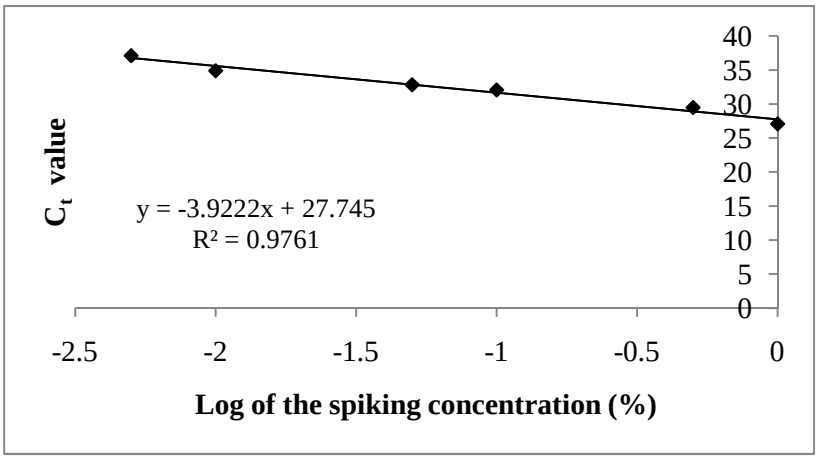

Figure 45: Standard curve for spiked crisp toast

Another matrix, grissini was used for spiking. The DNA extraction of spiked grissini samples was done thrice since in the first two attempts the appropriate DNA concentration was not obtained for certain spike levels. Even in the last case, the results of which are reported in table 13, sufficient DNA amounts could not be extracted for two spike levels, i.e. 0.5% and 0.001%.

Table 13: DNA extraction of spiked grissini samples

Spike level (%)	**DNA concentration** (ng/µL)	**Purity** (A_{260}/A_{280})
1	85.0	1.95
0.5	-	-
0.1	123.5	1.73
0.05	67.5	1.79
0.01	44.0	1.66
0.005	50.8	1.76
0.001	-	-

(-): The amount of DNA extracted was below the limit of detection.

The table represents the results from those samples which, out of the three samples for each spike level, proved to be the best in terms of concentration and purity. The DNA obtained from such samples was then used to carry out a PCR measurement. The results are depicted in figure 46.

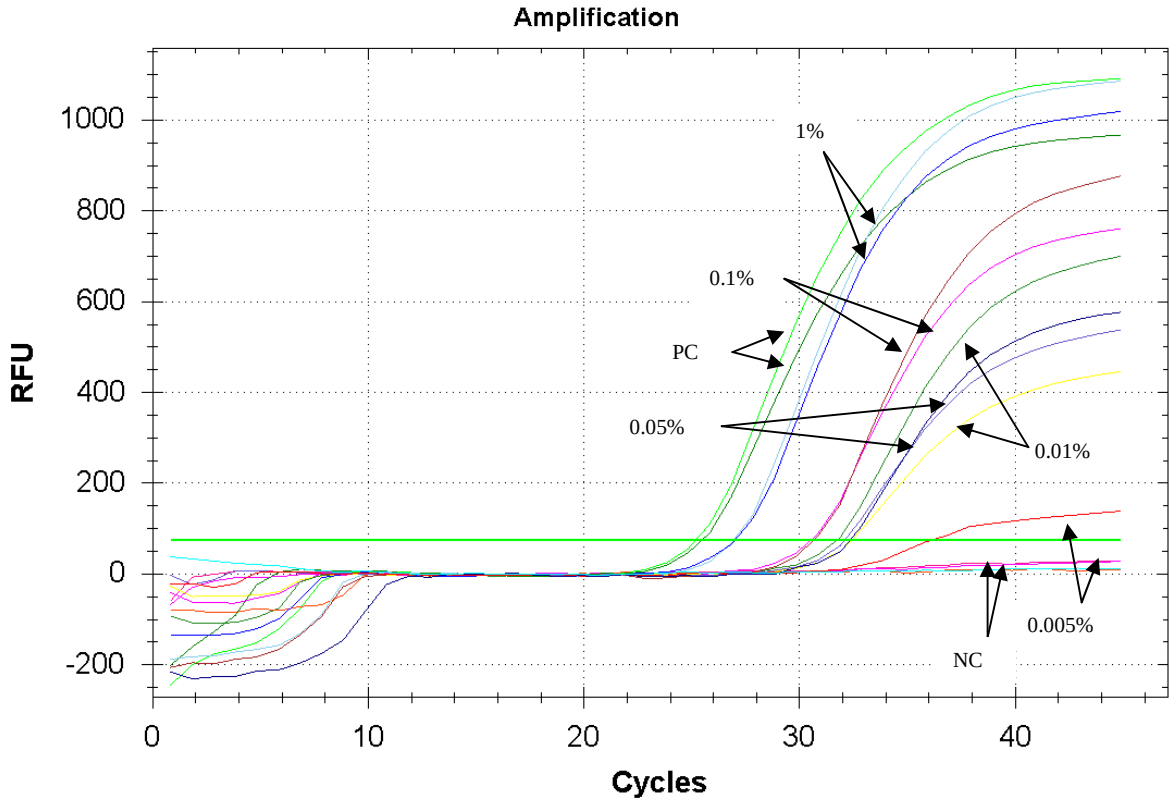

Figure 46: PCR analysis of spiked grissini samples

The figure indicates that the negative control does not show any amplification and the positive control resulted in a C_t value of 23. The spiked samples show a gradual shift in the C_t value corresponding to the spike level of poppy in the blank matrix. The C_t values of different spiked levels are depicted in table 14.

Table 14: C_t values obtained for spiked grissini samples

Spike level (%)	C_t value		Mean C_t value
1	26.93	27.00	26.96
0.1	30.52	30.65	30.58
0.05	32.33	32.08	32.20
0.01	32.39	31.72	32.05
0.005	-	-	-

(-): No increase in the signal until the 40th cycle.

A standard curve was obtained by plotting the obtained C_t values against the logarithm of different spiking levels. Figure 47 represents these results. The slope of the standard was -3.4145 which corresponds to the efficiency of 96.2%.

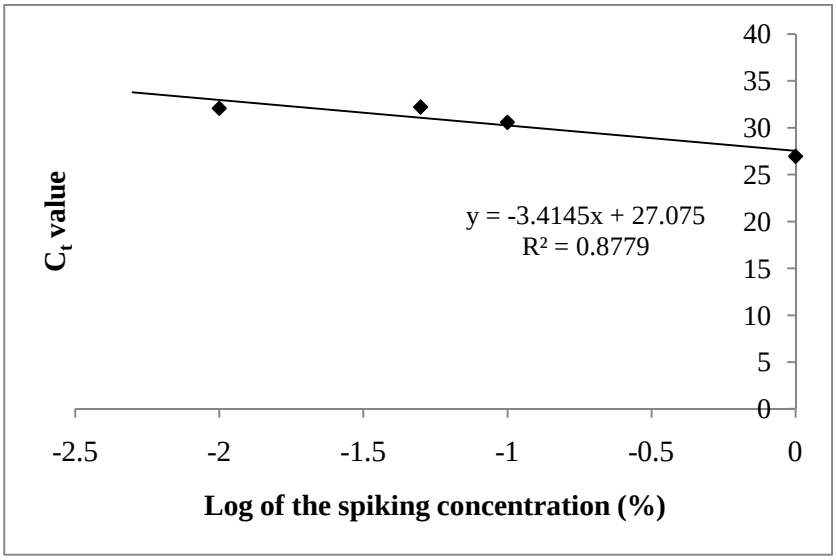

Figure 47: Standard curve for spiked grissini

4.3.6 Analysis of commercial food samples

The developed PCR method was checked for its applicability by analyzing commercial food samples. The analysis of commercial food samples was carried out on various food samples available locally; these included packaged food as well as fresh bakery products. The food

samples chosen were of two categories, one containing poppy and the other without any poppy labeling.

4.3.6.1 DNA extraction of poppy containing commercial food samples

The DNA was extracted from each food sample following the CTAB extraction method. DNA was extracted thrice from each food sample and the one with the appropriate purity and concentration was used for analysis by PCR. Results of the DNA extraction of food samples containing poppy are given in table 15.

Table 15: DNA extraction results of poppy containing commercial food samples

Sample	**DNA concentration** (ng/µL)	**Purity** (A_{260}/A_{280})
Poppy bread 1	45.5	1.82
Poppy strudel	270.0	1.90
Sesame cracker	73.8	1.98
Crackers	114.3	1.89
Poppy oil	45.5	1.00
Homemade poppy cake	320.3	2.01
Poppy crisp toast	370.3	1.95
Poppy cake	186.5	1.90
Poppy bagel	87.8	1.94
Poppy roll	120.5	1.93
Poppy bread 2	141.8	1.88
Poppy chocolate	43.0	1.05

Out of all the samples used for DNA extraction, sufficient purity and concentration was not obtained from the poppy chocolate. It was used as such for the PCR analysis, for all the rest dilutions were made so as to achieve a concentration of 45 ng/µL for PCR analysis.

4.3.6.2 PCR analysis of poppy containing commercial samples

PCR with the extracted DNA from the samples represented in table 15 was carried out and each sample was loaded on the plate in a concentration of 45 ng/mL. A positive control,

which is poppy DNA and a negative control was also loaded on the plate to rule out any false positive or negative results. Figure 48 represents the results obtained from this experiment.

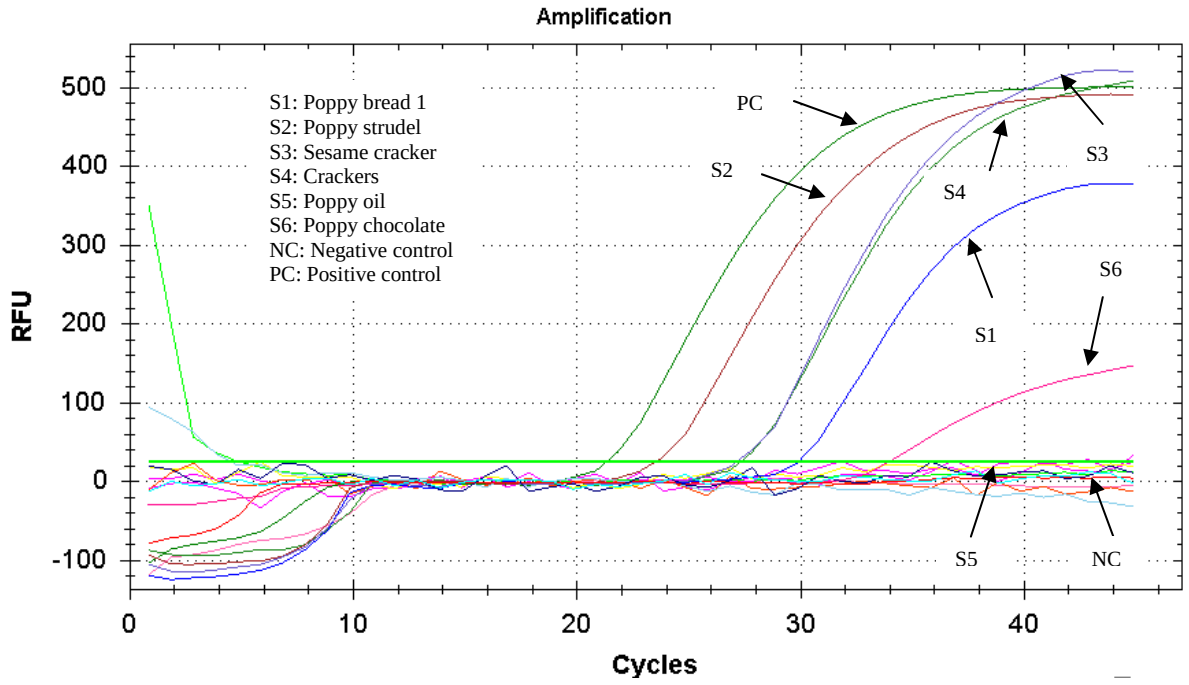

Figure 48: Analysis of commercially available poppy containing food samples I

As indicated by the figure the positive control as well as all samples containing poppy show amplification except poppy oil and the negative control. The rest of the samples were loaded on another PCR plate in the same manner and the results are depicted in figure 49.

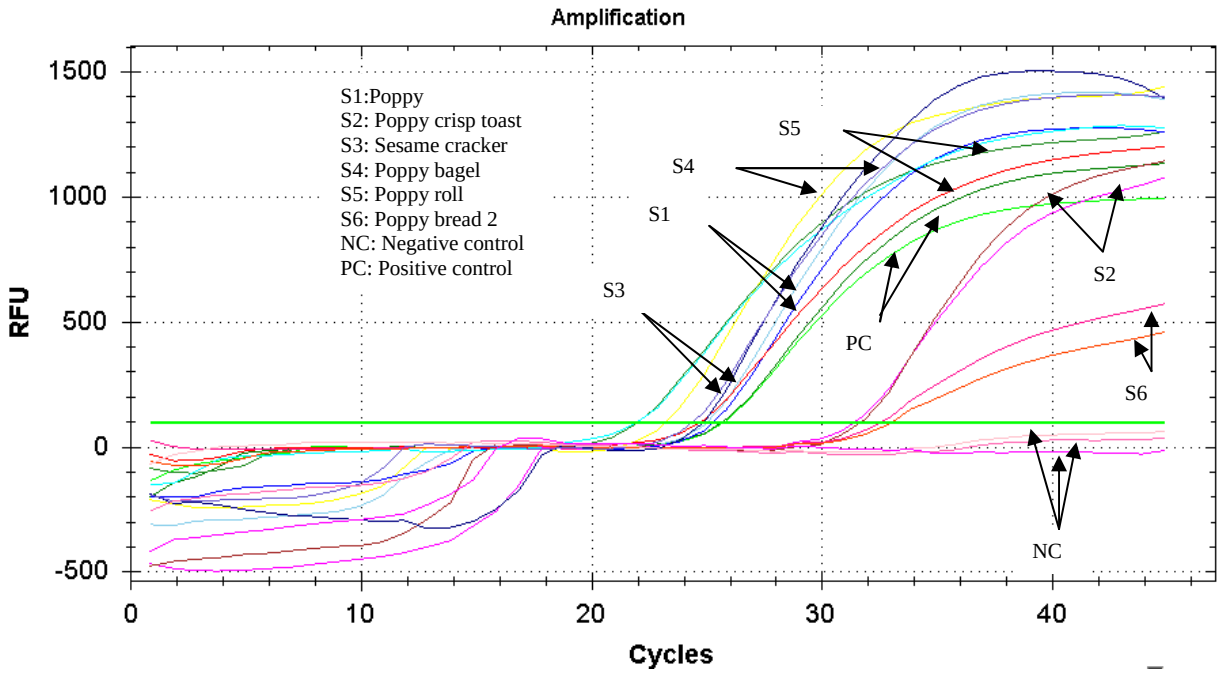

Figure 49: Analysis of commercially available poppy containing food samples II

As indicated by figures 48 and 49 all samples containing poppy seeds show certain amplification along with the positive controls whereas the negative controls show a negative response and hence no amplification.

4.3.6.3 DNA extraction of commercial food samples without poppy

The next category of commercial samples is of the samples free of poppy seeds. The DNA extraction for all these samples was conducted in the same way as explained before and the sample with an apt concentration and purity was chosen for analysis by PCR. The results from such samples are depicted in table 16.

Table 16: DNA extraction results of commercial samples without poppy

Sample	**DNA concentration** (ng/µL)	**Purity** (A_{260}/A_{280})
Crisp toast	271.2	1.74
Grissini	83.5	1.80
Fruit muesli 1	142.5	1.63
Biscuits	57.5	1.57
Gouda biscuits	69.2	1.79

Whole grain biscuits	323.0	2.08
Multigrain crisp toast	330.5	2.02
Sesame crisp toast	312.5	1.92
Sesame cracker	163.5	1.95
Fruit muesli 2	177.0	1.96
Fruit muesli 3	277.5	2.06

Since poppy is not included in the list of 14 food samples that must be labeled if present in a food product, the label of "may contain poppy" does not exist. Keeping this in mind several negative samples were analyzed and a positive signal in some cases was not surprising.
The results indicate that all samples gave a high enough DNA concentration and the purity was close to the required limit of 1.8-2.0.

4.3.6.4 PCR analysis of commercial samples not containing poppy

Figures 50 and 51 represent the results obtained from the experiment when the samples mentioned in table 16 were loaded on the PCR plate.

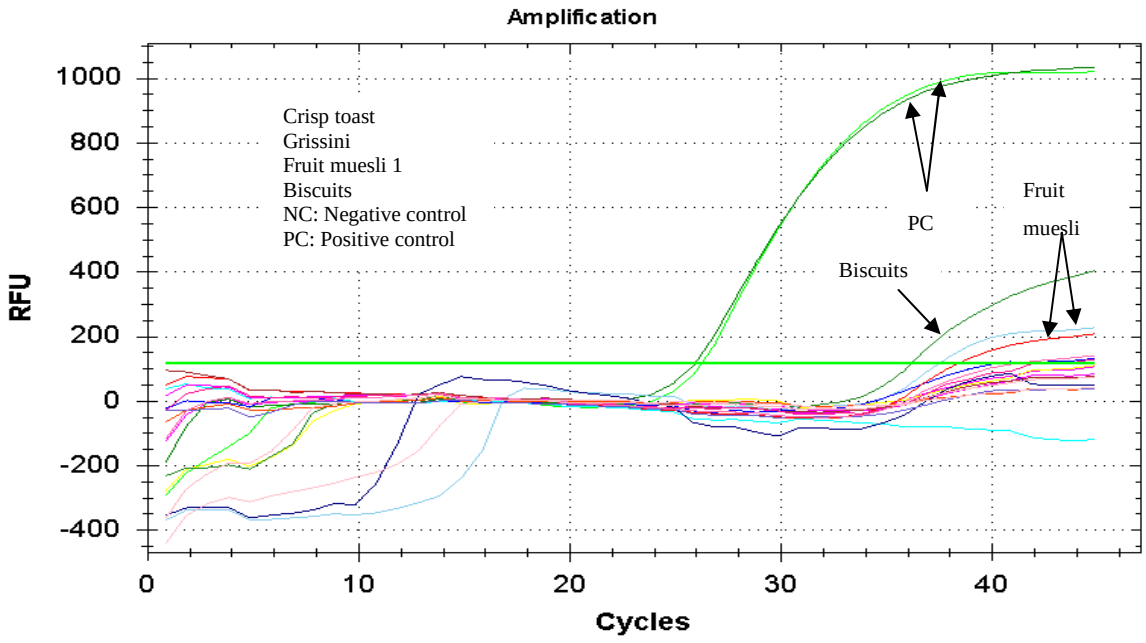

Figure 50: Analysis of commercially available food samples without poppy I

The samples were loaded after diluting them with bidistilled water to obtain a concentration of 45 ng/µL and analyzed. A positive control and a negative control were also loaded on the plate to rule out any false positive or negative results.

As the figure indicates that except biscuits and fruit muesli and the positive control all samples and the negative control show no amplification.

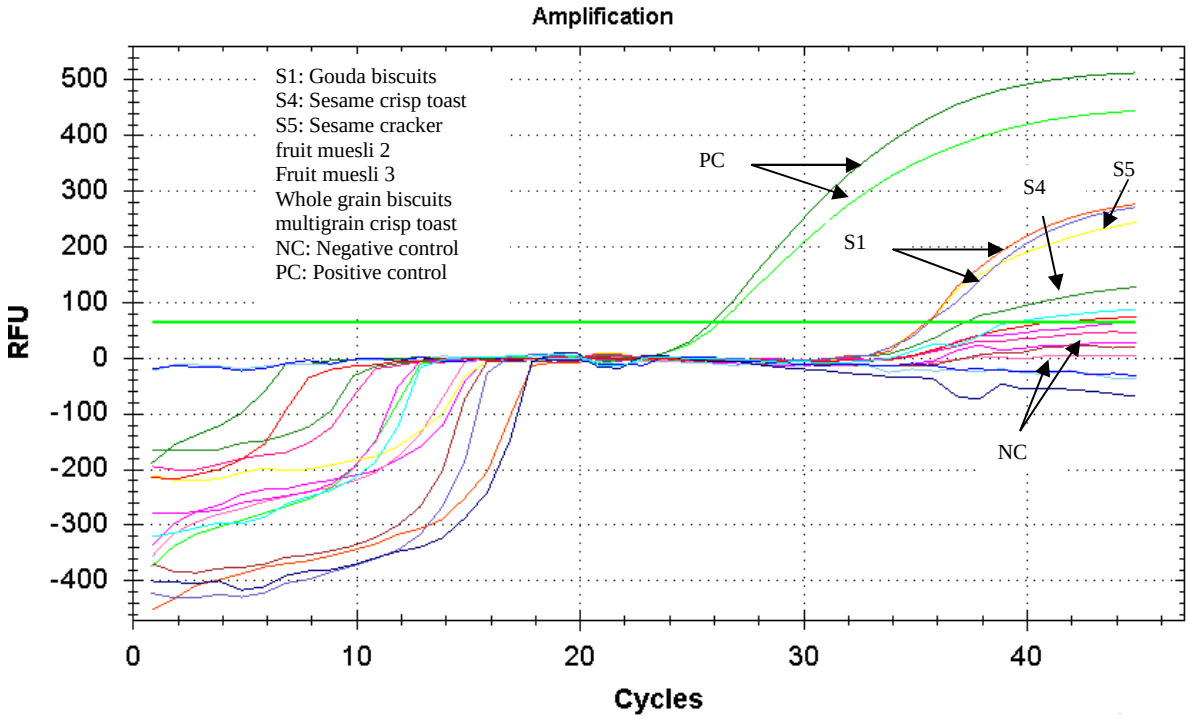

Figure 51: Analysis of commercially available food samples without poppy II

Similarly figure 51 indicates that not only the positive control but three more samples show a positive response. These include sesame cracker, sesame crisp toast and Gouda biscuits. It can be assumed that these food samples may have contained some traces of poppy as a result of cross contamination during manufacture or packaging. Since it is not a compulsion by law to label poppy and the label stating “may contain poppy” does not exist, these three food samples may lie in the category of such samples and hence a positive result was obtained for them.

4.4 Preliminary experiments for the development of an immuno-PCR method for lupine detection

In the last part of the thesis, preliminary tests were conducted for the development of an immuno-PCR method for the detection of lupine.

The immuno-PCR method was developed starting from the sandwich ELISA for lupine, developed by a colleague, Christina Ecker. Instead of using an enzyme labeled antibody for detection, a biotinylated antibody and streptavidin was used followed by the addition of biotinylated DNA. The biotinylated DNA was amplified by the polymerase chain reaction. For this purpose a sequence of the λ-phage DNA was selected.

4.4.1 Initial PCR experiments

Initially, simple PCR experiments were conducted in order to determine if the amplification of the λ-phage DNA worked or not and what concentration of the lambda phage DNA would be sufficient enough to result in an appropriate C_t value. The lambda phage sequence selected was:

5´- CCTGCAATGACCCCGCTGATGCTGGACACCTCCAGCCGTAAGCTGGTTGCG
TGGGATGGCACCACCGACGGTGCTGCCGTTGGCATTCTTGCGGTTGCTGCT
GACCAGACCAGCACCACG -3´

The forward and backward primers that were used were:

λ-phage forward: 5´-ACACCTCCAGCCGTAAGC-3´

λ-phage backward: 5´-AGCAGCAACCGCAAGAATG-3´

Beacon designer software was used to design the appropriate probe for these experiments. The experimental details of the first experiment are explained in section 5.4.4.1, which was carried out with the positive and negative controls only. The primer pair was used in a 200/200 nM concentration and the dye used was SYBR Green. Lambda phage DNA was used in two different concentrations, 0.2 and 2.0 ng/µL for the positive control, whereas, in the negative control the DNA was replaced by bidistilled water. The results indicated that the higher concentration of 2 ng/µL was not the best choice, therefore a similar experiment but

with lower DNA concentrations was carried out, in this case all variables were the same as in the previous experiment but the concentrations used for two positive controls were 2 pg/µL and 2 *f*g/µL respectively. The PCR experiment was carried out as explained in the experimental section 5.4.4.1 and the results are depicted by figure 52.

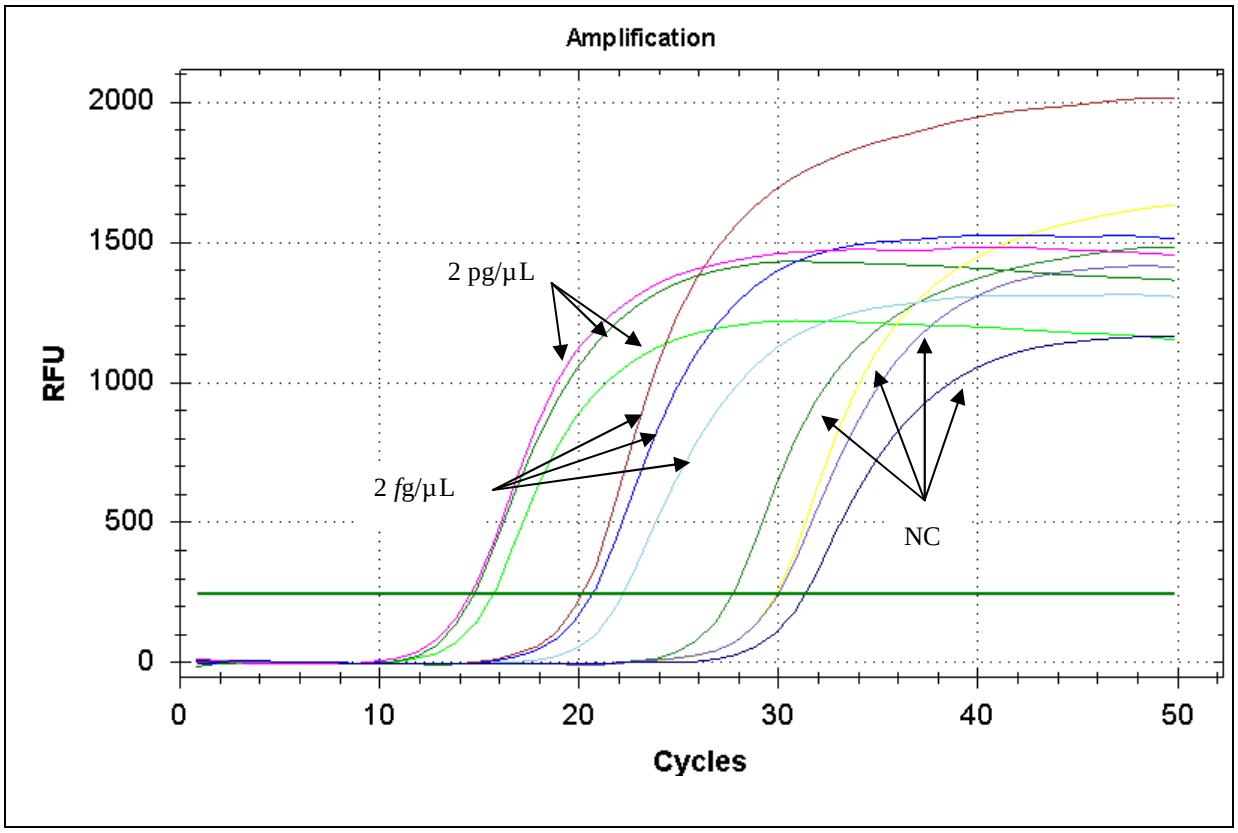

Figure 52: Positive and negative control test

It is evident from the figure that as the concentration of the DNA decreases there is a distinct change in the C_t values, for a concentration of 2 pg/µL the C_t values for the three samples loaded are 14.54, 14.74 and 15.69. Whereas for the concentration of 2 *f*g/µL the C_t values of the three samples loaded are 20.13, 20.67 and 22.17. All negative controls have C_t values higher than 27.0. The C_t values for negative controls are 27.70, 29.90, 29.98 and 31.33.

For TaqMan assay the probe in the concentration of 150 nM was included instead of using SYBR Green for the next experiment; the experimental details are as explained in section 5.4.4.2. In this case a total of four positive controls were loaded, two for each concentration

of 2 pg/µL and 2 *f*g/µL. Along with the positive controls, three negative controls were loaded. The results for the positive and negative controls were not reproducible and there was no correlation between the C_t value and the log of the concentration. The experiment was repeated thrice with the results depicting similar problems and the C_t value < 12 or <10 for all negative samples as well. Hence the experiment was repeated once more but with some changes. In this case difference being that the "incubation buffer", comprising of PBS buffer containing 1% (w/v) BSA and 0.05% (v/v) Tween 20, instead of bidistilled water or PBS was used for all sorts of dilutions needed at all levels of the experiment. The results are depicted in figure 53.

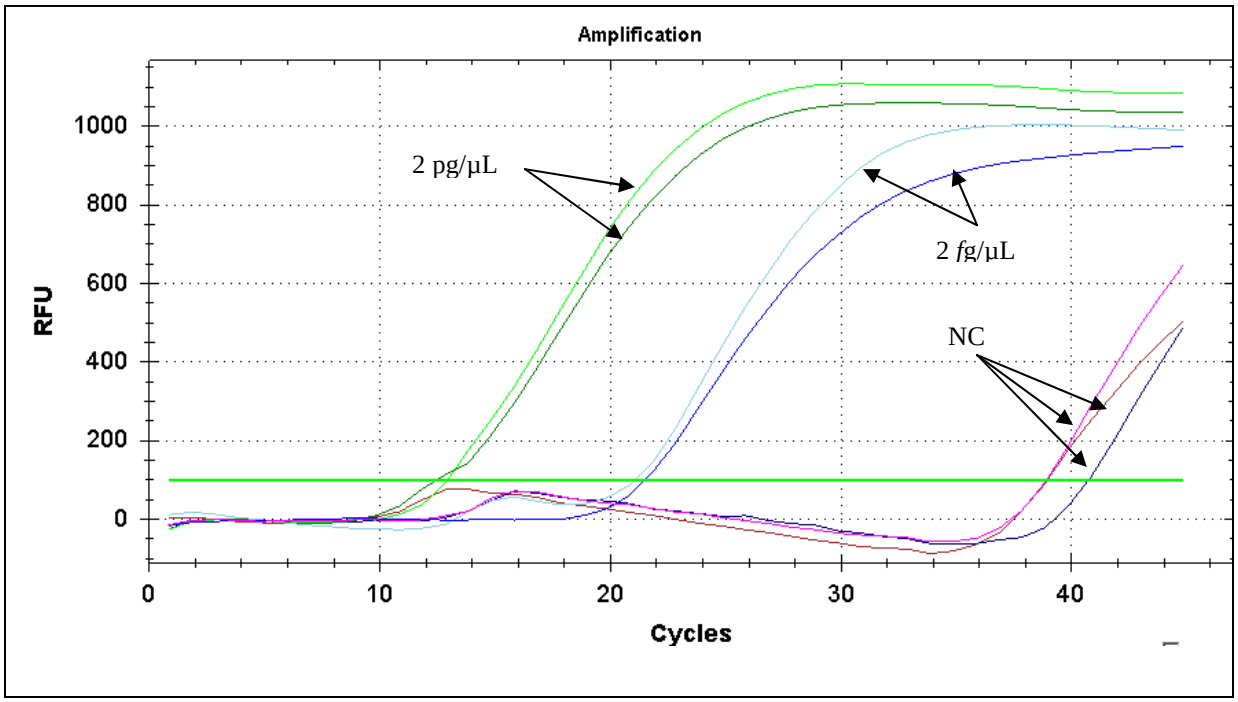

Figure 53: Positive and negative control test with the probe

It is quite clear from figure 53 that using the incubation buffer, prepared as explained in section 5.4.1, for all dilutions has a positive influence on the results; in this case the same concentration of the DNA resulted in the same threshold cycle and thus the curves representing them are close to each other. In addition a decrease in the concentration from 2 pg/µL to 2 *f*g/µL affects the C_t value. For 2 pg/µL the corresponding C_t values are 12.44 and

12.92 whereas for 2 *f*g/µL the C_t values are 21.05 and 21.47. The three negative controls have a very similar response and lead to C_t values of 38.90, 38.94 and 40.72.

4.4.2 Optimization experiments

In the next experiment, the optimization of the primer concentration and the annealing temperature was carried out according to the details given in section 5.4.5, figure 54 is a representative of these results.

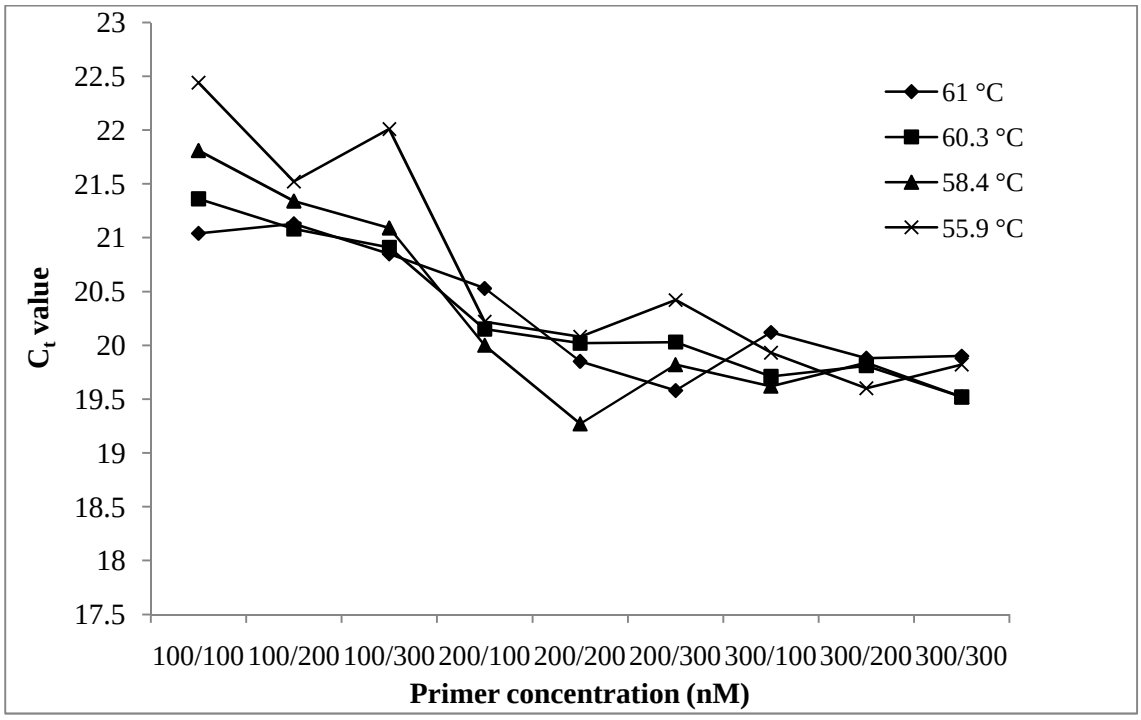

Figure 54: Primer pair and annealing temperature optimization

Inferring from the data obtained a primer pair concentration of 200/200 nM and an annealing temperature of 58.4 °C was selected to be the optimum one.

4.4.3 Immuno-PCR experiments

Later immuno-PCR experiments were carried out. The working principle similar to the one used by Lind et. al[4] was chosen. Coating, blocking and washing steps were carried out in the same way as was found to be optimum for the sandwich ELISA for lupine, except that the number of washing steps were increased and a biotinylated anti-rabbit antibody was used. The experimental procedure is explained in section 5.4.6. Lupine standards in a range of 100-

0.01 ng/mL of lupine protein were loaded along with negative controls without lupine protein. Blocking in this case was carried out at room temperature by 2% (w/v) casein solution in PBS. Following the same pipetting scheme as mentioned before for PCR experiments and using 150 nM of the probe and a 200:200 nM concentration of the forward and reverse primer the detection through PCR was carried out. Figure 55 depicts these results.

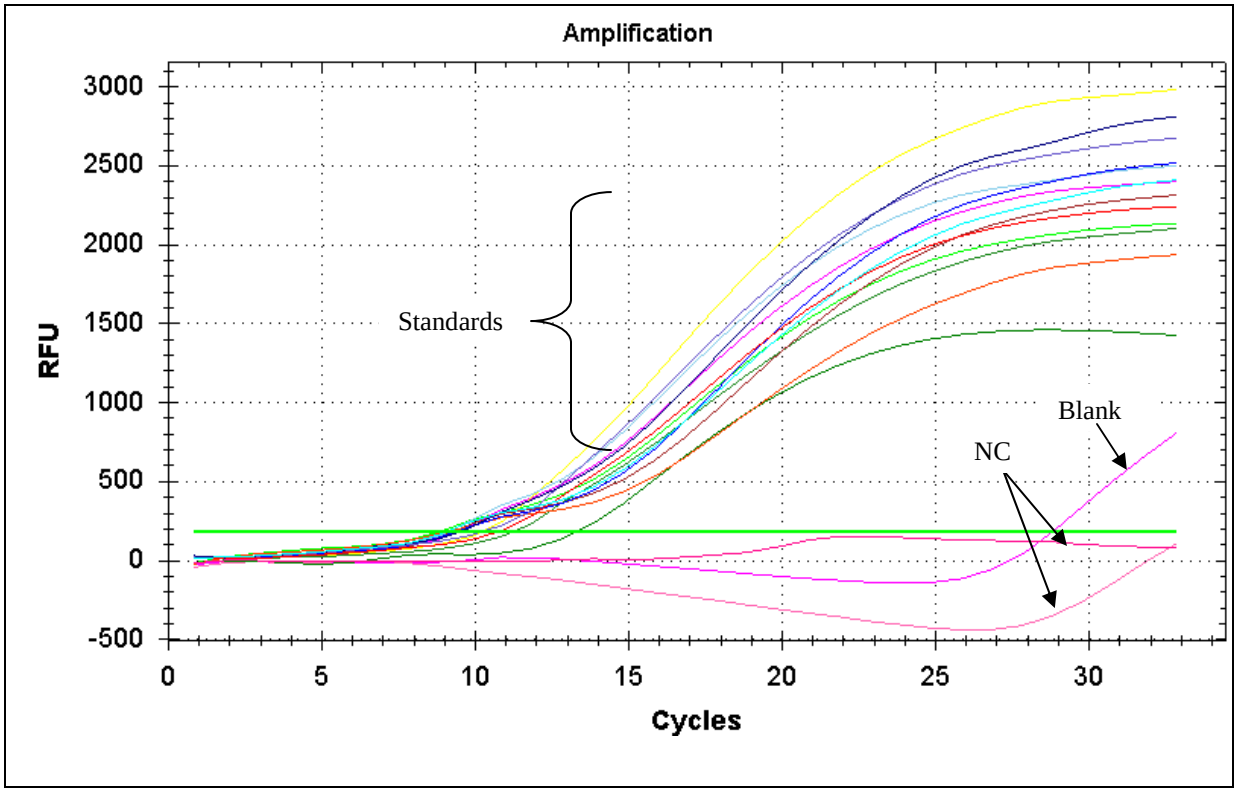

Figure 55: Immuno-PCR I

It is clear from the figure that there is no distinct difference in the C_t value obtained for the standards even in the case where there is a difference as big as 100 ng/mL to 0.01 ng/mL. All standards of different concentrations resulted in C_t values from 8.86 to 13.29. The blank, where no lupine proteins were added in the ELISA step, however show a C_t value > 28. The negative control, containing only the primer mix, water and super mix, that is the one loaded only for the PCR step, shows no amplification signal, thus eliminating a possibility of contamination.

Since it can be inferred from the results that there is no correlation between the C_t values and the lupine protein concentration, the same experiment was repeated with certain changes. In the next experiment, incubation buffer was used for blocking as well as for all dilutions during the ELISA steps instead of bidistilled water or PBS. The results were still not appropriate enough; therefore the experiment was repeated again but with certain changes for improving results.

In the next experiment two changes were incorporated. One was to carry out coating at room temperature instead of 4 °C, the other change was made in the washing buffer. Previously the washing buffer was the one used for the ELISA, as explained in 5.1.6 but for next immuno-PCR experiments the washing buffer containing Germal II was used, as explained in section 5.4.1. In this case three standards were loaded along with the negative controls. A certain difference in the amplification signal of the standards with the decrease in the concentration was observed but it was not as different as expected for such a change in the lupine protein concentration. Thus indicating, the method needed further improvement.

Blocking was modified by carrying out the blocking step using the incubation buffer for 1 h at 37 °C instead of at room temperature. The results obtained suggested that further improvements need to be made. For further improvements the coating was altered by using a 0.2 M NaH_2PO_4 solution and a higher antibody concentration of 20 µg/mL instead of 14.4 µg/mL. In addition, blocking was carried out using 2% (w/v) casein and 0.05% (v/v) Tween 20 in PBS at 37 °C instead of blocking with 2% (w/v) casein at room temperature. The results depicted in figure 56 represent this experiment.

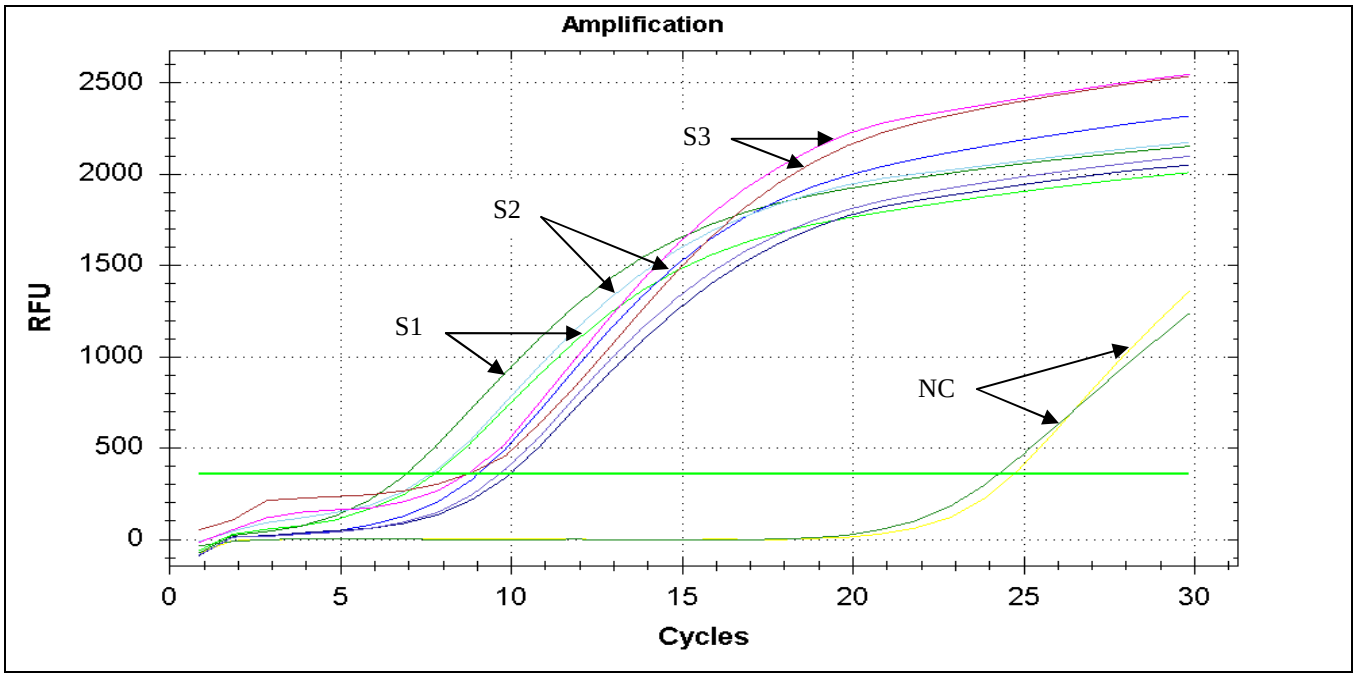

Figure 56: Immuno-PCR with a coating antibody concentration of 20 µg/mL

Concentration of lupine protein standards: S1: 100 ng/mL, S2:1 ng/mL and S3: 0.01 ng/mL.

The figure shows three pairs of positive standards, S1, S2 and S3 and a pair of negative controls. As indicated by the figure a substantial decrease in the lupine protein concentration does not result in a significant shift of the C_t value.

In the next experiments blocking was improved by incorporating 0.01% of salmon sperm DNA. Two blocking schemes were followed; in one instance 2% casein and in the other 1% BSA was used. The results showed that all positive samples as well as the negative controls resulted in C_t values very similar to each other. This indicated that the blocking needed further improvement. In order to achieve this, the experiment was repeated in the same way but with carrying out the blocking step overnight. Figure 57 is depictive of the results of the instance when BSA was incorporated in the blocking buffer and figure 58 illustrates the results when casein was used for blocking.

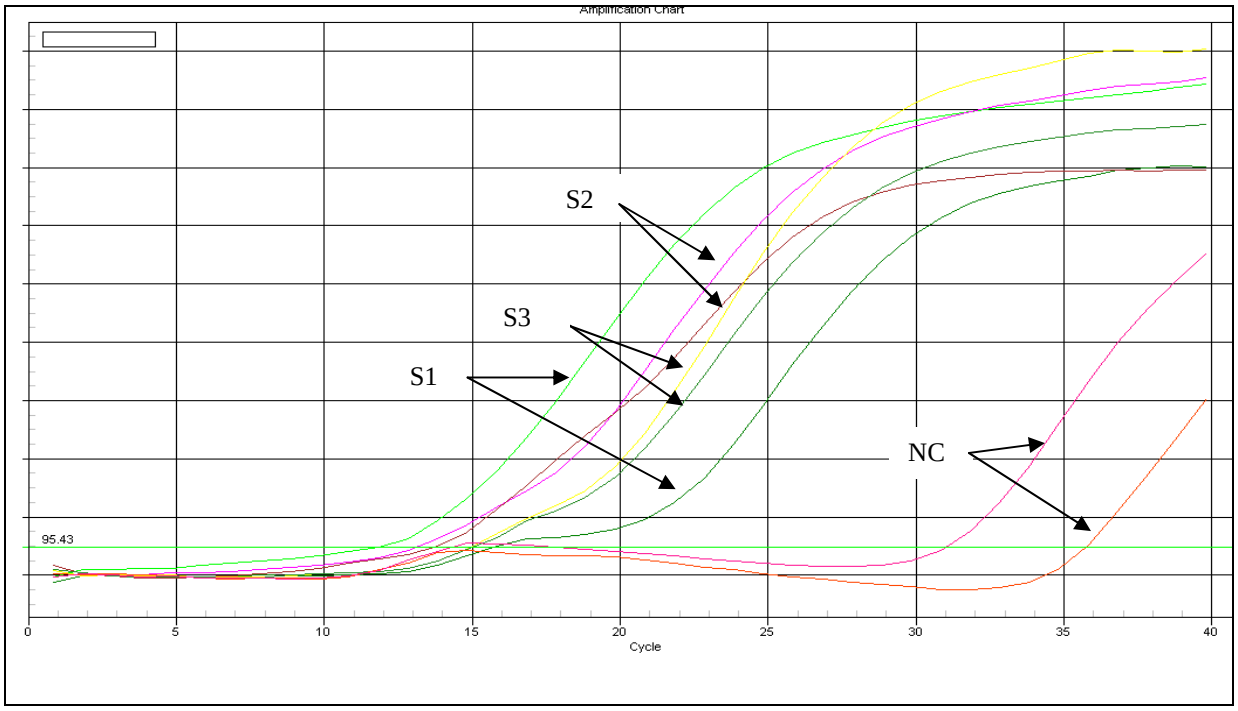

Figure 57: Immuno-PCR with blocking with 2% BSA, Tween 20 and salmon sperm DNA Concentration of lupine protein standards: S1: 100 ng/mL, S2: 1 ng/mL and S3: 0.01 ng/mL

As indicated by the figure the negative controls lead to a threshold cycle higher than the ones for the positive samples. The amplification curves obtained for S1 are wide apart and not similar to each other whereas the ones representing S2 and S3 are similar to each other and there is a slight shift in the C_t values with a decrease in concentration of lupine protein.

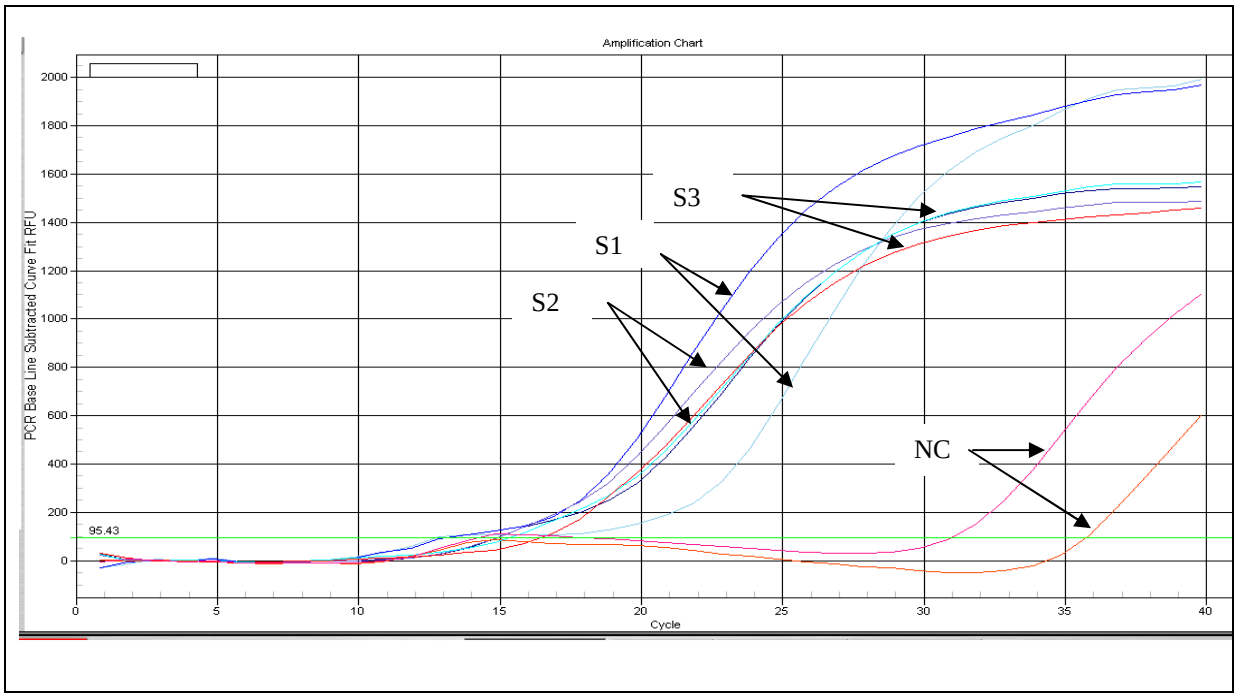

Figure 58: Immuno-PCR with blocking with 2% casein, Tween 20 and salmon sperm DNA Concentration of lupine protein standards: S1: 100 ng/mL, S2: 1 ng/mL and S3: 0.01 ng/mL

In this case the negative controls resulted in a C_t value higher than that of the positive samples. The curves representing S1 are not reproducible whereas the ones representing S2 and S3 are.

The afore mentioned experiments were repeated in exactly the same way using 96 well PCR plates instead of Thermoscientific Abgene PCR plates. Figures 59 and 60 represent the results when blocking was carried out for an hour and figures 61 and 62 are representatives of the results obtained in the case when blocking was carried out overnight.

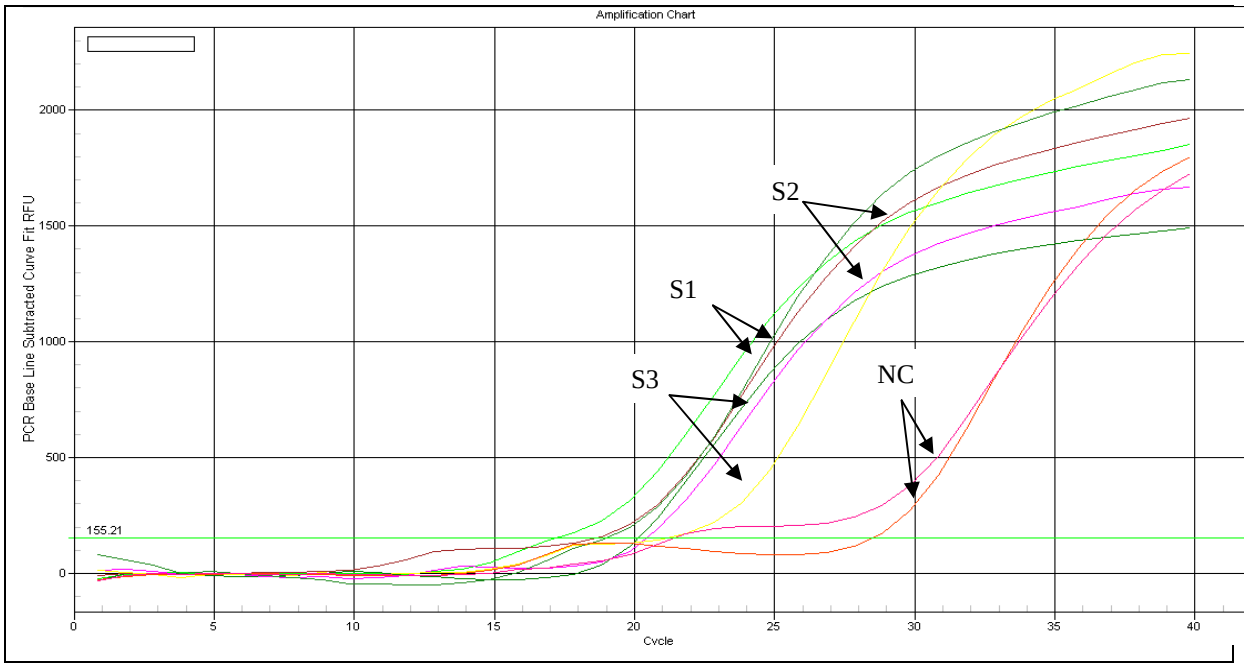

Figure 59: Immuno-PCR with blocking with 2% BSA, Tween 20 and salmon sperm DNA for 1 h

Concentration of lupine protein standards: S1: 100 ng/mL, S2: 1 ng/mL and S3: 0.01 ng/mL

As the figure indicates, the C_t value is not reproducible for the standard of the same concentration; the threshold cycle for the negative controls is significantly higher in comparison to that of the three standards. The experiment was repeated with a change in the blocking time.

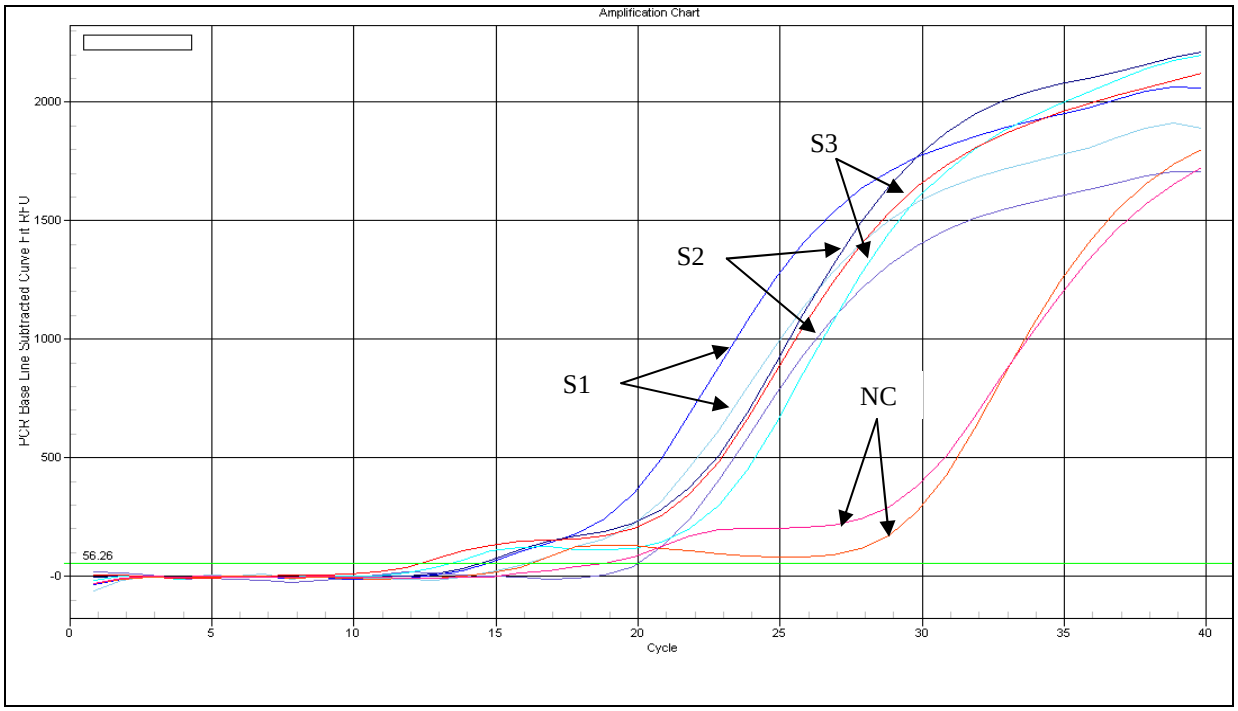

Figure 60: Immuno-PCR with improved blocking with 2% casein, Tween 20 and salmon sperm DNA for 1 h

Concentration of lupine protein standards: S1: 100 ng/mL, S2: 1 ng/mL and S3: 0.01 ng/mL

As the figure indicates, the C_t value for the negative controls is > 27. However, the two curves representing one of the same lupine protein concentration are very dissimilar to each other in terms of their threshold cycle.

The experiment was repeated by overnight blocking and yielded the results depicted by figures 61 and 62.

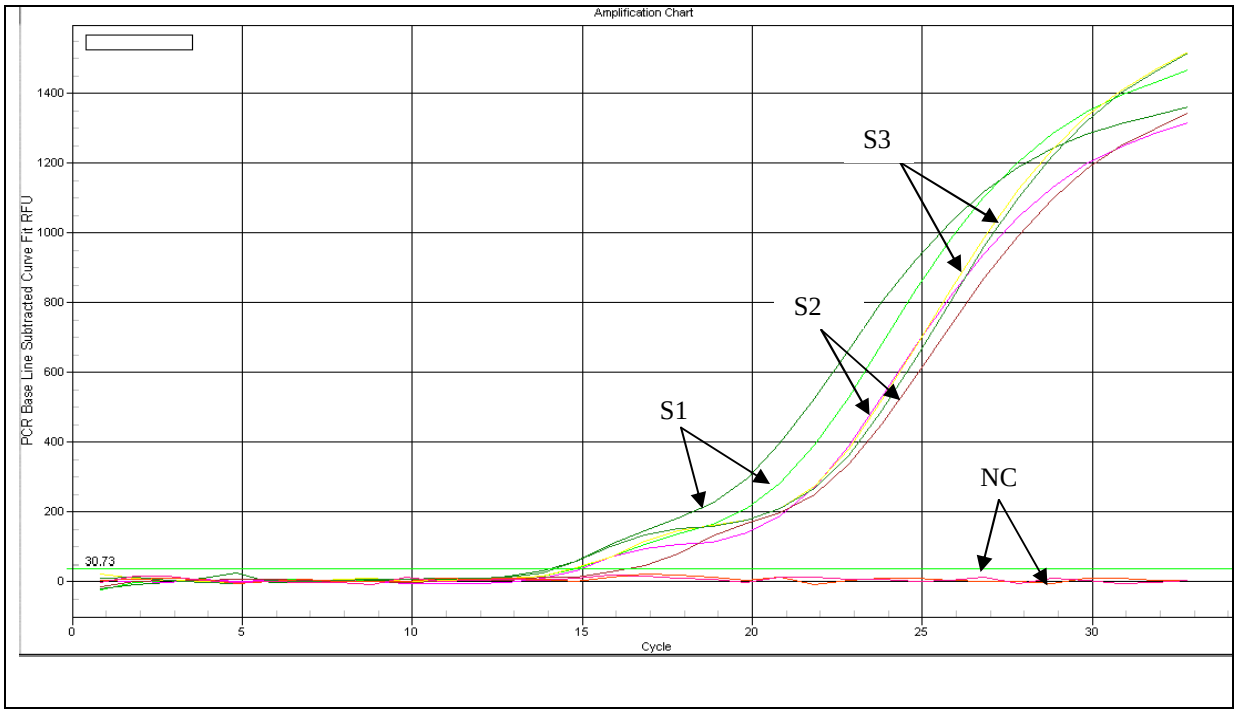

Figure 61:Immuno-PCR with common PCR plates and blocking overnight with 2% BSA, Tween 20 and salmon sperm DNA

Concentration of lupine protein standards: S1: 100 ng/mL, S2: 1 ng/mL and S3: 0.01 ng/mL

As the figure indicates, in this case the two curves representing each of the positive standards are somewhat similar to each other and there is also a slight shift in the C_t value. The negative controls did not result in an increase of the fluorescent signal.

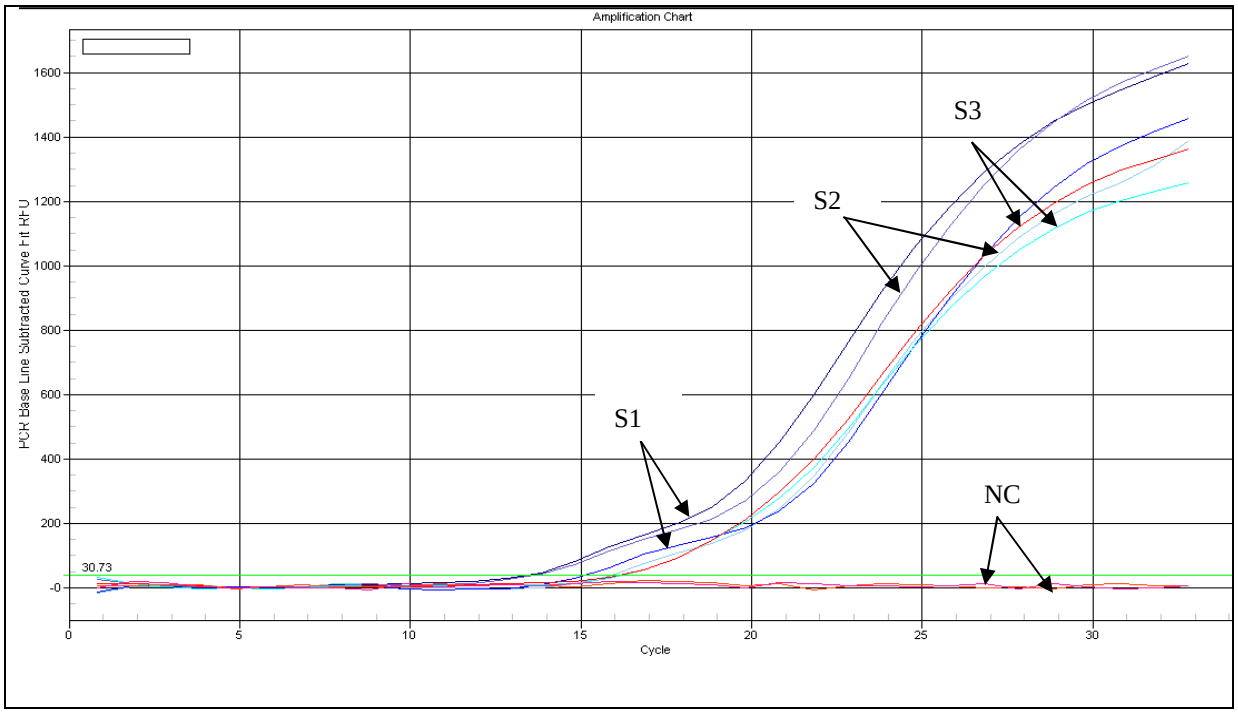

Figure 62: Immuno-PCR with common PCR plates and blocking overnight with 2% casein, Tween 20 and salmon sperm DNA

Concentration of lupine protein standards: S1: 100 ng/mL, S2: 1 ng/mL and S3: 0.01 ng/mL

As the figure indicates, in this case the two curves representing each of the positive standards are somewhat similar to each other and there is also a slight shift in the C_t value and the curves representing the negative controls are below the baseline.

Due to limited time, the experiments were discontinued at this stage although the method was not yet optimized.

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5 EXPERIMENTAL

5.1 Development, optimization and validation of a competitive enzyme linked immuno sorbent assay (ELISA) for sesame

5.1.1 Sesame extract preparation

The reagents used for sesame extraction included n-hexane and phosphate buffer saline (PBS) which was prepared by dissolving 21.25 g of NaCl, 31.15 g of Na_2HPO_4 x 2 H_2O and 3.9 g of NaH_2PO_4 x 2 H_2O in 2.5 L of water and the pH was adjusted to 7.6.

A sesame extract was prepared by grinding 4 g of white peeled sesame seeds, purchased from a local grocery store, in a grinding mill (Model MM 2000, Retsch, Haan, Germany) to get a smooth paste. The paste was then defatted using the soxhlet extraction method with n-hexane for 18 hours. After drying overnight at room temperature the defatted sesame was mixed with 30 mL of PBS buffer and stirred at room temperature for 2 h. This mixture was then centrifuged at 4000 rpm (1500 *g*) for 30 min at 4 °C using a centrifuge machine (Model 4K 10, Sigma, Vienna, Austria). The supernatant was saved and the pellet was thrown away. Several aliquots were prepared from this extract and stored at –18 °C.

5.1.2 Determination of protein concentration using Bradford

The required reagents included Brilliant Blue G dye, bovine serum albumin (BSA), 95% ethanol and 85% phosphoric acid. Two major solutions required were the Bradford stock solution and the Bradford working buffer. The Bradford stock solution was prepared by mixing 100 mL of 95% ethanol, 200 mL 85% phosphoric acid and 350 mg Brilliant Blue G dye. The Bradford working buffer was prepared using 425 mL of bidistilled water, 15 mL of 95% ethanol, 30 mL of 85% phosphoric acid and 30 mL Bradford stock solution.

The Bradford stock solution is stable indefinitely at room temperature. The Bradford working buffer was stored at room temperature after filtration through a black ribbon filter paper and could be reused for several weeks but it had to be refiltered from time to time.

A stock solution of 1 mg/mL BSA was prepared in PBS which was then diluted using PBS to make several standard solutions in the concentration range of 10 µg/mL to 200 µg/mL. The obtained sesame extract was also diluted using PBS. The sesame extract was used undiluted and in dilutions of 1:10 and 1:100. To 100 µL of the standard solution or the sesame extract, 1000 µL of the Bradford working buffer was added and the solution was vortexed. The blank was prepared by adding 1000 µL of the Bradford working buffer to 100 µL of PBS. After waiting for two min each sample was measured at 595 nm using a UV/VIS spectrophotometer (Genesys 10 UV, Thermo Scientific). A calibration curve was established by plotting the absorbance against the known concentration of each. From the curve, the concentration of the unknown sesame extract was calculated using the straight line equation.

5.1.3 SDS - PAGE

5.1.3.1 Reagents and solutions needed

Several reagents were used, these included acrylamide, N, N´- methylene-bisacrylamide, Tris hydroxy methyl amino methane (Trizma base/Tris), sodium dodecyl sulphate (SDS), ammonium persulphate, glycine, 2-mercaptoethanol, 1% bromophenol blue, glycine, Coomassie blue R-250, methanol, acetic acid, N, N, N´ N´- Tetramethylethylenediamine (TEMED) and bidistilled water. The solutions needed were prepared as described in the following text. The **SDS solution** was prepared in two concentrations of 1% and 10% by dissolving 1 g or 10 g sodium dodecylsulphate in 100 mL bidistilled water, respectively. For the **monomer solution**, 29.2 g acrylamide and 0.8 g N, N´- methylene-bisacrylamide were weighed and dissolved in bidistilled water in such a way that the total volume of the solution was 100 mL. A fresh **ammonium persulphate solution** was prepared for each experiment using 20 mg of ammonium persulphate dissolved in 200 µL water to get a 10% solution. A **separation gel buffer** was made using 36.3 g Tris and 2.0 mL 10% SDS, the volume was made up to 200 mL using bidistilled water and the pH was regulated at 8.8 using diluted HCl. The **stacking gel buffer** was prepared using 6.0 g Tris and 1.0 mL 10% SDS, the volume was made up to 100 mL using bidistilled water and the pH was adjusted at 6.8 using diluted HCl. The **denaturation mix** comprised of 2.5 mL of the stacking gel buffer, 2.0 mL of 10% SDS solution, 2.0 mL of glycine, 1 mL of 2-mercaptoethanol and 0.4 mL of 1% bromophenol blue. The volume of the mix was made up to 10 mL using bidistilled water.

The **running buffer** comprised of 0.025 M Tris, 0.192 M glycine and 0.1% SDS solution. For 2.5 L of the buffer, 7.5 g Tris, 35.4 g glycine and 2.5 g SDS was dissolved in bidistilled water in such a way that the total volume was 2.5 L and the pH was adjusted at 8.3. For the **staining solution** the reagents needed included 1% Coomassie blue R-250, 50% methanol and 10% acetic acid. For 2.5 L of the solution, 3.125 g of Coomassie blue R-250 was added to 250 mL acetic acid and 1.25 L methanol and the volume was made up to 2.5 L by adding water. The **de-staining solution I** was prepared using 50% methanol and 10% acetic acid. To make a 2.5 L solution, 250 mL acetic acid and 1250 mL methanol were mixed and the volume was made up to 2.5 L by adding bidistilled water. The **de-staining solution II** was prepared by using 5% methanol and 7% acetic acid, so for a 2.5 L solution 175 mL of acetic acid was added to 125 mL methanol and the volume was made up to 2.5 L using bidistilled water.

5.1.3.2 The gels – separation and stacking gels

Two gels were prepared, the separation gel and the stacking gel. For the separation gel, to 5 mL of the monomer solution, 3.75 mL of the separation gel buffer was added along with 150 µL of the 10% SDS solution. To this mixture 6.0 mL of bidistilled water was added and then it was degassed for 5 min using an ultra sonic bath. After that, 84 µL of freshly prepared 10% ammonium persulphate solution were added. As a catalyst 10 µL of TEMED were added and the total volume was 15 mL. The resulting solution was again degassed for one min using the ultra sonic bath.

The stacking gel was prepared by adding 700 µL of the monomer solution to 1.25 mL of the stacking gel buffer. To this mixture 50 µL of 10% SDS solution was added along with 3.0 mL of bidistilled water. The solution was then degassed using an ultra sonic bath. After degassing, 35 µL of 10% ammonium persulphate solution and 4 µL of TEMED were added. The solution was then degassed again for one min in an ultra sonic bath. The total volume of the solution was 5 mL.

5.1.3.3 Sample preparation for the gel

For preparing samples to load on the gel, 5 µL of the standard protein solution or the sesame protein fraction that had to be analyzed were taken, to this 5 µL of the denaturation mix was

added and heated for 5 min at 70 °C in the oven. A 3.5 µL aliquot was loaded onto a SDS polyacrylamide gel. Precision Plus Protein™ Standards (10-250 kDa; BioRad, Hercules, USA) and a protein mix containing conalbumin, BSA, ovalbumin, myoglobin and IgG, each in a concentration of 1 mg/mL in bidistilled water, were used as molecular weight markers.

5.1.3.4 Procedure for electrophoresis

Electrophoresis was performed by using a Mighty Small SE 250 apparatus (Hoefer Scientific Instruments, Holliston, MA, USA) and a Power Pac (BioRad). First of all a sandwich was prepared, which comprised of an alumina ceramic plate and a glass plate, separated by two spacers. Two such sandwiches were prepared and mounted on the gel caster.

The separation gel solution was filled into the two sandwiches using a syringe. To these two sandwiches, approximately 100 µL of bidistilled water was added to prevent the gel from drying while polymerizing. After 30 minutes, the gel polymerized and the water added was removed.

Then the stacking gel solution was pipetted in both sandwiches and combs were placed in order to make loading wells for the samples. After a 30 min polymerization time the gel caster was opened and the sandwiches were carefully removed and separated in such a way that each sandwich consisted of a glass plate, an alumina ceramic plate and two spacers and the gel was in between the glass and the alumina ceramic plate.

Each sandwich was then fixed, using two clamps on the electrophoresis unit in such a way that the glass plate was on the outer side. The cooling tubes were connected so that the water from the tap can circulate and cool the unit. The lower chamber was filled with the running buffer until 1 cm of each sandwich was submerged in it.

The loading wells were marked before the combs were taken out and then to each well 5 µL of the sample or the standard proteins were loaded.

After loading the samples, the electrophoresis unit was covered with the safety lid and the power cables were connected to the power supply. The electrophoresis was carried out at a current of 40 mA for 50 to 60 min. The run was stopped before the front marker reached the

buffer level in the chamber. The power was switched off and the two sandwiches were separated from the electrophoresis unit.

The glass plate was separated from the alumina ceramic plate, the spacers were also removed and the stacking gel was removed using a knife. The gels were carefully detached from the glass plates and placed into a china dish filled with the staining solution. The staining was carried out at 40 °C for 30 min. The staining solution was removed and the gels were flushed with bidistilled water. The gels were then placed in de-staining solution I for 1 hour and later in de-staining solution II until the background was low enough to see bands. Finally, a photograph was taken.

5.1.4 Immunization of hens to produce anti-sesame antibodies

IgY antibodies against sesame were produced at the Department of Biochemistry and Cell Biology, University of Vienna, Austria. Two hens were immunized with a fraction of the sesame extract in a concentration of 1 mg/mL in PBS for this purpose, repeating the initial injection every 30th day. The eggs were stored at 4 °C.

5.1.5 Isolation of antibodies (IgY)

From the yolks of the eggs, obtained a week after the immunization, the antibody fraction was then isolated and stored in small aliquots at -18 °C and later used for ELISA. The details of isolation are as follows.

After separating egg yolk and egg white and removing the vitelline membrane the volume of the egg yolk was measured. To the yolk an equal volume of PBS buffer was added and it was shaken for 30 min. The mixture was then centrifuged for 15 min at 4000 rpm (1500 *g*) using a centrifuge machine (Model 4K 10, Sigma, Vienna, Austria) at room temperature. After centrifugation the supernatant was filtered using a gauze bandage.

The antibodies were isolated from the filtrate using the ammonium sulphate/caprylic acid precipitation method.[1] The reagents required included 10 times concentrated PBS, 0.1 M NaOH, 1.0 M NaOH and 60 mM acetate buffer with pH adjusted to 4.0.

The separated yolk was centrifuged for 3 minutes to remove already precipitated proteins. To the supernatant, 60 mM acetate buffer was added; the volume of the acetate buffer added was

4 times the volume of the supernatant. The pH of the buffered supernatant was then adjusted to 4.5 using 0.1 M NaOH.

To this supernatant, caprylic acid (octanoic acid) was added drop wise. The volume of caprylic acid added was 25 µL per mL of the supernatant. The solution was then further stirred for 30 min. It was then centrifuged at 4000 rpm for 30 min to remove any insoluble material.

The supernatant obtained was then filtered using a black ribbon filter paper. To this purified supernatant, 10 times concentrated PBS buffer was added in such a way that 10 parts of the supernatant were mixed with one part of the 10 times concentrated PBS. Later, 1.0 M NaOH was added in order to adjust the pH to 7.4.

The supernatant was then cooled on ice for 15 min, to the cooled supernatant ammonium sulphate was added slowly in such a way that a 45% of the saturation concentration was achieved. For this, 0.277 g of ammonium sulphate was added per mL of the supernatant. The solution was stirred for 30 min and then stored overnight at 4 °C.

The precipitates obtained were then centrifuged the next day at 4000 rpm (1500 *g*) for 15 min. The pellet obtained after centrifugation was then dissolved in a very small volume, i.e. 200-500 µL of PBS.

The resuspended IgYs were then dialysed with PBS in order to remove ammonium sulphate. For this purpose dialysis tubes (cut off, 12000-14000 Da) were used, they were filled with the resuspended IgY using magnetic clamps to close the ends of the tube. These were then immersed in at least 500 mL of PBS and stirred at a very low speed. The PBS used to dialysis was changed every hour. After repeating this four times the dialysis tube was immersed in PBS and left as such overnight. The next day the volume was estimated and the concentration of the antibodies was determined using the Bradford assay in the same way as explained in section 5.1.2 with the exception that the Bradford assay was carried out using immunoglobulin G (Sigma) as standard. The antibodies were stored in small aliquots at -18 °C.

5.1.6 Titer determination

Titer determination was carried out with the yolks of eggs laid seven days after each immunization. Several buffers were also used; the coating buffer consisted of 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$ and 0.2 g of NaN_3 in 1 L of bidistilled water. The washing stock solution was prepared by adding 51 g of NaCl, 5.89 g of KH_2PO_4 and 30 mL of Tween 20 to 1 L of bidistilled water and the pH was maintained at 7.6. The washing buffer was prepared by taking 30 mL of this washing stock solution and making up the volume to 2 L using bidistilled water. The blocking buffer comprised of a 2% (w/v) casein solution in PBS. The citrate buffer was prepared by adding 46.04 g of potassium dihydrogen citrate and 0.1 g of sorbic acid in 1 L of water. Tetramethylbenzidine (TMB) solution was prepared by adding 0.375 g of TMB to 5 mL of DMSO and 20 mL of methanol. The substrate solution was made by adding 500 µL of TMB and 100 µL of 1% H_2O_2 to 25 mL of the citrate buffer. The stopping solution comprised of 0.5 M H_2SO_4. Flat-bottom polystyrene microtiter plates (Maxisorp F96, Nunc, Wiesbaden, Germany) were used. For washing the microtitre plate, an immuno wash (Bio-Rad model 1575) was used for washing each well four times. The microtitre plate reader was also from Bio-Rad (model 680 XR).

For the titer determination, each well of a plate was coated with 200 µL of the coating solution, for 16-18 hours at 4 °C. The coating solution comprised of sesame protein as the coating antigen in the coating buffer in a concentration of 0.5 µg/mL.

The plate was then washed using the washing buffer in the immuno wash, the binding sites were then blocked for 1 hour using a 2% (w/v) casein solution in PBS as the blocking buffer. After this, 100 µL of the isolated IgY from the egg yolk was loaded in each well and incubated for 30 minutes. The dilutions used ranged from 1:500 to 1:50000. IgY isolated from the egg yolk obtained before immunization was also loaded on the plate for comparison. Detection was carried out by using commercially available rabbit antibody raised against IgY, labeled with horseradish peroxidase. So, 200 µL of this secondary antibody were added, after the washing step, in a dilution of 1:30000 and incubated for 1 hour. After a washing step, 200 µL of the substrate solution was added to each well, after the development of the blue colour, 100 µL of the stopping solution was added and the OD was measured at 450 nm.

5.1.7 Sample preparation for protein extraction of food samples

The sample extraction buffer was prepared by dissolving 6.06 g of Tris and 11.69 g of NaCl in 1 L of water, adjusting pH to 8.2 using 1 M HCl.

All samples were purchased from local supermarkets. A wide range of commercially available food products were chosen to determine the applicability of the developed ELISA, these included samples which either contained sesame or were free of sesame. Some of the chosen samples were also labeled with “may contain sesame”. Various nuts, legumes and other food stuffs including peanut, hazelnut, walnut, Brazil nut, almond, sunflower seed, poppy seed, rice, wheat, rye, oat, chocolate and honey were analyzed for cross reactivity. Also, for spiking experiments, recovery studies and to investigate the matrix effect, the same sample extraction method was followed. To determine whether the matrix influences the ELISA results or not, different matrixes like fresh breads, crisp toasts, cookies and cracker were taken and the samples were extracted.

Each sample was grinded in a grinding mill for homogenization. To 10 g of the grinded sample, 50 mL of the sample extraction buffer was added. The resulting mixture was then homogenized using an ultra turrax (Model T25, IKA, Staufen, Germany) for 2 min. The acquired paste was then centrifuged at 4000 rpm (1500 *g*) for 30 min. The supernatant obtained was filtered using a black ribbon filter paper and again centrifuged at 10000 rpm for 5 min (Model 5424, Eppendorf, Hamburg, Germany). After filtering the resulting supernatant several aliquots were made and stored at -18 °C.

5.1.7.1 Preparation of roasted sesame

Thirty g of white, peeled sesame seeds was roasted at the Department of Food Sciences and Technology of the University of Natural Resources and Applied Life Sciences, Vienna, Austria. Roasting was carried out for 10 min at four different temperatures: 100 °C, 150 °C, 200 °C and 250 °C, using a baking oven (type 60/3 W, Manz Backtechnik, Münster, Germany). 10 g of the roasted sesame were then subjected to extraction as described above and the protein concentration in the extract was determined using the Bradford assay. The extracts were diluted 1:20 with PBS and analyzed by the ELISA.

5.1.8 ELISA - optimized procedure

For the competitive ELISA, various reagents, buffers and apparatus used are mentioned in 5.1.6.

For carrying out an ELISA, each well of the plate was first coated with 200 µL of the coating solution that comprised of 0.22 µg/mL sesame protein extract in the coating buffer. The coated plate was covered with para film and stored at 4 °C for 16-18 hours.

The plate was then washed with the washing buffer using the immuno wash. The binding sites of the wells were then blocked for 15 min with 200 µL of the blocking buffer, which comprised of a 2% (w/v) casein solution in PBS. This was done to prevent non specific binding. After blocking the plate, the competitive step was carried out by loading, in each well, 50 µL of the sesame protein standards, in the range of 0.001 ng/mL to 10000 ng/mL, or sample extracts, both in triplicates. All sample extracts were used in a dilution of 1:20 in PBS buffer. Next, 100 µL of the primary antibody was added in a dilution of 1:2000 in PBS buffer to all wells except the ones on the top and bottom outer sides. Along the outer sides of the plate, the wells were filled with 150 µL of PBS buffer for the determination of any non specific binding (NSB) or with 100 µL of the diluted primary antibody and 50 µL of the PBS buffer for the determination of the maximum signal (Bo) in the absence of any sesame protein. The incubation was carried out for 45 min at room temperature.

Subsequently the plate was washed in the immuno wash and 200 µL per well of the horse radish peroxidase labeled rabbit anti – IgY, in a dilution of 1:30000 in PBS, was added. After an incubation of 1 hour the plate was washed. Following washing, 200 µL of the substrate solution was added and the enzymatic reaction was carried out for 12-15 min until the blue colour was developed. After colour development, 100 µL per well of the stopping solution was added to stop further colour development. The optical density (OD) was measured at 450 nm using microtitre plate reader at a medium speed with a mix time of 5 s.

5.1.9 Data Analysis

The data obtained was evaluated using software, Sigma Plot 10.0. A sigmoid curve was obtained by plotting the OD values against the logarithm of the sesame protein concentration

and a sigmoidal four parameter logistic function was used for non-linear regression; the equation employed was:

$$y = yo + \frac{a}{1+(^{x}/_{xo})b} \qquad (6)$$

where y is the response, yo is the response at high dose, a is the response at zero dose, b is the slope factor, x is the calibrator concentration, x_0 corresponds to the value at 50% specific binding.

5.1.10 ELISA optimization

In the development of the ELISA, several parameters had to be optimized, e.g. concentrations of primary and secondary antibodies, concentration of the coating antigen, and coating temperature. Initially the concentration of the primary antibody was optimized. For this purpose an ELISA was carried out using four different primary antibody (PAB) dilutions, these included 1:5000, 1:7500, 1:10000, and 1:15000.

As a second step the coating antigen dilution and the coating temperature were optimized. In this case four different coating antigen dilutions were prepared, these included 1:6000, 1:12000, 1:18000, and 1:24000. These were then loaded onto two plates; one was kept overnight at 36 °C in an oven and the other at 4 °C in a refrigerator.

After that the various incubation periods were also optimized. First of all the blocking time was optimized for this purpose, a blocking time of 15 min, 30 min or 45 min was used on a single plate. Then the primary antibody incubation time was optimized, for this purpose incubation was carried out for 30 min, 45 min and 60 min.

5.1.11 Cross reactivity studies

For cross reactivity tests the extracts were acquired from several nuts and legumes such as peanuts, hazelnut, walnut, Brazil nut, almond, sunflower seeds, poppy seed, rice, wheat, rye and oat and other ingredients such as chocolate and honey. The food extracts were prepared by the extraction method described in section 5.1.7. The protein concentration of these extracts was determined by the Bradford assay, the extracts were then diluted to achieve concentrations between 0.001 ng/mL - 10000 ng/mL. An ELISA was then carried out by loading diluted extracts and the sesame standards in the range between 0.001 ng/mL - 10000

ng/mL. In order to calculate the cross reactivity, the absorbance (A) at 450 nm was normalized using the following equation:

$$A_{corr} = \frac{A - NSB}{A_o - NSB} \qquad (7)$$

A is the absorbance obtained, NSB (non specific binding) was determined by carrying out the immunoassay in the absence of the primary antibody as well as the analyte with only PBS buffer to make up for the volume. A_o was determined by carrying out the immunoassay with the primary antibody and no sesame protein at all. Cross reactivity (%) was then calculated by dividing the 50% binding concentration of sesame by the 50% concentration of the cross reactant and multiplying by 100.

5.1.12 Recovery studies

In order to investigate the recovery of sesame protein in different food matrices, recovery studies were carried out on commercially available blank food samples which did not contain any sesame and were spiked with known amounts of sesame protein before analysis. Spiking of samples was carried out in two ways, one method was to spike the sample extract just prior to the ELISA. The second method was to spike samples with sesame protein before sample extraction.

5.1.12.1 Spiking with sesame proteins after sample extraction

Ten g of the blank sample were homogenized and the extraction was carried out with 50 mL of the extraction buffer as described in section 5.1.7. The extract was diluted 1:20 with PBS and then sesame protein extract was added to obtain a sesame protein concentration of 200 µg/g. From this spike level, the other spike levels of 100 µg/g, 50 µg/g and 25 µg/g were obtained by diluting with the 1:20 diluted extract of the certain food matrix. The recovery was calculated from the ratio of the amount of sesame protein determined to the amount of sesame protein actually added to the sample.

5.1.12.2 Spiking with sesame proteins before sample extraction

After grinding, 10 g of the homogenized blank food were spiked with sesame protein at levels of 25 µg/g, 50 µg/g, 100 µg/g and 200 µg/g.Following an incubation step of 15 min, the protein fraction was extracted from the spiked samples as described in section 5.1.7. After diluting 1:20 with PBS, the extracts were subjected to ELISA analysis. The recovery was calculated from the ratio of the amount of sesame protein determined to the amount of sesame protein actually added to the sample.

5.1.13 Method validation

5.1.13.1 Repeatability

For the validation of the developed ELISA, same samples were loaded on the plate on 3 or 4 different days to check for repeatability. Also, at times the same loading scheme was used twice on the same plate.

5.1.13.2 Limit of detection and limit of quantification

In order to determine the limit of detection (LOD) and the limit of quantification (LOQ) of the ELISA, the extracts of blank food matrices which were diluted 1:20 with PBS were filled in six wells of a microtiter plate and subjected to ELISA analysis. The LOD of the ELISA was calculated by subtracting three times the standard deviation of the obtained OD values from the mean OD value and calculating the corresponding concentration by using the equation of the calibration curve established with sesame protein standard solutions. The LOQ was calculated in the same way but by subtracting ten times the standard deviation of the OD value.

5.1.14 Analysis of commercial food samples

To demonstrate the applicability of the developed ELISA, 28 commercially available food samples, with different declarations, such as “may contain sesame”, “contains sesame” and some with no mention of sesame in the ingredient list were analyzed. Commercial food stuffs were extracted as described above. The extracts were diluted with extracts from the certain blank food matrices, 1:20 diluted with PBS. The dilution factors ranged from 1:5 to 1:500.

5.2 Development and validation of a QCM immuno sensor for the detection of potentially allergenic sesame proteins

5.2.1 Anti-sesame antibodies

For the development of the immuno sensor, anti-sesame IgY antibodies were used. The antibodies were prepared by immunizing hens with sesame protein extract, the details of which can be found in section 5.1.4. The antibodies were isolated from the egg yolks and purified using the ammonium sulphate precipitation method; details are given in section 5.1.5.

5.2.2 The quartz, sieve designing and silk–screen printing

AT-cut quartz crystals with a fundamental resonance frequency of 10 MHz were purchased from Zheijiang Quartz Crystal Electronic Company, Shanghai, China.

A silk screen was produced using the optical lithographical procedure. A 21 µm mesh size silk filter was glued to a hard metalloid frame. Then Azocol Poly plus S by KIWO, which is a UV sensitive polymer, was spread over the screen and dried for 20-30 minutes in the dark. The desired area was then exposed to UV lamp for 30 minutes; warm water was used to remove the unhardened lacquered area. This screen was then used for printing purposes.

Particular electrode geometry was generated on the quartz via silk-screen printing of the gold paste with the help of a designed sieve. Figure 63 shows one such setup.

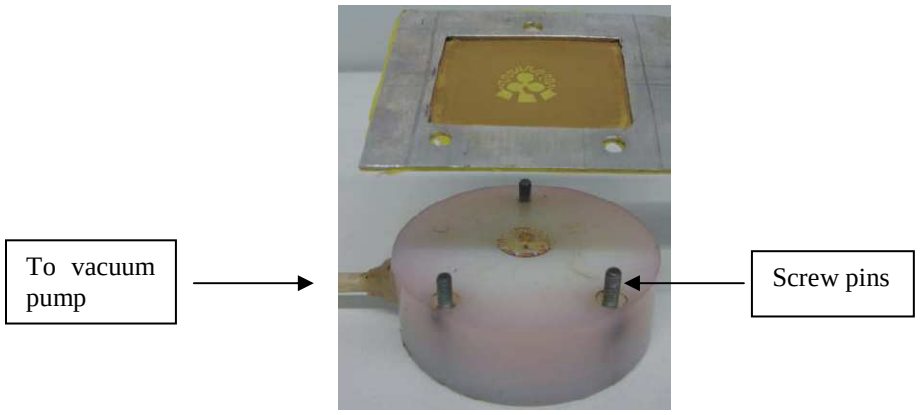

Figure 63: Setup for quartz coating

Quartz was placed onto a Teflon block; the prepared screen was placed over it and adjusted by using screw pins and holes on the block. A vacuum pump was connected to the Teflon block which when switched on, prevented the quartz from moving.

5.2.3 Quartz coating with gold paste

After fixing the quartz, a layer of gold paste was finely spread over the screen surface in such a way that a very thin gold layer was coated on the quartz. After coating the quartz from one side it was heated in the oven for 3 hours at 400 °C. After cooling, the other side of the quartz was coated in the same way to get the double electrode structure. The prepared quartz was then cleaned with 1-methyl-2-pyrolidone, in order to get rid of any organic components since the gold paste consisted of gold in organic oil. One side of the coated quartz was cleaned with the diamond paste; this side was later used for antibody immobilization.

5.2.4 Checking quartz for damping

The size of electrodes on either side of the quartz was different; on one side it was larger than the other. This was done in order to reduce the damping. The same size of electrodes on both sides of the quartz results in higher damping as in this case, crossing of field lines into the quartz is higher. The frequency of the prepared quartz was measured with the help of a network analyzer of the ENA series of Agilent Technologies (model no. E5062A). The network analyzer helped to determine the damping of the quartz. Figure 64 shows the setup of a network analyzer measurement and the damping spectra of a quartz microbalance.

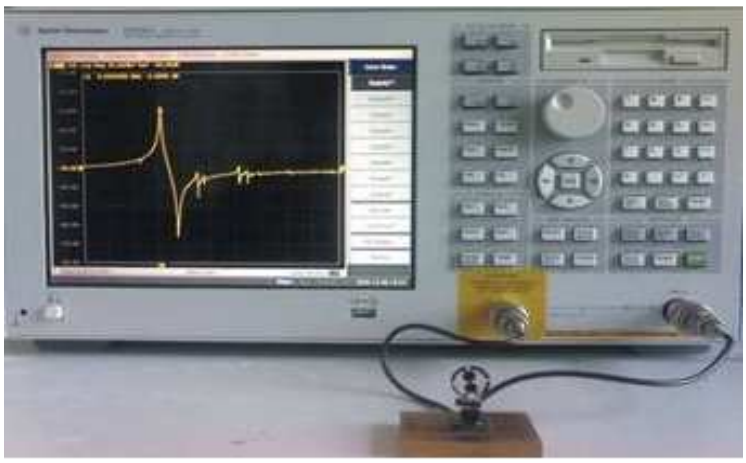

Figure 64: Network analyzer setup and damping spectra

5.2.5 The measurement cell

The prepared quartz was mounted in the measurement cell. The measurement cell protected the quartz crystal microbalance (QCM) from external influences such as thermal or mechanical, ensuring a stable environment for carrying out measurements. Figure 65 shows a quartz mounted in such a measurement cell with an inlet and outlet for the solution and the connections to the oscillator circuit.

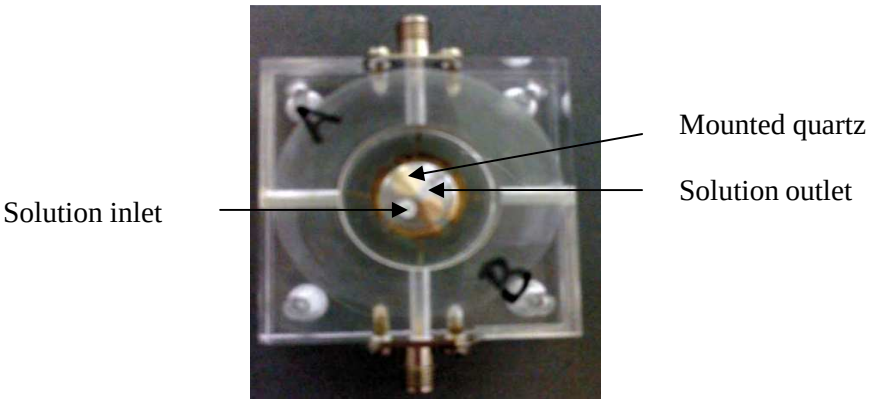

Figure 65: Measurement cell

5.2.6 Mass-sensitive measurements

The designed QCM sensor consisted of a dried and heat treated quartz microbalance, a sensitive layer on one electrode and a reference layer on the other. Using the measurement cell shown in figure 65, mass sensitive measurements were carried out. The solution inlet was used to inject the solution; approximately 180 µL of the solution was injected using a pipette. The solution outlet was used to remove the solution from the measurement cell. During the measurement only one face of the QCM came in contact with the sample solution i.e. the aqueous solution. Therefore, the damping of the quartz was half to what the damping would be if quartz would be in contact with the liquid on both sides. After injecting the solution, as the solution or water flushed through, it took a few minutes to get the frequency signal stable.

5.2.6.1 Antibody immobilization and formation of the antibody-antigen bond

Initially deionized water was injected in the measurement cell, after the frequency signal stabilized the water was removed and an anti-sesame IgY antibody solution was added in a concentration of 2 mg/mL. After removing the antibody solution, sesame protein extract in the concentration of 1.5 mg/mL was injected. Both the solutions of the antibody and the antigen were made using deionized water.

5.2.6.2 Elution step and regeneration of the antibodies

After the formation of the antibody-antigen complex, the sesame protein solution was removed using a pipette and a 2 M guanidine hydrochloride solution was injected in the cell. After waiting for five min, it was removed; deionized water was then injected three times to wash away any remaining guanidine hydrochloride. After removing the water, PBS was added to regenerate the antibodies. This was done for 5 min and then PBS was removed and the cell was washed with deionized water again before the next experiment was carried out. For leaving the sensor overnight, PBS was injected and the sensor was stored at 4 °C.

5.2.6.3 Washing step

For measurements concerning cross reactivity tests and testing commercial samples a washing step was included. For this, the sample extract was added, after 5 min it was removed and deionized water was added and the measurement was carried out. In this way all measurements were carried out in the same medium.

5.2.6.4 Cross reactivity tests

For cross reactivity studies, fourteen different foods and food ingredients were tested. These included almond, Brazil nut, peanut, hazelnut, walnut, sunflower seeds, poppy, rice, oat, chocolate, rye, wheat, honey and soybean. All of the above mentioned extracts were injected in the measurement cell in a concentration of 1 mg/mL. In a cross reactivity experiment sesame extract was injected in the beginning, the concentration of sesame protein was varied; it was 1 mg/mL in certain cases and 3.5 mg/mL and 0.88 µg/mL in other instances. Then a sample of the food extract from the fourteen samples was injected followed by the injection of a spiked food extract of the same sample and then the measurement was carried out.

Spiking of the blank food extract was done by adding sesame protein extract in a certain concentration corresponding to the concentration of the sesame extract added in the beginning. In between each measurement, the washing step and the elution step was also incorporated.

5.2.6.5 Analysis of roasted sesame

Sesame seeds were roasted and extracted as explained in section 5.1.7.1. For mass sensitive measurements, the extract was then injected in the measurement cell in the concentration of 1 mg/mL for sesame roasted at all temperatures except the one roasted at 250 °C, which was injected undiluted. After sample injection the measurement was carried out. In between each injection of the sesame extract, the elution step was incorporated to break the antibody-antigen bond.

5.2.5.6 Validation of the sensor - Reproducibility and repeatability

The inter day repeatability was determined by injecting the sesame protein extract in a concentration of 1 mg/mL twice on one day and twice on the other day and the signal response was measured.

The batch to batch reproducibility or the inter assay reproducibility was determined by immobilizing a new antibody layer and injecting sesame protein extract in a concentration of 500 µg/mL in the measurement cell on three days and comparing the sensor response.

To determine the limit of detection two experiments were carried out. In one, sesame extract in PBS buffer in concentrations ranging from 3.5 mg/mL to 0.007 mg/mL was added in the measurement cell and the mass sensitive measurement was carried out. In between each addition elution was carried out using 2 M guanidine hydrochloride solution and PBS was added and incubation was carried out for 5 min to regenerate the antibodies. For the other experiment, sesame extract was added in a concentration range from 3.5 mg/mL to 0.007 mg/mL but it was diluted with a blank matrix of whole grain bread which was prepared using the extraction method explained in section 5.1.7. In this experiment an elution step was incorporated in between each addition of the sesame extract. Also a washing step was included to remove the matrix and to carry out the measurement in deionized water.

5.2.5.7 Analysis of commercial food stuffs

The selected samples were of three types, depending on their labeling. Some samples were positive i.e. they contained sesame, these included sesame balls, sesame peanut cookies and sesame cashew nut cookies. Other samples chosen had a labeling 'may contain sesame', these included muesli and biscuits. The last category was of negative samples i.e. samples without any sesame, these included cereals and crackers.

Samples were extracted as explained in section 5.1.7, the extracted sample was injected through the solution inlet. After 5 min it was followed by the washing step that is removing the extract and injecting water, the measurement was then carried out.

5.3 Development and validation of real-time polymerase chain reaction (PCR) method for poppy *(papaver somniferum)*

5.3.1 Reagents and buffers

The reagents required include sodium chloride (NaCl), ethylenediaminetetraaceticacid (EDTA), trishydroxylmethylaminomethane (Tris), 70% ethanol, chloroform, isopropanol and bidistilled water that was purchased from Fluka while all other reagents were purchased from Sigma. The **CTAB extraction buffer** was prepared by making a solution of 20 g of cetyltrimethylammoniumbromide (CTAB) in 1 L bidistilled water (H_2O_{dd}) along with 1.4 M NaCl prepared by dissolving 81.03 g of NaCl in 1 L of water and 0.1 M Tris and 0.2 M EDTA. HCl was used to maintain the pH at 8. The **CTAB precipitation buffer** was made by making a solution of 5 g/L of CTAB and 0.04 M NaCl. It was prepared by dissolving 2.34 g of NaCl and 5 g of CTAB in 1 L bidistilled water. The **proteinase K** solution was made by dissolving 100 mg of proteinase K in 4 mL of 50% glycerine in 10 mM Tris-HCl with pH adjusted to 8. It was then stored at -20 °C. The **RNase** was obtained from Sigma in a concentration of 29 mg/mL, it was then diluted to get a concentration of 20 µg/mL using 50% glycerine in 10 mM Tris-HCl with pH adjusted to 8.

5.3.2 DNA extraction

For all PCR experiments the DNA was isolated from food stuffs following the CTAB extraction method with some modifications concerning the incubation times, the details of these modifications are given in the following text. For each sample three extractions were carried out and the best ones were chosen with regards to highest purity and yield. The extraction procedure is explained in detail in the following text.

First of all, the sample was homogenized, for this purpose, depending on the nature of the sample, mortar and pestle or a kitchen blender was used. Then 100 mg of the homogenized sample was weighed, to it 500 µL of the CTAB extraction buffer was added. It was incubated at 65 °C for one hour instead of the 30 min incubation time for the normal CTAB method. To this 15 µL of the proteinase K solution was added and incubated at 50 °C overnight, instead of the 1 hour incubation time followed in the normal CTAB method. After vortexing 20 µL of RNase was added to each sample and vortexed again. After waiting for 4 min, each sample was centrifuged for 5 min at 10000 rpm. After centrifugation, the pellet was thrown away and the supernatant was transferred into another Eppendorf tube. To each sample then 200 µL of chloroform was added and each sample was vortexed for at least 45 s. Each sample was again centrifuged for 5 min at 10000 rpm and the supernatant was collected and transferred in another eppendorf tube. To this the CTAB precipitation buffer was added such that the volume of the precipitation buffer was twice to that of the sample obtained after centrifugation. Samples were then vortexed followed by an incubation time of 2 h. After the incubation period was over, each sample was again centrifuged at 10000 rpm for 5 min. After centrifugation the supernatant was discarded ant the pellet was saved. To the pellet 350 µL of 1.2 M NaCl and 350 µL of chloroform was added followed by vortexing and eventually centrifugation at 10000 rpm for 5 min. After centrifugation the upper aqueous phase was saved and the chloroform phase was discarded. Then isopropanol was added to each sample in a volume 0.6 times of the sample collected, followed by vortexing and centrifugation at 10000 rpm for 5 min. The pellet was saved and isopropanol was discarded. 500 µL of ice cold 70% ethanol was then added followed by vortexing and centrifugation at 10000 rpm for 5 min. The ethanol was then removed and samples were dried overnight at room temperature.

5.3.2.1 Determination of concentration and purity of the extracted DNA

The next step was the determination of concentration and purity of the obtained DNA extracts. For this purpose a spectrophotometric determination was carried out. 16 µL of the DNA extract and 64 µL of H_2O_{dd} were directly added to a UV microcuvette so the total volume for each was 80 µL after a 1:5 dilution. H_2O_{dd} was used for all dilutions and as well as a blank. The absorbance was measured at 260 nm and at 280 nm using a UV spectrophotometer. The concentration of each sample was determined using the formula given below

$c\ [\mu g/ml] = A_{260} * V * F$

c = concentration

A_{260} = absorbance at 260 nm

V = dilution factor, which in this case was 5

F = is a constant i.e. the multiplication factor for a double stranded DNA, is equal to 50.

The purity was measured using the following equation:

$Purity = A_{260}/A_{280}$

Where A_{260} is the absorbance at 260 nm and A_{280} is the absorbance at 280 nm.

5.3.3 Real-time PCR

All PCR experiments were performed in an iCycler thermocycler, with IQ5 multicolor real-time PCR detection system, both the thermocycler and the detection system were from BioRad. As a general approach, a total volume of 25 µL was pipetted into the 96 well PCR plates; this was then closed with an adhesive foil before being placed in the thermocycler. All analyses were carried out loading doublets and for each analysis two positive controls and at least two negative controls were loaded onto the plate. For the DNA to be analysed, the desired concentration was obtained by carrying out dilutions using autoclaved double-distilled water.

5.3.4. Primer and probe design

Three primer pairs were designed using computer software programs. The allergen sequence for poppy is not known so initially NCBI, National Centre for Biotechnology Information was used via www.ncbi.nlm.nih.gv and the accession number was obtained. By clicking on BLAST the Basic Local Alignment Search Tool and then on nucleotide BLAST and entering the accession number nucleotide search was also carried out.

The software used was Beacon Designer 7.0 by Premier Designed Biosoft International. Using BLAST, homology to other plant and animal sequences was checked and excluded. Three different primer pairs including forward and reverse primers were designed using the software. The primers were synthesized by Sigma Genosys (Steinheim, Germany).

The criteria for primer selection includes that the primers should comprise of 18-30 basepairs and the GC content should be 40-60%. Not more than 4 identical bases are to be used as this could lead to an unspecific binding. The melting temperature should be between 55-80 ° C such that an amplicon size of 75-150 bp is obtained.

The probe, complimentary to the sequence of the DNA template was designed using Beacon designer. The TaqMan-probe should have a length of 20-30 bases and a GC content of 40-60%. There should not be more than 3 consecutive identical bases, especially not Gs. A string with more Cs than Gs in the probe sequence would be preferable. At the 5' end of the probe there should be no G and at the 3' end there should be no more than 3 Cs or Gs within the last 5 bases. The probe was labeled with the fluorophor FAM at the 5' end and with the quencher BHQ1 at the 3' end. Care was taken that there would be no complementarity to the primers and no secondary structure to primers or the probe in the target area. The melting temperature should be 5 to 10 °C higher than the one of the primers.

5.3.5 General pipetting scheme

On each well of the PCR plate 12.5 µL of the supermix was added, then 7.5 µl of the mastermix was added and finally either 5 µL of poppy DNA for positive control or that of sample DNA or 5 µL of H_2O_{dd} were added for negative control. For every measurement positive and negative controls were always loaded on the plate.

5.3.6 SYBR Green assay

SYBR Green assay was conducted for the optimization of the poppy primer concentrations and the annealing temperature; it was also used for testing the primer pairs for any cross reactivity with other food stuffs.

5.3.6.1 Primer concentration and annealing temperature optimization

For primer pair concentration and annealing temperature optimization, 12.5 µl of SYBR Green supermix, 7.5 µl mastermix and 5 µL sample or H_2O_{dd} was used. The mastermix is the mixture that contains the primers in appropriate concentrations and H_2O_{dd}. The 5 µl of H_2O_{dd} was used for the negative control. The PCR run was carried out in a two-step real-time PCR program. The initial denaturation was carried out at 95 °C for 3 min. Then 45 cycles were run, each cycle consisting of denaturation at 95 °C for 20 s, followed by primer annealing at 61.5 °C for 40 s and elongation at 72 °C for 11 s. The temperature program in this case comprised of 81 cycles at the temperature range of 55 - 95 °C for 10 s. The increase in the signal is detected at the elongation stage whereas the melt point analysis detects the decrease in signal. Both the C_t values and the T_m values were used for evaluation of the results obtained.

For the optimization experiment nine different primer pair concentrations and six different annealing temperatures T_m, were analysed for figuring out the optimum T_m and primer concentrations. For the primer pairs, the primers were dissolved in H_2O_{dd} according to the directions by the supplier to obtain a concentration of 100 µM and diluted 1:10 prior to use in the PCR. Table 17 indicates the different primer concentrations that were used. The six temperatures used were 62.0, 60.3, 58.4, 55.9, 54.1 and 52.8 °C. The volumes indicated in the table were added per well of the PCR plate (PCR plates 96 well by BioRad).

Table 17: Pipetting scheme for primer pair concentration and T_m optimization

Reagents	Primer pair concentration (forward/ reverse) (nM)								
	100/100	**100/200**	**100/300**	**200/100**	**200/200**	**200/300**	**300/100**	**300/200**	**300/300**
Forward primer (µL)	0.25	0.25	0.25	0.5	0.5	0.5	0.75	0.75	0.75
Reverse primer (µL)	0.25	0.5	0.75	0.25	0.5	0.75	0.25	0.5	0.75
H_2O_{dd} (µL)	7.00	6.75	6.5	6.75	6.5	6.25	6.5	6.25	6.00
Total volume (µL)	25								

5.3.6.2 Cross reactivity tests

For cross reactivity tests various kinds of food stuffs were chosen, these included various nuts and legumes including sesame, black sesame, oat, wheat, lentils, corn, macadamia, peacan, hazelnut, peanut, pistachio, chocolate, soya, cashew, sunflower, chick pea, rice, almond, walnut, rye, Brazil nut, peas, barley, pine nut, pumpkin seed, coffee, lupine and white beans. For each of them the DNA was extracted and a PCR analysis based on SYBR Green including a melt curve analysis was conducted to rule out the possibility of a cross reaction. In each well of the PCR plate, 12.5 µL of SYBR Green supermix, 7.5 µl mastermix and 5 µL sample DNA in a concentration of 20 ng/µL was added. For a positive control 5 µL of poppy DNA was added and for a negative control 5 µL of H_2O_{dd} was added. The results obtained for all samples were compared with the positive and negative controls. The melt point analysis was included in the program in this case. The PCR run was carried out in a three-step real-time PCR program. The initial denaturation was carried out at 95 °C for 3

min. Then 50 cycles were run, each cycle consisting of denaturation at 95 °C for 20 s, followed by primer annealing at 60.3 °C for 40 s and elongation at 72 °C for 10 s. The melting temperature program in this case comprised of 81 cycles at the temperature range of 55 - 95 °C for 10 s.

5.3.6.2.1 Agarose gel electrophoresis

Agarose, 10 mg/mL ethidium bromide (EtBr), 50x Tris-acetate-EDTA (TAE) buffer prepared by dissolving 242 g Tris, 57.1 mL acetic acid, 100 mL of 0.5 M EDTA in 1 L H_2O_{dd}. This was diluted 1:50 prior to use. The loading buffer was prepared by mixing 50 mM Tris-HCl, 25% glycerol, 5 mM EDTA, 0.2% bromophenol blue and 0.2 % xylene cyanol.

A 3% gel was prepared by dissolving 4.5 g of agarose in 150 mL of 1x TAE buffer by heating in the microwave oven for 5 min. Afterwards 2.25 µL of EtBr solution was added. This mixture was poured in the gel casket and allowed to gel for 1-1.5 hours. The samples comprising of a mixture of 10 µL of the PCR product and 10 µL of the loading buffer were loaded on the gel. The marker, molecular ruler 20 bp by BioRad was loaded at least twice on the gel. The electrophoresis was conducted at 150 volts for 45-55 min and then the gel was photographed.

5.3.7 TaqMan assays

5.3.7.1 Probe optimization

Generally 12.5 µL IQ5 supermix, 7.5 µL mastermix comprising of forward and reverse primers and probe in the corresponding concentrations and H_2O_{dd} and 5 µL of the sample (or H_2O_{dd} for negative control), was added to the PCR plate. A temperature program consisting of denaturation, annealing and elongation steps was used. The PCR run was carried out in a three-step real-time PCR program. The initial denaturation was carried out at 95 °C for 3 min. Then 45 cycles were run, each cycle consisting of denaturation at 95 °C for 20 s, followed by primer annealing at 60.3 °C for 40 s and elongation at 72 °C for 11 s. The detection was carried out during the elongation step and C_t values obtained were used for evaluation. For optimization of the probe concentration six different probe concentrations were tested, these included 25 nM, 50 nM, 75 nM, 100 nM, 150 nM and 200 nM. The 100

µM probe was diluted 1:10 before hand. Table 18 represents the pipetting scheme for this experiment. For this experiment the primer pair concentration of 200/300 nM was used.

Table 18: Pipetting scheme for probe optimization experiments

Reagents	**Probe concentration (nM)**					
	25	**50**	**75**	**100**	**150**	**200**
Forward primer (µL)	0.50	0.50	0.50	0.50	0.50	0.50
Reverse primer (µL)	0.75	0.75	0.75	0.75	0.75	0.75
Probe (µL)	0.625	1.25	1.875	2.50	3.75	5.00
H_2O_{dd} (µL)	5.625	5.00	4.375	3.75	2.50	1.25
Total volume (µL)	25					

5.3.7.2 Primer pair concentration, T_m and probe optimization

A probe concentration of 150 nM was used and the primer pair concentration was optimized along with the probe with the TaqMan assay. For this experiment nine different primer pair concentrations were analysed and four annealing temperatures, 61.5 °C, 60.3 °C, 58.4 °C and 55.9 °C were also checked for the optimum conditions of probe, T_m and primer concentration. Table 19 represents the pipetting scheme that was followed.

*Table 19***:** *Pipetting scheme for primer pair, probe and Tm optimization experiments*

Reagents	**Primer pair concentration (forward/ reverse) (nM)**								
	100/100	**100/200**	**100/300**	**200/100**	**200/200**	**200/300**	**300/100**	**300/200**	**300/300**
Forward primer (µL)	0.25	0.25	0.25	0.50	0.50	0.50	0.75	0.75	0.75
Reverse primer (µL)	0.25	0.50	0.75	0.25	0.50	0.75	0.25	0.50	0.75
Probe (µL)	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
H_2O $_{DD}$ (µL)	3.25	3.00	2.75	3.00	2.75	2.5	2.75	2.50	2.25
Total volume (µL)	25								

5.3.8 Efficiency measurement and LOD

To determine the efficiency and the LOD of the developed PCR method, for the first experiment, a positive control of 20 ng/µL was loaded on the PCR plate along with different dilutions of the positive control including 1:10, 1:100; 1:1000, 1:10000 and 1:100000 which were made by using bidistilled water to obtain the standards of varying concentrations, these included 2 ng/µL, 0.2 ng/µL, 0.02 ng/µL and 0.002 ng/µL. These standards along with the positive and negative controls were loaded on to the PCR plate for a PCR run to measure the efficiency. Later, experiments were carried out using a positive control of 45 ng/µL and also for making standards of various concentrations as explained before.

5.3.9 Spiking of samples

Spiking of samples was carried out using blank food matrices i.e. samples free of poppy seeds. Two blank matrices, crisp toast and grissini, were used for this purpose. Spiking was carried out by adding poppy seeds to the matrix followed by homogenization in the kitchen blender. Subsequent spiking levels were made using the previous ones. Each was then homogenized for 20 min in the kitchen blender. After spiking, three aliquots from each spike level were taken and DNA was extracted from each of them. The one that proved to be the most suitable one in terms of appropriate concentration and purity was chosen to carry out a PCR measurement. Table 20 illustrates the details of preparing spiked samples.

Table 20: Spiking scheme for poppy seeds into blank food matrices

Sample number	**Spiking level (%)**	**Spiking method**
1	1	99 g matrix + 1 g poppy seeds
2	0.5	50 g matrix + 50 g of sample 1
3	0.1	90 g matrix + 10 g of sample 1
4	0.05	50 g matrix + 50 g of sample 3
5	0.01	90 g matrix + 10 g of sample 3
6	0.005	50 g matrix + 50 g of sample 5
7	0.001	90 g matrix + 10 g of sample 5

5.3.10 Analysis of food samples

All commercial food samples were purchased from local supermarkets for analysis by the developed PCR method. These included samples labeled with containing poppy seeds and those with no mention of poppy seeds and a wide range of nuts and legumes for cross

reactivity tests. The food samples containing poppy seeds included packaged food as well as fresh bakery products, the samples analysed included poppy bread, poppy strudel, poppy chocolate, poppy cake, poppy bagel, poppy roll, poppy oil, poppy cake prepared by a lab colleague, crisp toast, a sesame snack and crackers. All samples were homogenized either using a pestle and mortar or a kitchen blender, depending on the nature of the sample, prior to the DNA extraction which was carried out as explained in section 5.3.2. The samples were stored at -18 °C whereas the isolated DNA from these samples was stored at 4 °C. The concentration and purity of all samples was measured and the best out of the three of each food was chosen for analysis by PCR.

The food samples that did not contain any poppy included various kinds of crisp toasts, fruit muesli and biscuits and crackers. DNA was extracted in the same way as explained before and the extracted DNA with the appropriate concentration and purity was chosen for PCR analysis.

5.4 Preliminary experiments for an immuno-PCR method for the detection of lupine

Preliminary experiments for the development of an immuno-PCR method for the detection of lupine in food stuffs were carried out, the details of these experiments are discussed in the following sections.

5.4.1 Reagents, buffers and apparatus required

Various reagents were required at various steps of the experimental procedure, these included casein, NaCl, NaH_2PO_4, Tween 20, Na_2CO_3, $NaHCO_3$, NaN_3, KH_2PO_4, Germal II, BSA, Tris, streptavidin, biotinylated anti-rabbit antibody, biotinylated DNA and bidistilled water.

Several buffers were used during the experimental process the details of which are as follows: the **incubation buffer** comprised of PBS buffer containing 1% (w/v) BSA and 0.05% (v/v) Tween 20. The **washing buffer** consisted of 0.154 M NaCl, 5 mM Tris, 0.005% Tween 20 and 0.1% diazolidinyl urea (Germal II). The pH was regulated at 7.75. The **coating** and **blocking buffers** were prepared as explained in section 5.1.6.

For the immuno-PCR experiments the apparatus and instruments used were the same as used for ELISA and PCR the only difference was that the Thermoscientific Abgene PCR plates were used in this case.

5.4.2 Lambda phage DNA, primer and probe design

A biotinylated DNA was required, therefore using the following lambda phage sequence a biotinylated DNA was ordered at Sigma with biotin at the 5´ end.

5´- CCTGCAATGACCCCGCTGATGCTGGACACCTCCAGCCGTAAGCTGGTTGCG
TGGGATGGCACCACCGACGGTGCTGCCGTTGGCATTCTTGCGGTTGCTGCT
GACCAGACCAGCACCACG -3´

The forward and backward primers that were used are:

λ - phage forward: 5´-ACACCTCCAGCCGTAAGC-3´

λ - phage backward: 5´-AGCAGCAACCGCAAGAATG-3´

Using the software Beacon designer, the corresponding probe was designed and ordered at Metabion. The sequence of the probe was:

5´-FAM-ACCGTCGGTGGTGCCATCCCA-BHQ-1-3´.

5.4.3 Producing IgG antibodies

The lupine anti-serum was produced at BioGenes (Berlin, Germany) by immunizing two rabbits; this was achieved by injecting lupine protein extract in a concentration of 1 mg/mL four times with an interval of seven days. Twenty one days after the fourth immunization a last immunization procedure was carried out and seven days later the animals were bled to obtain the sera. The antibodies produced were isolated using the caprylic acid/ammonium sulphate precipitation method as explained in 5.1.5 and characterized by Bradford assay as explained in section 5.1.2.

5.4.4 Initial PCR experiments

Initially a few experiments were carried out only to see if the amplification of the template DNA works and what concentration of the biotinylated lambda phage DNA would be sufficient enough for appropriate amplification. Therefore, a positive and negative control test was conducted using SYBR Green and later one with the probe and thus using IQ 5 supermix.

5.4.4.1 Positive and negative control experiment

For the first experiment, the reverse and forward primers in concentration of 200/200 nM were used. So 0.5 µL of both reverse and forward primers and 6.5 µL of water were needed per well, the primer mix was prepared accordingly. Thus each well contained 7.5 µL of the primer mix, 12.5 µL of the supermix SYBR Green, 5 µL of either water for negative control or DNA for the positive control. Two wells were loaded with a DNA concentration of 20 ng/µL and two with a concentration of 0.2 ng/µL along with two negative controls containing water instead of the DNA.

In another similar experiment, the same loading scheme was followed with the only difference being the concentration of the DNA loaded for positive controls, which were 2 pg/µL and 2 fg/µL, respectively. Each positive control was loaded on the plate thrice whereas the negative control was loaded four times on the plate; this was done to rule out any false results. The PCR plates used for immuno-PCR experiments were ABgene PCR plates, thermofast SSK 96 *x*.

5.4.4.2 Positive and negative control experiment with the probe

In the next experiment the probe in a concentration of 150 nM was also included; the reverse and forward primers in 200:200 nM were used. The primer mix was prepared by adding 0.5 µL of both reverse and forward primers, 3.75 µL of the 1:10 diluted 100 µM probe and 2.75 µL of water per well. Each well was loaded with 7.5 µL primer mix, 12.5 µL of the super mix IQ 5 and 5 µL of DNA or water for positive and negative controls, respectively. For this experiment, four positive controls were used; the concentration of DNA loaded was 2 pg/µL and 2 fg/µL for each two. The experiment also included three negative controls.

The experiment was repeated again using the incubation buffer for all dilutions at all stages of the experiment. A similar experiment without using the probe was also carried out.

5.4.5 Primer concentration and temperature optimization

For the primer concentration and temperature optimization experiment, primer pairs in different ratios; 100:100 nM, 100:200 nM, 100:300 nM, 200:100 nM, 200:200 nM, 200:300 nM, 300:100 nM, 300:200 nM and 300:300 nM were used. For each well, 7.5 µL of primer mix with one of these ratios, 12.5 µL of the super mix i.e. SYBR Green and 5 µL of DNA were loaded in each well. Using the software four different temperatures, 61.5, 60.3, 58.4 and 55.9 °C were checked for the optimum annealing temperature.

5.4.6 Development of the immuno-PCR

Using a sandwich ELISA developed in the laboratory by a colleague, Christina Ecker, for her doctoral research, an immuno-PCR experiment was conducted.

The procedure followed had several steps, the first one being **coating**, each well was coated with 25 µL of the coating solution containing 1:5000 of the IgY in the coating buffer and stored overnight at 4 °C. This was followed by manually **washing** six times with 200 µL of the wash buffer. The next step was **blocking** each well with 25 µL of the blocking buffer overnight. After washing 6 times with 200 µL of the wash buffer 25 µL of the lupine protein as the **antigen** in different concentrations such as 100 ng/mL, 10 ng/mL, 1 ng/mL, 0.1 ng/mL and 0.001 ng/mL was added and incubated for 1 hour. Following washing as before, 25 µl of **IgG** in a dilution of 1:15000 was added and incubated for 30 min; this was followed by washing again. Later 25 µL of **biotinylated anti-rabbit antibody** in a concentration of 2.3 nM was added followed by an incubation period of 1 h at room temperature. After washing 25 µL of **streptavidin** in a concentration of 5 nM were added following an incubation of 30 minutes. Washing was then followed by the addition of 25 µL of **biotinylated DNA** in a concentration of 0.7 pM and incubating for 1 hour. Finally the wells were washed six times with 200 µL of the wash buffer followed by washing 10 times with 200 µL of water. Finally a PCR was run under the conditioned explained before.

5.4.7 Stepwise improvements

In the first immuno-PCR experiment, afore mentioned procedure was carried out, the lupine standards in a range of 100-0.01 ng/mL and two blanks without lupine protein were loaded. Blocking was carried out at room temperature by 2% (w/v) casein solution in PBS. Following the same pipetting scheme as before, using 150 nM of the probe and a 200:200 nM concentration of the forward and reverse primer detection through PCR, along with negative controls was carried out.

In the next experiment two changes were incorporated, blocking was carried out by using the incubation buffer and secondly all dilutions during the ELISA steps were made using the incubation buffer instead of bidistilled water or PBS.

A similar experiment was carried out with all steps as developed for ELISA but with some changes in the washing buffer and in the coating. Coating in this case was carried out overnight at room temperature instead of overnight at 4 °C. The washing buffer used in the previous experiment was prepared as explained in section 5.1.6 but for next immuno-PCR experiments the washing buffer used consisted of 0.154 M NaCl, 5 mM Tris, 0.005% Tween 20 and 0.1% diazolidinyl urea (Germal II), the details are explained in section 5.4.1.

In the next experiment standards were analyzed, with some changes in the coating step. Coating was carried out overnight at 4 °C but the coating buffer was changed, in this case the buffer used for coating was a 0.2 M NaH_2PO_4 solution as reported by Lind et.al. [2] and blocking was carried out with the incubation buffer at 37 °C as before.

Later coating was improved by using a coating buffer comprising of a 0.2 M NaH_2PO_4 solution and the coating antibody concentration of 20 µg/mL was used to coat at 4 °C overnight. Blocking was also altered by replacing the 1% BSA by 2% casein in the incubation buffer, hence it was carried out using 2% casein and 0.05% Tween 20 in PBS at 37 °C. The incubation time for both antigen addition and IgG was 30 minutes.

In the next experiment, coating was carried out overnight at 4 °C with 20 µg/mL of the IgY. Salmon sperm DNA was incorporated in the blocking buffer to improve blocking efficiency. For blocking, two different schemes were followed at 37 °C. In one case 1% BSA, 0.05%

Tween 20 and 0.01% of the salmon sperm DNA in the incubation buffer was used. In the other case 2% casein, 0.05% Tween 20 and 0.01% salmon sperm DNA in the incubation buffer was used. The same experiment was repeated in the same way but with carrying out the blocking step overnight. The afore mentioned experiments were repeated in exactly the same way using common PCR plates (PCR plates 96 well by BioRad).

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1. M. M. McKinney, A. Parkinson. A Simple Non-Chromatographic Procedure to Purify Immunoglobulins from Serum and Ascites Fluid. Journal of Immunological Methods. **1987**;96:271-278.

2. K. Lind, M. Kubista. Development and Evaluation of Three Real-Time Immuno-PCR Assemblages for Quantification of PSA. Journal of Immunological Methods. **2005**;304(1-2):107-116.

Conclusion

The thesis investigates and discusses the development and applicability of various analytical methods designed for the detection of three different allergenic foods namely, sesame, poppy and lupine in view of the ever growing problem of food allergy.

A significant percentage of the population suffers from food allergies according to the epidemiological studies published over the last 10-15 years. Due to the continually increasing prevalence, with the exact cause still unknown, it has become an important health problem all over the world. Since allergen avoidance is the key to avoid an allergic reaction legislations have been formulated for consumer protection. Annex IIIa of the Amendment Directive EC 2003/89 of EC Directive 2000/13 issued by the European Commission includes a list of food ingredients and products thereof classified as possible allergenic foods. Sesame and its products are on the list as well as lupine and its products, which have been added to the list in 2006. Poppy has yet to make its way to this list since allergy to poppy, although severe, is very rare. Due to the evolving legislations allergen detection in food and food stuffs at all levels is gaining importance and reliable analytical methodology is required to detect allergenic proteins.

In the present thesis, three analytical methods were developed: an indirect competitive enzyme linked-immunosorbent assay (ELISA) and a quartz crystal microbalance immuno sensor for the detection of traces of sesame as well as a real-time polymerase chain reaction (PCR) method for the detection of poppy in food.

Firstly a competitive ELISA, using anti-sesame IgY, was developed and optimized for the detection of trace amounts of sesame in different food stuffs. Various parameters were optimized and the optimized conditions that were used for future ELISA experiments were coating at 4 °C with 1.22 µg/mL of the coating antigen solution, blocking with 2% (w/v) casein solution for 15 min and incubation for 45 min with a 1:10000 dilution of the primary antibody from an antibody solution with a concentration of 29 mg/mL. After optimization cross reactivity studies were carried out, the ELISA did not show any cross reactivity with 12

from the 13 food ingredients tested. For chocolate a low cross reactivity of 0.7% was observed. Matrix influences were eliminated by preparing the sesame protein standard solutions in a matrix diluted 1:20 with PBS, similar to the one that had to be analyzed. For instance a blank cracker extract, diluted 1:20 with PBS was used for preparing standard solutions for analyzing all kinds of crackers and cookies. The accuracy and precision of the ELISA were determined in recovery studies performed by spiking blank food matrices in a range of 25 µg/g to 200 µg/g with sesame protein. The recovery of sesame protein in commercially available blank food stuffs, spiked after extraction, was in the range of 82 to 114%. The recovery of sesame protein in commercially available blank food stuffs spiked prior to extraction was in the range of 85% to 126%, with the exception of multi grain crisp toast, which gave high recoveries ranging from 117% to 160% and whole grain bread, that yielded low recoveries ranging from 70% to 85%. In crisp bread, cracker, cereals and snacks the LOD was found to be 5 µg sesame protein/g food, in fresh breads and rolls, the LOD was 11 µg sesame protein/g food. To demonstrate the applicability of the developed ELISA, 28 commercially available samples, with different declarations, such as “may contain sesame”, “contains sesame” or with no sesame declaration were analyzed. The results were found to be in accordance with the labeling declaration. The results obtained for this study have been published, consult appendix B.

In the next part of the research a quartz crystal microbalance immuno sensor to detect sesame proteins was developed and optimized. In this case, the same anti-sesame IgY antibodies were used as for ELISA. The antibodies were immobilized on the quartz surface and measurements were carried out. A change in frequency or the signal response after the addition of the sesame extract results from a mass change and indicates the formation of the anti-sesame IgY- sesame protein complex. To reuse the sensor this complex was successfully dissociated without disturbing the antibody layer using 2 M guanidine hydrochloride. The sensor showed high interday repeatability, when same measurements were carried out on different days, and a high batch to batch reproducibility when same measurements were carried out with a different antibody layer immobilized. In order to eliminate matrix effects a washing step was incorporated in all measurements ensuring the measurement medium to be the same in all cases. The LOD was found to be 35 µg of sesame protein per gram.

In the next section of the research a real-time polymerase chain reaction (PCR) method for the detection of poppy seed in food matrixes was developed. The DNA extraction was carried out using a CTAB protocol. SYBR Green assay was carried out for cross reactivity measurements and for the optimization of the primer pair concentration and the annealing temperature. For further experiments the TaqMan assay was used. For cross reactivity measurements twenty eight different food ingredients were tested by PCR, no further investigation was carried out for those food stuffs for which a distinct negative was obtained in the PCR analysis. For others agarose gel electrophoresis was carried out to ensure the absence of any cross reactivity. Initially, three primer pairs were used, depending on the highest suitability one of them, primer pair 1, was chosen. The developed method was then validated by determining the efficiency and the LOD. Standards solutions with the concentrations of 45 ng/µL, 0.45 ng/µL, 0.045 ng/µL 0.0045 ng/µL and 0.00045 ng/µL of poppy DNA were tested and the efficiency was found to be 88.2%. Next, spiking experiments were conducted by spiking blank matrixes with poppy seeds in a range of 0.001% - 1% and then extracting DNA for PCR analysis. In case of spiking crisp toast the efficiency was found to be 79.9% and in case of grissini it was 96.2%.

In the last part of this dissertation, preliminary experiments for the development of an immuno-PCR method for the detection of lupine were carried out. For this, the experimental setup was initiated using the sandwich ELISA developed for lupine detection in our laboratory. During these experiments the coating, washing and blocking conditions were improved but the development and optimization was discontinued due to limited time.

Abstract

Food allergy is considered the fourth most important public health problem by the WHO. Since strict avoidance of the allergenic food is the only therapy for allergic patients it is important to develop sensitive analytical methods for the detection of allergens in food.

In the present thesis, three analytical methods were developed: an indirect competitive enzyme linked-immunosorbent assay (ELISA) and a quartz crystal microbalance immuno sensor for the detection of traces of sesame as well as a real-time polymerase chain reaction (PCR) method for the detection of poppy in food.

The ELISA did not show any cross reactivity with twelve of thirteen food ingredients tested (including several nuts and seeds), for chocolate a low cross reactivity of 0.7% was observed. Recovery of sesame protein in food samples ranged from 85%-126%, with the exception of multi grain crisp toast, yielding higher recoveries (117%-160%), and whole grain bread, yielding lower recoveries (70%-85%). For crisp bread, cracker and snacks the limit of detection (LOD) was 5 µg sesame protein/g food, in fresh breads and rolls the LOD was 11 µg sesame protein/g food.

The immuno sensor did not show any cross reactivity with fourteen food ingredients tested. The LOD was found to be 35 µg of sesame protein per gram food in whole grain bread. The sensor showed a high interday repeatability and high batch to batch reproducibility and could be reused at least ten to twelve times after regenerating the antibodies.

The real-time PCR method was specific for poppy and did not show any cross reactivity with the twenty eight food ingredients tested. The amplification efficiency of the method was found to be 79.9% in crisp toast and 96.2% in grissini.

All three methods developed are applicable to detect traces of allergens in complex and highly processed foods.

Abstract (German)

Lebensmittelallergien sind laut der Weltgesundheitsbehörde (WHO) das viertwichtigste Gesundheitsproblem in der Bevölkerung. Da für Lebensmittelallergiker eine strikte Vermeidung der Aufnahme der Allergene die einzige Chance ist, allergische Reaktionen zu verhindern, benötigt man empfindliche und zuverlässige Analysenmethoden, welche die Detektion von Allergenen in Lebensmitteln ermöglichen.

In der vorliegenden Doktorarbeit wurden drei Analysenmethoden zum Nachweis von allergenen Lebensmitteln entwickelt: ein indirekter kompetitiver Enzymimmunoassay (ELISA) und ein Quartz Crystal Microbalance (QCM) Immunsensor zur Bestimmung von Sesam und eine real-time PCR Methode zum Nachweis von Mohn in Lebensmitteln.

Der ELISA zeigte keine Kreuzreaktivität mit 12 von 13 getesteten Lebensmitteln bzw. Lebensmittelinhaltsstoffen, nur für Schokolade wurde eine Kreuzreaktivität von 0,7% ermittelt. Die Wiederfindung für Sesamprotein in Lebensmitteln lag zwischen 85 und 126%, nur in Mehrkornknäckebrot wurde eine höhere (117-160%) und in Vollkornbrot eine niedrigere Wiederfindung (70-85%) erhalten. In Knäckebrot, Crackers und Knabbergebäck betrug die Nachweisgrenze (LOD) 5 µg Sesamprotein / g Lebensmittel, in frischem Brot und Semmeln 11 µg Sesamprotein /g Lebensmittel.

Von vierzehn Lebensmitteln bzw. Lebensmittelzutaten zeigte keine(s) eine Kreuzreaktivität im Immunsensor. Die Nachweisgrenze des Sensors betrug 35 µg Sesamprotein / g Lebensmittel Vollkornbrot. Der Sensor zeigte eine hohe Interday- und Batch-zu-Batch Reproduzierbarkeit. Durch Regeneration der Antikörper nach der Dissoziation des Antigen-Antikörper-Komplexes konnte der Sensor mindestens zehn bis zwölf mal verwendet werden.

Die real-time PCR Methode war spezifisch für Mohn und zeigt keine Kreuzreaktivität mit 28 Lebensmitteln bzw. Lebensmittelzutaten. Die Amplifikationseffizienz der Methode betrug 79.9% in Knäckebrot und 96.2% in Grissini.

Die drei im Rahmen der Dissertation entwickelten Analysenmethoden sind geeignet, um Spuren von Allergenen in komplexen und stark verarbeiteten Lebensmitteln nachzuweisen.

List of Figures

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List of Abbreviations

A	Absorbance
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
BAW	Bulk acoustic wave
BC	Background control
BLAST	Basic Local Alignment Selection Tool
bp	Base pair
BSA	Bovine serum albumin
CDR	Complimentary determining regions
C_p	Crossing point
C_t	Threshold cycle
CTAB	Cetyltrimethylammoniumbromide
DBPCFC	Double blind placebo controlled food challenges
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotidtriphosphates
E	Efficiency
EC	European Commission
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immuno sorbent assay
EU	European Union
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPCR	Immuno polymerase chain reaction
IT	Immuno therapy
IUPAC	International Union of Pure and Applied

	Chemistry
kDa	Kilodaltons
LOD	Limit of detection
LOQ	Limit of quantification
MED	Minimal eliciting dose
MHC	Major histocompatibility complex
MPa	Megapascal
NCBI	National Centre For Biotechnology Information
NSB	Non specific binding
NTC	No template control
OAS	Oral allergy syndrome
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
QCM	Quartz crystal microbalance
RNA	Ribonucleic acid
SAW	Surface acoustic wave
SDS-PAGE	Sodium dodecyl sulfate-poly acrylamide gel electrophoresis
SPT	Skin prick test
TEMED	N, N, N´ N´ Tetramethylethylenediamine
T_m	Melting temperature
TMB	Tetramethylbenzidine
USA	United States of America
UV	Ultra violet
WHO	World Health Organization

List of Chemicals

1-Methyl-2-pyrolidone, VWR

2-Mercaptoethanol, Sigma

Acetic acid, Fluka

Acryamide, Sigma

Ammonium persulphate, SERVA

Ammonium sulphate, Sigma

Bidistilled water, Fluka

Biotinylated DNA, Sigma

Bovine serum albumin (BSA) Sigma

Brilliant Blue G dye, Sigma

Bromophenol blue, Sigma

Casein from cow’s milk, Sigma

Cetyltrimethylammoniumbromide (CTAB), Sigma

Chloroform, Roth

Coomassie blue R-250, Sigma

Dimethyl sulfoxide (DMSO), Sigma

Ethanol, Austrian AlkohandelsGmbH

Ethylenediaminetetraacetic acid (EDTA), VWR

Germal II (diazolidinyl urea), Sigma

Glycine, Sigma

Guanidine hydrochloride, Sigma

Hydrochloric acid (HCl), 37%, VWR

Hydrogen peroxide (H_2O_2), Sigma

Immunoglobulin G (Sigma)

IQ™ Supermix, BioRad

Isopropanol, VWR

$K_2HPO_4.3H_2O$, VWR

$KH_2PO_4.H_2O$, VWR

Methanol, VWR

N, N, N´ N´ Tetramethylethylenediamine (TEMED), Sigma

N, N´- Methylene-bisacrylamide, Sigma

Na_2CO_3, Sigma

$Na_2HPO_4.2H_2O$, VWR

NaCl, VWR, Sigma

$NaH_2PO_4.2H_2O$, VWR

$NaHCO_3$, VWR

NaN_3, VWR

NaOH, VWR

n-Hexan, Roth

Octanoic acid, VWR

Phosphoric acid, VWR

Potassium dihydrogen citrate, Fluka

Precision Plus Protein^{TM} Standards (10-250 kDa; BioRad)

Primer, Sigma-Genosys

Proteinase K, Sigma

RNase, Sigma

Salmon sperm DNA, Sigma

Sodium acetate, Fluka

Sodium dodecyl sulphate (SDS), Sigma

Sorbic acid, Sigma

Streptavidin, Thermoscientific

Sulphuric acid, VWR

TaqMan probe, Metabion

Tetramethylbenzidin (TMB), Sigma

TRIZMA Base, Sigma

Tween 20, Sigma

List of Apparatus

Analytical balance: Mettler AT 400

Baking oven: Type 60/3 W, Manz Backtechnik, Münster, Germany

Centrifuge machines: Model 4 K 10 Sigma, Vienna, Austria

Model 5424, Eppendorf, Hamburg, Germany

Detection system: IQ^{TM} 5 Multicolor Real-time PCR Detection System, BioRad

Dialysis tubes: Cut off, 12000-14000 Da

Drying oven: Memmer Modell 500

Gel electrophoresis apparatus: Mighty Small SE 250 apparatus (Hoefer Scientific Instruments, Holliston, MA, USA) and Power Pac (BioRad).

Grinding mill: Model MM 2000, Retsch, Haan, Germany

Immuno wash: BioRad, Modell 1575

Microplate reader: BioRad, Model 680 XR

Microtiter plate shaker: MTS4, IKA, Staufen, Germany

Microtiter plates: Maxisorp F96, Nunc, Wiesbaden, Germany

Network analyser: Model E5062A, ENA series, Agilent Technologies

PCR plates: 96 well, BioRad

pH-Meter: Metrohm 691

Photometer: Genesys 10 UV, Thermo Spectronic

Pipette: Eppendorf research 5000 µL

Eppendorf 100 - 1000 µL

Gilson P200, 200 µL

BioRad 0.5-10 µL

Brand Transferpette-8 20-200 µL

Quartz crystals: AT-cut quartz crystals (10 MHz), Zheijiang Quartz Crystal Electronic Company, Shanghai, China

Thermocycler: iCycler BioRad

Thermoscientific Abgene PCR plates, thermofast SSK 96 *x*

Ultra sonic bath: Bandelin SONOREX SUPER RK103H

Ultra-Turrax: Model T25, IKA

Vortex: Model VF2, IKA

Resume

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Journal Publications

- F. T. Husain, I. E. Bretbacher, A. Nemes, M. Cichna-Markl; "Development and validation of an indirect competitive ELISA for the determination of potentially allergenic sesame (Sesamum indicum) in food," Journal of Agriculture and Food Chemistry, 1434–1441, Vol.58, 2010.
- R. Schirhagl, A. Seifner, F. T. Husain, M. Cichna-Markl, P. A. Lieberzeit, F. L. Dickert; "Antibodies and their Replicae in Microfluidic Sensor Systems – Label free Quality Assessment in Food Chemistry and Medicine," Sensor Letters,399-404, Vol.8, 2010.

- G. Redl, F. T. Husain, I. E. Bretbacher, A. Nemes, M. Cichna-Markl; "Development and validation of a sandwich ELISA for the determination of potentially allergenic sesame (Sesamum indicum) in food," Analytical and Bioanalytical Chemistry, 1735-1745, 398, 2010.

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- F. T. Husain, M. Cichna-Markl; "Applicability of a commercial ELISA to detect potentially allergenic sesame in food," 3rd International Symposium on Recent Advances in Food Analysis, Prague, Czech Republic, 7-9th November 2007.

- F. T. Husain, M. Cichna-Markl; "Development of a competitive enzyme linked immunosorbent assay (ELISA) to determine potentially allergenic sesame in food," 1st. Meeting of the Vienna Research Platform of Nutrition and Food Sciences. Vienna, Austria, 25th April 2008.

- F. T. Husain, M. Cichna-Markl; "Competitive ELISA to determine potentially allergenic sesame in food," Österreichische Lebensmittelchemiker Tage, Eisenstadt, Austria, 28-30th May 2008.

- G. Redl, F. T. Husain, M. Cichna-Markl; "Optimierung eines Sandwich-ELISAs zur Bestimmung von Spuren von potentiell allergenem Sesam in Lebensmitteln," Österreichische Lebensmittelchemiker Tage, Eisenstadt, Austria, 28-30th May 2008.

- F. T. Husain, M. Cichna-Markl, R. Schirhagl & F. L. Dickert; "Development Of A Quartz Crystal Microbalance (QCM) Immuno sensor To Detect Potentially Allergenic Sesame In Food" presentation at 13th Österreichische Chemietage, 24-27th August 2009.

- F. L. Dickert, R. Schirhagl, P. A. Lieberzeit, F. T. Husain, M. Cichna-Markl, "Replicae of Antibodies – Robust Mass-Sensitive Sensors for allergens and other Bioanalytes," Presentation at Sensor + Test Conference, Nürnberg (Germany), 26-28 May 2009.

- R. Schirhagl, F. L. Dickert, M. Cichna-Markl, F. T. Husain; Kunststoffimmunglobuline zur Detektion von Sesamproteinen; Presentation at 6. German Biosensor Symposium, Freiburg (Germany) March 29- April 1st 2009.

- F. L. Dickert, R. Schirhagl, A. Seifner, F. T. Husain, P.A. Lieberzeit, M. Cichna-Markl; "Antibodies and their Replicae in Microfluidic Sensor Systems – Label free quality assessment in Food Chemistry and Medicine," Presentation at European Material Research Meeting (EMRS), Strasbourg (France), 8-12th June 2009.

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- F. T. Husain, M. Cichna-Markl, R. Schirhagl & F. L. Dickert; "Development of a Quartz Crystal Microbalance (QCM) Immuno sensor To Detect Potentially Allergenic Sesame In Food," Österreichische Lebensmittelchemiker Tage, Seggau, 19-21 May 2010.

- F. T. Husain, M. Cichna-Markl, R. Schirhagl & F. L. Dickert; "Development of a Quartz Crystal Microbalance (QCM) Immuno sensor To Detect Potentially Allergenic Sesame In Food" Federation of Analytical Chemistry and Spectroscopic Societies (FACCS) Raleigh, North Carolina, USA, 17-21 October 2010.

Appendix A: Posters Presented

Applicability of a Commercial ELISA to Detect Potentially Allergenic Sesame in Food

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Introduction

Sesame (*Sesamum indicum*) is one of the 14 allergenic foods which have to be given in the list of food ingredients according to European Union legislation. It is reported to be a severe allergen capable of causing life threatening anaphylaxis. Analytical measurements have to be carried out to verify if sesame containing food products are labelled in accordance with the regulations and if non-labelled food products are actually free of sesame. The detection is not easy as the allergens are present in minute amounts in complex food matrices. Therefore, the analytical method should be specific for the food allergen and sensitive enough to enable the detection of allergen amounts which are sufficient to elicit allergic reactions in highly sensitized patients. Although enzyme linked immunosorbent assays (ELISAs) are frequently used to determine allergens in foods, up to now no study has been published dealing with the development of a sesame specific ELISA. However, some test kits for detecting sesame in food are commercially available.

Sesame plants

White sesame seeds

Objectives

To investigate the applicability of a commercial test kit to detect sesame in different food samples

Experimental

- All samples were purchased from local Austrian stores
- Some samples were labelled that they contain sesame, others with may contain sesame and others with no sesame at all
- Samples which did not contain sesame were spiked with known amounts of sesame (0.5%)

Dry sesame seeds

Sample preparation

Grinding of the sample in a grinding mill

↓

Weighing exactly 10.0 g of the grinded sample

↓

Mixing the sample with the sesame extraction buffer using an ultra turrax for exactly two minutes

↓

Centrifugation at 4000 rpm for 30 min at room temperature

↓

Decanting the supernatant and centrifuging it again at 10000 rpm for 5 min at room temperature

Commercial ELISA

- For commercial ELISA, **Sesame Protein Test Kit by BIO KITS** was used.
- According to the manufacturer the test is **highly sensitive** and can detect as low as 6 ppm of sesame in food samples.
- The reagents were declared to give a **negative response** with over seventy single commodities (seeds, nuts, grains, fruits).

Procedure

- The standards were made using the **sesame stock solution (sesame high control)**, provided by the manufacturer.
- Each extract was then diluted with the **working diluent** (1:50).
- The **low control, high control, working diluent** and **diluted sample extracts** were loaded on the wells and incubated for one hour (100 µL each).
- **Anti-sesame biotin** was then added (50 µL) and incubated for 1 hour.
- **Avidin peroxidase** was then added (50 µL) and incubated for 15 min.
- At the end the **TMB substrate** was added (50 µL) and incubated for 45 min.
- At the end the **stop solution** (50 µL) was added.
- In between all steps the wells were washed with the **working wash solution.**

Results and Discussion

Sample No.	Samples Tested	Declaration	Result
1	Sesame oil	+	-
2	Black sesame	+	+
3	Schnitzel fried	+	+
4	Crisp toast 1	+	+
5	Black sesame cookies	+	+
6	Salty sesame snack 1	+	+
7	Salty sesame snack 2 spiked (0.5%)	+	+
8	Crisp toast 2 spiked (0.5%)	+	+
9	Peanut cookies packed with sesame cookies	+/-	+
10	Cereal	+/-	+
11	Cashew nut cookies packed with sesame cookies	+/-	+
12	Muesli	+/-	+
13	Crisp toast 2	-	-
14	Crisp toast 3	-	-
15	Salty sesame snack 2	-	-

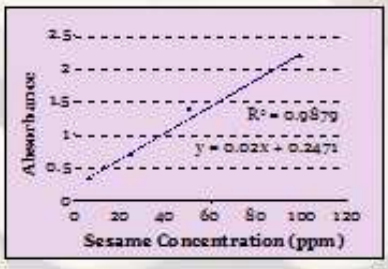

Figure 1: Sesame Protein Test Kit standard curve

Conclusion

- Sesame could not be detected in **sesame oil**.
- In all products declared with **"contains sesame"**, sesame could be detected.
- In all products declared with **"may contain sesame"**, sesame could be detected.
- In all products declared with **"do not contain sesame"**, a negative signal was obtained.

Future Plans

To develop an ELISA using an anti-sesame IgY produced by immunizing hens.

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Competitive ELISA to Determine Potentially Allergenic Sesame in Food

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Introduction

Sesame (*Sesamum indicum*) has become one of the most common causes of severe allergic reactions to food. It is one of the 14 allergenic foods which have to be given in the list of food ingredients according to European Union legislation. Analytical measurements have to be carried out to verify if sesame containing food products are labelled in accordance with the regulations and if non-labelled food products are actually free of sesame. The detection is not an easy task as the allergens are present in minute amounts in complex food matrices. Therefore, analytical methods should be specific for the food allergen and sensitive enough to enable the detection of allergen amounts which are sufficient to elicit allergic reactions in highly sensitized patients. Although enzyme linked immunosorbent assays (ELISAs) are frequently used to determine allergens in foods, up to now no study has been published dealing with the development of a sesame specific ELISA.

Sesame plants

White sesame seeds

Objectives

To develop a competetive sesame specific ELISA
To determine the applicability of the optimized ELISA to detect sesame in different food matrices
To validate the developed ELISA

Experimental

Sample preparation

Grinding of the sample in a grinding mill

↓

Weighing exactly 10.0 g of the grinded sample

↓

Mixing the sample with the sesame extraction buffer using an ultra turrax for exactly two minutes

↓

Centrifugation at 4000 rpm for 30 min at room temperature

↓

Decanting the supernatant and centrifuging it again at 10000 rpm for 5 min at room temperature

ELISA procedure

- The plate was coated with **sesame** protein in **coating buffer** (sesame protein concentration 1.22 µg/mL, 200 µL) and stored at 4°C for 16-18 h.
- The plate was then blocked with the **blocking buffer** for 15 min. (300 µL).
- The **standards** and **samples** were loaded on the wells (50 µL each).
- **Primary antibody** was then added (100 µL) and incubated for 45 min.
- **Secondary antibody** was then added (200 µL) and incubated for 1 hour.
- At the end the **TMB substrate** was added (200µL).
- The **stop solution** (100 µL) was added to stop the colour development.
- In between all steps the wells were washed with the **working wash solution**.

Dry sesame seeds

Results and Discussion

ELISA OPTIMIZATION

For developing the ELISA several factors were optimized. Figure 1 shows that a dilution of 1:10000 of the primary antibody gave a calibration curve with the highest slope; therefore, it was chosen to be the preeminent one. It is clear from Figure 2 that the optimum incubation time for the primary antibody was 45 minutes.

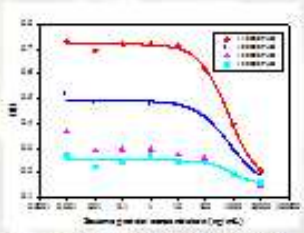

Fig 1: *Influence of the primary antibody (PAB) concentration on the calibration curve*

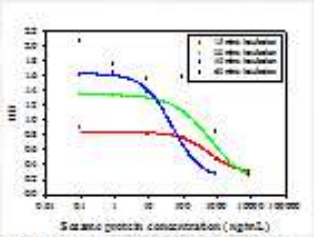

Fig 2: *Influence of the primary antibody (PAB) incubation time on the calibration curve*

Recovery results

Samples which did not contain sesame were spiked with sesame proteins and analysed. A few recovery results are shown in Table 1.

Cross reactivity tests

The optimized ELISA proved to be specific for sesame and did not show any cross-reactivity with peanuts, hazelnut, walnut, Brazil nut, poppy seed, wheat, rye, oat, chocolate, honey and several other products. Figure 3 is a representative figure of various cross-reactivity tests.

Table 1: Recovery results of sesame protein in a commercially available spiked crisp bread

Spiking Concentration (ng/mL)	Recovery (%)				Mean recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
	Day 1	Day 2	Day 3	Day 4			
250	87	105	96	91	95	7	8
500	102	99	108	104	103	3	3
1000	91	97	106	110	101	8	8
2000	98	94	96	81	92	7	8

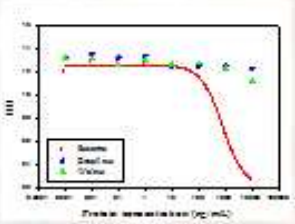

Fig 3: *Cross-reactivity studies*

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F. T. Husain, M. Cichna-Markl; "Development of a competitive enzyme linked immunosorbent assay (ELISA) to determine potentially allergenic sesame in food," 1st. Meeting of the Vienna Research Platform of Nutrition and Food Sciences, Vienna, Austria, 25th April 2008

Developing a Competitive ELISA to Determine Potentially Allergenic Sesame in Food

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Introduction

Food allergies create serious problems for the society in several ways and have a remarkable clinical impact. Sesame (*Sesamum indicum*) has become one of the most common causes of severe allergic reactions to food. It is one of the 14 allergenic foods which have to be given in the list of food ingredients according to European Union legislation. Analytical measurements have to be carried out to verify if sesame containing food products are labelled in accordance with the regulations and if non-labelled food products are actually free of sesame. The detection is not an easy task as the allergens are present in minute amounts in complex food matrices. Therefore, analytical method should be specific for the food allergen and sensitive enough to enable the detection of allergen amounts which are sufficient to elicit allergic reactions in highly sensitized patients. Enzyme linked immunosorbent assays (ELISAs) are frequently used to determine allergens in foods. However, development of a sesame specific ELISA has not been published yet.

Sesame plant

White sesame seeds

Dry sesame seeds

Objectives

To develope a competitive ELISA which is sesame specific

To demonstrate the applicability of the ELISA to detect sesame in different food matrices

Experimental

Sample preparation

Grinding of the sample in a grinding mill

↓

Weighing exactly 10.0 g of the grinded sample

↓

Mixing the sample with the sesame extraction buffer using an ultra turrax for exactly two minutes

↓

Centrifugation at 4000 rpm for 30 min at room temperature

↓

Decanting the supernatant and centrifuging it again at 10000 rpm for 5 min at room temperature

ELISA procedure

- The plate was coated with sesame protein in **coating buffer** (0.22 µg/mL; 200 µL) and stored at 4°C for 16-18 hours.
- The plate was then blocked with the **blocking buffer** for 45 min (200 µL).
- The **standards** and **samples** were loaded on the wells (50 µL each).
- **Primary antibody** was then added (100 µL) and incubated for 45 min.
- **Secondary antibody** was then added (200 µL) and incubated for 1 hour.
- At the end the **TMB substrate** was added (200 µL).
- The **stop solution** (100 µL) was added to stop the colour development.
- In between all steps the wells were washed with the **working wash solution.**

Cross reactivity tests

The optimized ELISA proved to be specific for sesame and did not show any cross-reactivity with nuts, legumes and other food stuff. Figure 3 is a representative figure of various cross-reactivity tests.

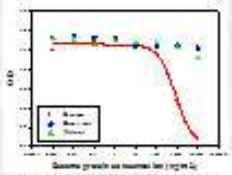

Fig 3: Cross-reactivity studies

Results and Discussion

ELISA Optimization

For developing the ELISA several factors were optimized. Figure 1 shows that a dilution of 1:10000 of the primary antibody gave a calibration curve with the highest slope, therefore, it was chosen to be the preeminent one. It is clear from Figure 2 that the optimum coating condition was overnight at 4°C.

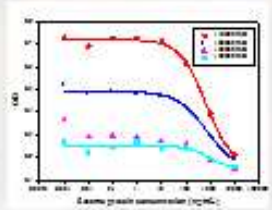

Fig 1: Influence of the primary antibody (PAB) concentration on the calibration curve

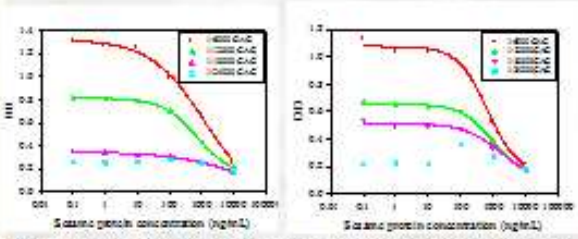

Fig 2: Influence of the coating antigen concentration (CAC) on the calibration curve. (a) Coating temperature: 36°C (b) Coating temperature: 4°C

Recovery results

Samples which did not contain sesame were spiked with sesame proteins and analysed. A few recovery results are shown in Table 1.

Table 1: Recovery results of sesame protein in a commercially available crisp bread

Spiking Concentration (µg/g)	Recovery (%)				Mean Recovery (%)	Standard Deviation (%)	Relative Standard Deviation (%)
	Day 1	Day 2	Day 3	Day 4			
25	87	105	96	91	95	7	8
50	102	99	108	104	103	3	3
100	91	97	106	110	101	8	8
200	98	94	96	81	92	7	8

Conclusion

- The optimized ELISA proved to be specific for sesame and showed no cross reactivity.
- Samples without any sesame gave good recovery results when spiked with sesame.

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F. T. Husain, M. Cichna-Markl; "Competitive ELISA to determine potentially allergenic sesame in food," Österreichische Lebensmittelchemiker Tage, Eisenstadt, Austria, 28-30th May 2008.

Optimierung eines Sandwich-ELISAs zur Bestimmung von Spuren potentiell allergenen Sesams in Lebensmitteln

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Einleitung

14 Nahrungsmittelallergien und deren Produkte listen besonders häufig Allergien aus. Oft verlangt die akute allergische Reaktion eine notfallmedizinische Behandlung des betroffenen Menschen. Aus diesem Grund müssen die allergenen Nahrungsmittelzutaten nach EU-Richtlinie 2007/68/EG deklariert werden, eines davon ist Sesam (Sesamum indicum).

Die Detektion der Allergene in komplexer Nahrungsmittelmatrix verlangt nach spezifischen und kostengünstigen Tests mit niedrigen Nachweisgrenzen. ELISAs (Enzyme-linked immunosorbent assay) spielen dabei eine wichtige Rolle. Bisher wurden keine Methoden zur Bestimmung von Sesam publiziert.

Ziel

Ziel der vorliegenden Arbeit sind Entwicklung und Optimierung eines Sandwich-ELISAs zur Detektion von Sesam-Proteinen in Lebensmitteln.

Methode

Die **Optimierung des Assays** wurde durch Variation u.a. folgender Parameter erreicht: Konzentration der immobilisierten Antikörper, Konzentration des sekundären Antikörper, Temperatur während des Coatens und während des Blockens, Art und Konzentration der Blocklösung.

Ergebnisse

Im Rahmen der Studie konnte eine Methode zur Detektion von Sesam-Proteinen in komplexen Nahrungsmitteln entwickelt werden.

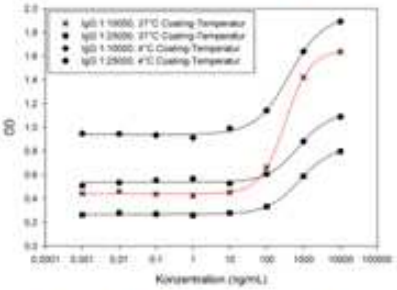

Abb. 1: Einfluss der Antikörper-Konzentration (IgG aus Kaninchen) und der Coating-Temperatur auf die Kalibrierfunktion

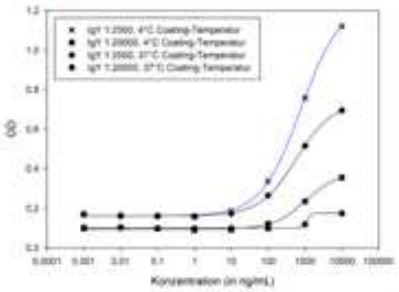

Abb. 2: Einfluss der Antikörper-Konzentration (IgY aus Eidottern) und der Coating-Temperatur auf die Kalibrierfunktion

Die in Abbildung 1 rot dargestellte Kalibrierfunktion zeigt die größte Steigung und die größte Differenz zwischen maximalem und minimalem Signal (= hohe Empfindlichkeit, großer Arbeitsbereich). Allerdings zeigt die blau dargestellte Kalibrierfunktion in Abbildung 2 – verglichen mit der roten – ein viel niedrigeres Leerwertsignal. Darum wurde die IgY aus Huhn als primäre und die IgG aus Kaninchen als sekundäre Antikörper verwendet.

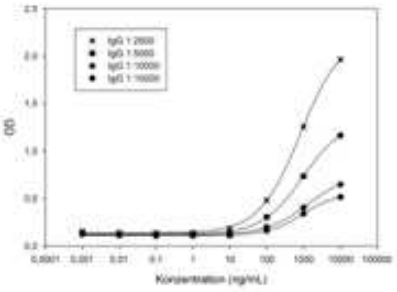

Abb. 3: Einfluss der Konzentration des sekundären Antikörpers auf die Steigung und Höhe der Kalibrierfunktion

Mit höherer Konzentration des sekundären Antikörpers konnte ein höherer Signalabstand erzielt werden (Abb.3).

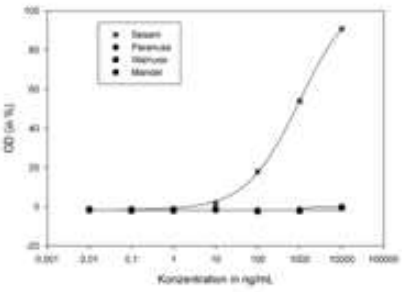

Abb. 4: Ergebnis der Kreuzreaktivitätsbestimmung

Kreuzreaktivität

Die entwickelte Methode zur Detektion von Sesam-Proteinen ist spezifisch für Sesam und zeigt mit keiner der übrigen getesteten Lebensmittelzutaten (Paranuss, Walnuss, Mandel, Erdnuss, Haselnuss, Sonnenblume, Sojabohne, Mohn, Weizen, Roggen, Hafer, Schokolade und Honig) eine Kreuzreaktivität (Abb. 4).

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Development Of A Quartz Crystal Microbalance (QCM) Immunosensor To Detect Potentially Allergenic Sesame In Food

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Introduction

Food allergy is a hypersensitive immune response to the exposure of food allergens, roughly estimated the prevalence is 5 to 6% in children and about 2% in adults. Sesame (*Sesamum indicum)* , one of the 14 allergenic foods which have to be given in the list of food ingredients according to European Union legislation is reported to be a severe allergen capable of causing life threatening anaphylaxis. Analytical methods to verify if sesame related labeling in food products is of vital importance and these should be specific for the food allergen and sensitive enough to enable the detection of allergen amounts which are sufficient to elicit allergic reactions in highly sensitized patients. QCM sensors offer several advantages in such studies as they do not require labeled reagents and the analyte can be detected in real-time. In QCM immunosensors the analyte is recognized by antibodies immobilized on a thin layer on a crystal surface. The resulting mass change is then transformed into an electronically measurable quantity.

Objectives

To develop and investigate the applicability of a QCM immunosensor to detect sesame in different food samples

Experimental

- All samples were purchased from local Austrian stores and had different sesame related labeling
- Poly clonal antibodies were prepared by immunizing hens

Sample preparation

Grinding of the sample in a grinding mill

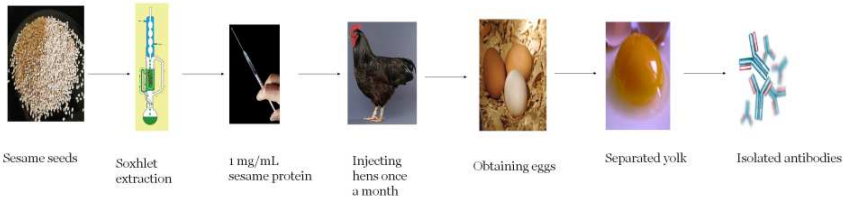
Weighing exactly 10.0 g of the grinded sample

Mixing the sample with the sesame extraction buffer using an ultra turrex for exactly two minutes

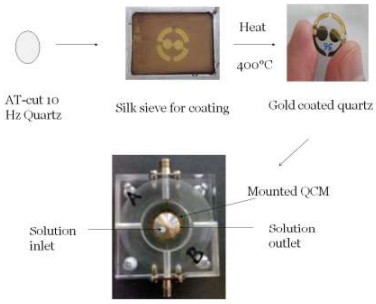
Centrifugation at 4000 rpm for 30 min at room temperature

Decanting the supernatant and centrifuging it again at 10000 rpm for 5 min at room temperature

Production of polyclonal antibodies



Preparing the immunosensor



Results

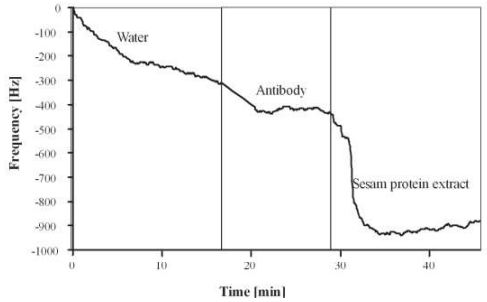


Figure 1: Sensor response to the antibody solution and to the sesame protein extract (concentration 1 mg/mL)

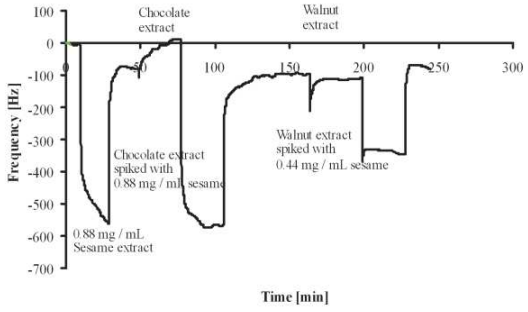


Figure 2: Results obtained in cross reactivity tests; sensor response to sesame, chocolate and walnut

Conclusion

- The sensor gave a significant response for sesame.
- The developed sensor could be reused several times after elution.
- The developed sensor was specific for sesame and did not show any cross reactivity for the fourteen food samples that it was tested with.

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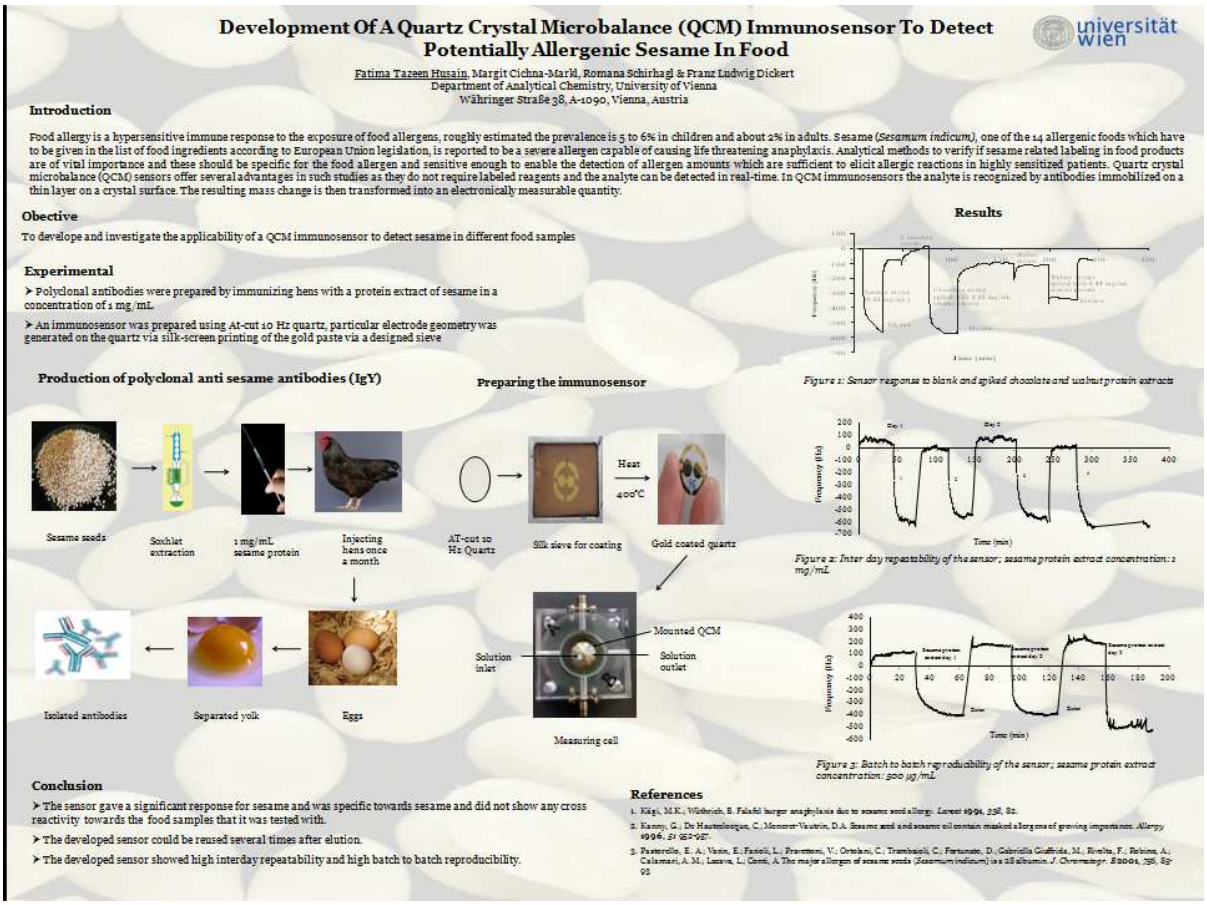

F. T. Husain, M. Cichna-Markl, R. Schirhagl & F. L. Dickert; “Development Of A Quartz Crystal Microbalance (QCM) Immuno sensor To Detect Potentially Allergenic Sesame In Food” Federation of Analytical Chemistry and Spectroscopic Societies (FACCS), Raleigh, North Carolina, USA, 17-21 October 2010.

Appendix B: Journal Publications

1434 J. Agric. Food Chem. 2010, 58, 1434–1441
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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Development and Validation of an Indirect Competitive Enzyme Linked-Immunosorbent Assay for the Determination of Potentially Allergenic Sesame (*Sesamum indicum*) in Food

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This study was designed to develop an indirect competitive enzyme linked-immunosorbent assay (ELISA) to detect traces of sesame in food. Antibodies against sesame were prepared by immunizing a hen with a protein extract of white, peeled sesame. The ELISA did not show any cross-reactivity with 12 of 13 food ingredients tested, only for chocolate was a low cross-reactivity of 0.7% observed. To eliminate matrix effects, sesame protein standard solutions were prepared by diluting the sesame extract with blank food matrix (1:20 diluted with PBS). Recovery of sesame protein in food samples (crisp toasts, snacks, and rolls) spiked with different sesame protein concentrations ranged from 85% to 120%, with the exception of multigrain crisp toast, resulting in too high recoveries (117%−160%) and whole grain bread, yielding too low recoveries (70%−85%). In crisp bread, cracker, cereals, and snacks the limit of detection (LOD) was found to be 5 μg of sesame protein/g of food, in fresh breads and rolls, the LOD was 11 μg of sesame protein/g of food.

KEYWORDS: Sesame; *Sesamum indicum*; allergen; enzyme linked immunosorbent assay (ELISA); food

INTRODUCTION

According to rough estimations, the prevalence of allergic reactions to food is between 6% and 8% in children younger than 3 years of age and about 3% in adults (*1*). The exact prevalence of food allergies is, however, unknown since there is a lack of appropriate epidemiological studies. In addition, not every diagnosis is based on double-blind placebo controlled food challenge (DBPCFC) tests. Instead of using this "golden standard", diagnosis is often only made by the perception of allergic symptoms (*2*).

In the past, sesame (*Sesamum indicum*) allergy was only common in Eastern countries, for example, in Israel, where sesame containing foods are frequently consumed early in life (*3*). Papers, however, indicate that an increasing number of Europeans is confronted with health problems due to allergic reactions to sesame (*3*−*5*). The higher prevalence of sesame allergy is associated with the increasing consumption of sesame which has become a common ingredient in bakery products, fast food, and vegetarian and ethnic dishes (*6*). Sesame allergens are known to be very potent, causing particularly severe reactions in sensitized persons with a high risk of life threatening anaphylaxis (*4*, *5*, *7*, *8*). Some of them have already been identified: a sulfur poor 2S albumin (molecular weight 10 kDa, Ses i 1), a sulfur rich 2S albumin (7 kDa, Ses i 2), a 7S vicillin-like globulin (45 kDa, Ses i 3) (*9*, *10*), two oleosins (17 kDa, Ses i 4; 15 kDa, Ses i 5) (*11*), and two 11S globulins (Ses i 6 and Ses i 7) (*12*).

Recent studies raise the hope that in the future new strategies, such as sublingual and oral immunotherapy, will be effective in reducing sensitivity to allergens (*13*). However, currently the only option for allergic individuals is to avoid the certain allergenic food completely. In many countries the presence of allergenic substances has therefore to be declared on the food label. According to European Union legislation, 14 allergenic foods and food ingredients, including sesame seeds and products thereof, have to be given in the list of food ingredients (*14*). Analytical measurements are needed to verify if sesame containing food products are labeled in accordance with the regulations and if nonlabeled food products are actually free of sesame. The detection is, however, not an easy task since the allergens are present in minute amounts in complex and often highly processed food. Several review articles cover the latest developments in food allergen analysis (*15*−*17*). Analytical methods developed so far can be divided into protein-based and DNA-based methods. Among the protein-based methods, immunoassays, in particular enzyme linked immunosorbent assays (ELISAs), play the most important role. ELISAs have already been developed to detect potentially allergenic hazelnut (*18*, *19*), peanut (*20*), walnut (*21*), soy (*22*), and lupine (*23*). Up to now, only a few papers report the development of dipsticks (*24*,*25*) or immunosensors (*26*) allowing the detection of allergenic food. An overview on commercial immunoanalytical methods for the detection of allergenic food is given in a recent review article (*27*).

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PCR methods are based on the amplification of specific DNA sequences which can be detected either by agarose gel electrophoresis or in real-time by using fluorescently labeled probes. Real-time PCR methods have already been published for the detection of hazelnut (*28*), peanut (*29*), lupine (*30*), sesame (*31*,*32*), or the simultaneous determination of sesame and hazelnut in food (*33*). In addition, several PCR-based test kits are commercially available.

To our knowledge, an ELISA for the detection of sesame in food has not been published in a peer reviewed journal so far.

It was therefore the aim of the present study to produce polyclonal antibodies against sesame proteins by immunizing a hen and to use the resulting antibodies in the development and validation of an indirect competitive ELISA allowing the detection of traces of sesame in food. Hens were selected for immunization because in general large amounts of antibodies can be obtained from the egg yolks, thus avoiding the necessity of bleeding the animals.

MATERIALS AND METHODS

Reagents and Buffers. Polyclonal antisesame antibodies (IgY) were produced by immunizing two hens with a protein extract of sesame as described below. The secondary antibody was a rabbit antibody raised against IgY, labeled with horseradish peroxidase (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

The sample extraction buffer was prepared by dissolving 6.06 g of Tris and 11.69 g of NaCl in 1 L of water, adjusting the pH to 8.2 with 1 M HCl. Phosphate-buffered saline (PBS), pH 7.6, was prepared by dissolving 21.25 g of NaCl, 31.15 g of $Na_2HPO_4 \cdot 2H_2O$ and 3.9 g of $NaH_2PO_4 \cdot 2H_2O$ in 2.5 L of water.

For the ELISA, the following buffers and solutions were used. The coating buffer, pH 9.6, consisted of 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 in 1 L of water. The washing buffer, pH 7.6, was prepared by dissolving 51 g of NaCl, 5.89 g of KH_2PO_4, and 30 mL of Tween 20 in 1 L of water. A 2% (w/v) casein solution in PBS was used as blocking buffer. The citrate buffer was prepared by dissolving 46.04 g of potassium dihydrogen citrate and 0.1 g of sorbic acid in 1 L of water. The tetramethylbenzidine solution was prepared by dissolving 0.375 g of tetramethylbenzidine (Sigma, Vienna, Austria) in 5 mL of dimethylsulfoxide and 20 mL of methanol. The substrate solution was made by adding 500 μL of tetramethylbenzidine solution and 100 μL of 1% H_2O_2 to 25 mL of the citrate buffer. A 0.5 M H_2SO_4 solution was used as stop solution. All solutions and buffers were prepared with bidistilled water.

Extraction of Sesame Seed. Sesame seeds and food samples were purchased from local supermarkets. The sesame extract was prepared by grinding 4 g of white, peeled sesame in a model MM 2000 grinding mill (Retsch, Haan, Germany) to get a smooth paste. The paste was then defatted by Soxhlet extraction with *n*-hexane for 18 h. After drying overnight at room temperature, the defatted sesame was mixed with 30 mL of PBS buffer and stirred at room temperature for 2 h. This mixture was then centrifuged at 1500*g* for 30 min in a model 4K 10 centrifuge (Sigma, Vienna, Austria). The protein concentration of the supernatant was determined with the Bradford assay using bovine serum albumin (BSA) as standard. Small aliquots of the extract were stored at −18 °C.

For immunizing the hens, the sesame extract was diluted with PBS to a concentration of 1 mg protein/mL. Sesame protein standard solutions used in the ELISA were prepared by diluting the sesame extract with PBS to concentrations from 0.001 to 10000 ng protein/mL.

Extraction of Food Samples. Food samples were homogenized in a grinding mill. To 10 g of the ground sample, 50 mL of the sample extraction buffer were added. The resulting mixture was then mixed with a T25 Ultra Turrax (IKA, Staufen, Germany) for 2 min and centrifuged at 1500*g* for 30 min. The supernatant was filtered through a black ribbon filter paper (Schleicher & Schuell, Dassel, Germany) and centrifuged at 10000 rpm for 5 min in a model 5424 centrifuge (Eppendorf, Hamburg, Germany). After the supernatant was filtered through a black ribbon filter paper, aliquots were stored at −18 °C.

Production of Antibodies. Antibodies against sesame were produced at the Department of Biochemistry and Cell Biology, University of Vienna, Austria. Two hens (one laying brown eggs ("hen 1"), the other one laying white eggs ("hen 2")) were immunized by subcutaneously injecting the sesame extract (1 mg protein/mL PBS) containing Complete Freund's adjuvant, repeating the initial injection every 30th day. Hen 1 was immunized five times. Hen 2, however, stopped laying eggs after the third immunization. The eggs were stored at 4 °C. Titer determination was carried out with the yolks of eggs laid seven days after each immunization. For titer determination, the microtiter plates were coated with sesame extract and dilutions of the egg yolks were incubated for 30 min. IgY bound to sesame protein were detected with the commercially available rabbit antibody raised against IgY, labeled with horseradish peroxidase.

Isolation of Antibodies. Since hen 2 stopped laying eggs after the third immunization, the ELISA was developed with the antibodies produced by hen 1. Antibodies were isolated from the egg laid seven days after the fifth immunization. After egg yolk and egg white were separated and the vitelline membrane was removed, the volume of the yolk was measured. An equal volume of PBS buffer was added to the yolk, and the mixture was shaken for 30 min. After centrifugation at 1500*g* at room temperature for 15 min, the supernatant was filtered using a gauze bandage. Antibodies were then isolated from the filtrate by the ammonium sulfate precipitation method at a saturation concentration of 50% (w/v) (*34*). The concentration of the isolated antibodies was determined with the Bradford assay using immunoglobulin G (Sigma) as standard. The antibodies were stored in small aliquots at −18 °C.

SDS PAGE and Immunoblotting. A 5 μL portion of the sesame extract (1 mg/mL) was mixed with 5 μL of a 125 mM Tris-HCl buffer pH 6.8 containing 10% mercaptoethanol, 4% sodium dodecyl sulfate (SDS), 0.04% bromophenol blue, and 20% glycerol and heated to 60 °C for 30 min. A 3.5 μL aliquot was loaded onto a SDS polyacrylamide gel (T = 10.0%, C = 2.7%). Precision Plus Protein Standards (10−250 kDa; BioRad, Hercules, USA) and a protein solution containing conalbumin, BSA, ovalbumin, and IgG (concentration of each: 1 mg/mL in bidistilled water) were used as molecular weight markers. The electrophoresis buffer contained 0.025 M Tris−HCl pH 8.3, 0.1% SDS, and 0.192 M glycine. Electrophoresis was performed by using a Mighty Small SE 250 apparatus (Hoefer Scientific Instruments, Holliston, MA, USA) and a Power Pac (BioRad) set at 40 mA for 1.5 h. After the gel was equilibrated for 1 h in the blotting buffer (0.025 M Tris−HCl pH 8.3 and 0.192 M glycine) the proteins were transferred from the gel to a nitrocellulose membrane (7 × 8.5 cm, pore size 0.45 nm, BioRad) under cooling and stirring by using a Mini Trans-Blot electrophoretic transfer cell (BioRad) at 400 mA for 2 h. Then, the membrane was cut into strips.

After they were blotted, the proteins were stained with 0.1% amido black, 25% (v/v) isopropyl alcohol, and 10% (v/v) acetic acid in water. The proteins were made visible with the destaining solution, (25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid in water.

For immunoblotting, the strips were blocked with a solution of Tris-buffered saline, pH 7.5 (TBS: 10 mM Tris-HCl and 150 mM NaCl) and 3% (w/v) BSA at room temperature for 1 h. The strips were then incubated with the antisesame antibody in 0.5% BSA/TBS solution at 4 °C overnight. Bound IgY were detected by using commercial antichicken IgY labeled with peroxidase (Pierce), diluted 1:1000 with 0.5% BSA/TBS solution. After incubation for 2 h, staining was carried out with a 4-chloro-1-naphthol substrate solution. Between each step, the strips were washed thoroughly three times with TBS buffer for 10 min. The washing steps and the second incubation step were performed under gentle shaking at room temperature.

ELISA Procedure. Flat-bottom polystyrene Maxisorp F96 microtiter plates (Nunc, Wiesbaden, Germany) were coated with 200 μL/well of the coating solution comprising 1.22 μg/mL sesame extract in coating buffer. The plate was covered with parafilm and stored at 4 °C for 16−18 h. The plate was then washed three times with 300 μL of the washing buffer per well using a model 1575 immuno wash (BioRad). Remaining binding sites of the wells were then blocked with 200 μL/well of the blocking buffer at room temperature for 15 min. After washing the plate, the wells were filled with 50 μL of the sesame protein standards, in a range between 0.001 and 10000 ng/mL, or sample extracts, both in triplicates. Sesame protein standard solutions were prepared by diluting the sesame extract with the extract of the certain blank food matrix which had previously been diluted 1:20 with PBS.

Next, 100 μL/well of the primary antibody were added in a dilution of 1:10000 in PBS. To determine any nonspecific binding (NSB), 150 μL of PBS were added to six wells on the outer sides of the plate (instead of filling them with 50 μL of sample extract/sesame protein standard and 100 μL of the primary antibody). To determine the maximum signal (B_o) six wells on the outer sides of the plate were filled with 100 μL of the primary antibody solution and 50 μL of PBS. The incubation was carried out for 45 min at room temperature.

After washing the plate, 200 μL of the secondary antibody (horseradish peroxidase labeled anti-IgY antibody, diluted 1:30000 in PBS) were added to each well. After an incubation period of 1 h the plate was washed again.

A 200 μL portion of the substrate solution was added to each well. The enzymatic reaction was stopped after 12−15 min by adding 100 μL of the stop solution. The absorbance at 450 nm was measured using a model 680 XR microtiter plate reader (BioRad). Calibration curves were obtained by plotting the absorbance against the logarithmus of the sesame protein concentration. Nonlinear regression was carried out with SigmaPlot using a sigmoidal four parameter logistic function.

Cross-Reactivity Studies. Extracts of food used for cross-reactivity studies were prepared as described above. The following foods and food ingredients were extracted: peanut, hazelnut, walnut, Brazil nut, almonds, sunflower seed, poppy seed, rice, wheat, rye, oat, chocolate, and honey. The protein concentration of these extracts was determined by the Bradford assay using BSA as standard. Before the extracts were loaded onto the microtiter plate, they were diluted with PBS to achieve protein concentrations between 10 ng/mL and 1 mg/mL.

To calculate the cross-reactivity, the absorbance (A) at 450 nm was normalized using the following equation:

$$A_{corr} = \frac{A - NSB}{B_o - NSB}$$

Cross-reactivity (%) was then calculated by dividing the 50% binding concentration of sesame by the 50% binding concentration of the cross reactant and multiplying by 100.

Spiking of Samples and Recovery Studies. Recovery studies were performed with food samples the sesame protein concentration of which was below the limit of detection of the ELISA. The food matrices included snacks, crisp toast, freshly baked breads, crackers, whole wheat cookies, and muesli.

Spiking was carried out by two different methods.

Spiking with Sesame Proteins. After being ground, 10 g of the homogenized blank food sample (crisp toast, snack, roll, or whole grain bread) was spiked with sesame protein at the following levels: 25, 50, 100, and 200 μg/g. Following an incubation step of 15 min, the protein fraction was extracted from the spiked samples as described above. After diluting 1:20 with PBS, the extracts were subjected to ELISA analysis. The recovery was calculated from the ratio of the amount of sesame protein determined to the amount of sesame protein actually added to the sample.

Spiking with Sesame Seeds. Blank whole wheat cookies and muesli were spiked with white, peeled sesame seeds. The following spike levels were used: 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% (w/w) sesame in food matrix. First, sesame seeds were chopped in a small kitchen blender and ground with a mortar and pestle. The blank food matrix was chopped in a big kitchen blender. For the 1% spike level, 99 g of the chopped food sample and 1 g of homogenized sesame were mixed for 10 min in the big kitchen blender. After the mixture was blended with a spoon, the spiked food matrix was mixed for further 10 min. Before the kitchen blender was used again, it was washed properly with ethanol. For the 0.5% spike level, 50 g of the food matrix spiked with 1% sesame were mixed with 50 g of unspiked food matrix. For the 0.1% spike level, 10 g of the food matrix spiked with 1% sesame were mixed with 90 g of unspiked food matrix. The other spike levels were prepared in the same manner by "diluting" the spiked food matrix with the unspiked one.

After extracting the protein fraction as described above, the extract was diluted with PBS, the dilution factor depending on the spike level (from 1:2 to 1:500). For quantification, the absorbance closest to the inflection point of the calibration curve was used.

Limit of Detection and Limit of Quantification. To determine the limit of detection (LOD, S/N = 3) and the limit of quantification (LOQ, S/N = 10) of the ELISA, the extracts of blank food matrices (diluted 1:20 with PBS) were filled in six wells of a microtiter plate and subjected to ELISA analysis. The LOD of the ELISA was calculated by subtracting three times the standard deviation of the obtained absorbance from the mean absorbance and calculating the corresponding concentration by using the equation of the calibration curve established with sesame protein standard solutions. The LOQ was calculated in the same way but by subtracting 10 times the standard deviation of the absorbance.

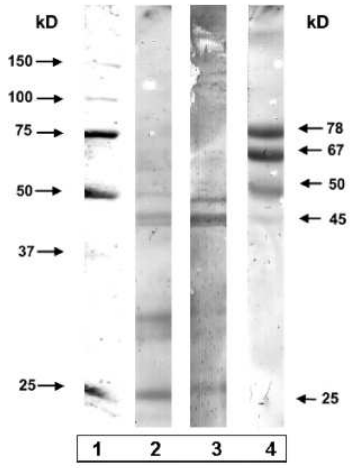

Figure 1. Characterization of the antisesame IgY. Lane 1 and lane 4: Blot of molecular weight markers, stained with amido black. Protein sizes (kDa) are indicated on the side of the strips (lane 1: precision Plus Protein Standard (10−250 kDa; BioRad), lane 4: protein solution containing conalbumin, BSA, ovalbumin and IgG). Lane 2: Blot of the sesame extract, stained with amido black. Lane 3: Immunoblot of the sesame extract with antisesame IgY.

Analysis of Roasted Sesame Seeds. A 30 g portion of white, peeled sesame seeds was roasted at the Department of Food Sciences and Technology of the University of Natural Resources and Applied Life Sciences, Vienna, Austria. Roasting was carried out for 10 min at four different temperatures: 100, 150, 200, and 250 °C, using a type 60/3 W backing oven (Manz Backtechnik, Münster, Germany). A 10 g aliquot of the roasted sesame was then subjected to extraction as described above, and the protein concentration in the extract was determined using the Bradford assay. The extracts were diluted 1:20 with PBS and analyzed by the ELISA.

Analysis of Commercial Foodstuffs. Commercial foodstuffs were extracted as described above. The extracts were diluted with PBS in the range from 1:5 to 1:500. For quantification, the OD value closest to the inflection point of the calibration curve was used.

RESULTS AND DISCUSSION

Production and Characterization of Antisesame Antibodies. Extraction of white, peeled sesame seeds yielded an extract with a protein concentration of 7.3 mg/mL. For antibody production, two hens were immunized with the extract previously diluted with PBS to a concentration of 1.0 mg/mL. The initial injection was repeated every 30th day. However, after the third immunization hen 2 stopped laying eggs. The ELISA was therefore developed with the antibodies produced by hen 1. Antibodies were isolated from eggs laid 5−7 days after the fifth immunization. In an average, 95 mg of antibodies were isolated per egg yolk. The ELISA was developed with antibodies isolated from the egg laid 7 days after the fifth immunization.

SDS PAGE and immunoblotting were performed to investigate which proteins of the sesame seed extract were recognized by

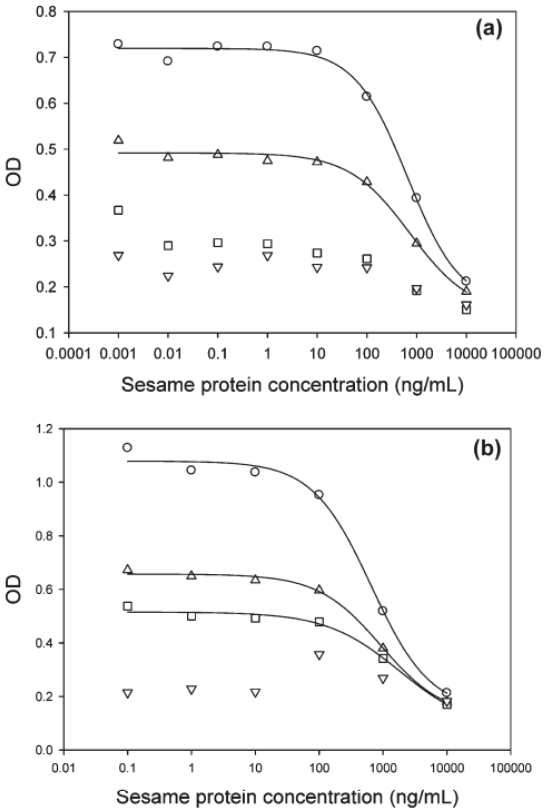

Figure 2. Influence of the concentration of the primary antibody (**a**) and the coating solution (**b**) on the calibration curve. (**a**) The primary antibody solution was diluted 1:10000 (○), 1:20000 (△), 1:30000 (□) or 1:50000 (▽). (**b**) The concentration of the coating solution was 1.22 (○), 0.61 (△), 0.41 (□), or 0.31 μg/mL (▽). Coating temperature: 4 °C.

the IgY. Staining the blotted proteins with amido black (**Figure 1**, lane 2) resulted in protein bands at approximately 25, 29, 31, 45, and 48 kDa. These molecular weights are in agreement with those published by Beyer et al. (*10*). Lane 3, showing the immunoblot of the sesame extract with IgY, indicates that the IgY reacted with sesame proteins of 25, 45, and 48 kDa.

Development and Optimization of the ELISA. In the development of the ELISA, the following parameters were optimized: concentration of the coating antigen, coating temperature, blocking time, concentration of the primary antibody and incubation time of the antigen and the primary antibody.

Initially the concentration of the primary antibody was optimized by diluting the primary antibody solution from 1:10000 to 1:50000. The influence of the primary antibody concentration on the calibration curve is shown in **Figure 2A**. Dilution factors of 1:10000 and 1:20000 resulted in slopes of 0.8179 and 0.7110 mL/ng, respectively. In all further experiments, the primary antibody solution was therefore diluted 1:10000. Incubation time was varied from 15 min to 1 h; the optimum time was found to be 45 min.

To optimize the coating antigen concentration and the coating temperature, two microtiter plates were coated with four different coating antigen concentrations, one plate at 36 °C and the other one at 4 °C. The influence of the coating antigen concentration on the calibration curve is shown in **Figure 2B**. Coating antigen

Table 1. Cross-Reactivity of Selected Foods and Food Ingredients in the Sesame ELISA

food/ingredient	protein concentration (mg/mL)	cross-reactivity (%)[a]
peanut	12.3	n.d.
hazelnut	13.4	n.d.
walnut	2.5	n.d.
brazil nut	48.5	n.d.
almonds	51.9	n.d.
sunflower seed	16.6	n.d.
poppy seed	18.0	n.d.
rice	2.0	n.d.
wheat	13.1	n.d.
rye	17.0	n.d.
oat	14.4	n.d.
chocolate	9.2	0.7
honey	0.05	n.d.

[a] n.d. = below the LOD.

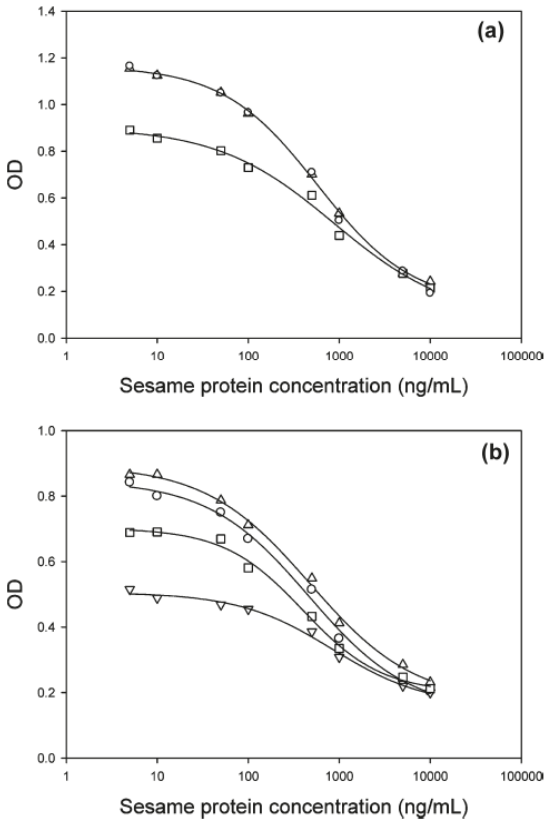

Figure 3. Influence of the matrix on the calibration curve. (**a**) (△) sesame protein standards were prepared in PBS; (□) sesame protein standards were prepared with the extract of blank crackers, which had been 1:20 diluted with PBS; (○) the highest concentrated standard solution was prepared by diluting the sesame extract with the extract of blank crackers, which had been 1:20 diluted with PBS; further dilutions were prepared by dilution with PBS. (**b**) Sesame protein standard solutions were prepared either in PBS (△), the extract of blank crackers (○), the extract of blank cereals (□), or the extract of blank crisp toast (▽). All food extracts had previously been diluted 1:20 with PBS.

concentrations of 1.22, 0.61, and 0.41 μg/mL yielded calibration curves with slopes of 0.9424, 0.8848, and 0.7561 mL/ng,

Table 2. Recovery of Sesame Protein in Commercially Available Blank Samples Spiked Prior to Extraction

		recovery (%)					
sample	spiking concentration (μg/g)	day 1	day 2	day 3	day 4	mean recovery (%)	interassay relative standard deviation (%)
crisp toast 1	25	87	105	96	91	95	8
	50	102	99	108	104	103	3
	100	91	97	106	110	101	8
	200	98	94	96	81	92	8
crisp toast 2	25	78	91	89	–	86	8
	50	88	119	97	–	101	16
	100	93	102	100	–	98	5
	200	101	139	105	–	115	18
multigrain crisp toast	25	186	180	146	128	160	17
	50	127	141	137	134	134	4
	100	98	148	134	145	131	17
	200	77	93	151	150	117	32
snack	25	85	115	105	93	99	13
	50	85	110	97	112	101	12
	100	108	123	135	138	126	10
	200	72	72	76	85	76	8
roll	25	80	115	86	98	94	16
	50	96	118	77	115	101	19
	100	95	116	78	117	101	19
	200	88	102	75	109	93	16
whole grain bread	25	77	57	76	–	70	16
	50	78	70	99	–	82	18
	100	67	76	114	–	85	29
	200	51	68	99	–	73	33

–: not determined.

respectively. In contrast to the coating antigen concentration, the coating temperature did not have any influence on the calibration curve. In all further experiments coating was carried out at 4 °C.

After the coating step, the remaining binding sites of the wells were blocked with a 2% (w/v) casein solution in PBS. To optimize the blocking time, a microtiter plate was divided into four parts and the blocking time varied from 15 min to 1 h. Since the slopes of the calibration curves were rather similar, in all further experiments a blocking period of 15 min was applied.

As already mentioned above, optimization experiments were carried out with antibodies isolated from only one egg. We have, however, carried out additional experiments using antibodies isolated from different eggs laid in the same time period. The calibration curves obtained did not differ significantly.

Cross-Reactivity of the ELISA. To determine the cross-reactivity of the ELISA, the protein fraction of food ingredients commonly found in sesame containing foodstuffs was extracted. The food ingredients and the protein concentrations of the extracts are listed in **Table 1**. The extracts were diluted with PBS to obtain protein concentrations between 10 ng/mL and 1 mg/mL and subjected to analysis by the ELISA. **Table 1** indicates that the ELISA did not show any cross-reactivity with 12 from the 13 food ingredients tested. Only for chocolate was a low cross-reactivity of 0.7% determined. It is, however, probable that the decrease in the OD value was caused by nonspecific interference of the chocolate matrix and not due to specific interactions with the antisesame antibodies.

Matrix Influence. To test for any matrix influences, the ELISA was carried out with sesame protein standard solutions which had been prepared by three different methods. In one case, the standard solutions were prepared by diluting the sesame extract with PBS. In the next case, the sesame extract was diluted with the extract of blank crackers (diluted 1:20 with PBS) to obtain the highest concentrated standard solution and then serially diluted with PBS to obtain standard solutions of lower concentrations. And in the last case, all standard solutions were prepared by diluting the sesame extract with the extract of blank crackers (diluted 1:20 with PBS).

The standard curves obtained with the different sesame protein standard solutions are shown in **Figure 3A**. Preparing the standard solutions by diluting the sesame extract with the extract of blank crackers (1:20 diluted with PBS) yielded a calibration curve which differed from the other two calibration curves, indicating a matrix effect.

To check the influence of other food matrices a series of experiments was carried out by diluting the sesame extract with extracts of various blank food matrices, for example, cracker, crisp toast, and cereals. Each of the extracts was used 1:20 diluted with PBS. **Figure 3B** shows that the food matrices influenced the calibration curve to a different degree. Among the matrices tested, crisp toast showed the biggest influence.

To take these matrix effects into account, further experiments were carried out with sesame protein standard solutions obtained not by diluting the sesame extract 1:20 with PBS but by diluting it with the extract of blank matrix (1:20 diluted with PBS).

Table 3. Recovery of Sesame in Blank Muesli Spiked with Sesame Seeds

spiking level (%)	dilution of the extract	sesame protein concentration (μg/g)	sesame concentration (%)	recovery (%)
0.001	1:2	5	0.003	300
0.005	1:2	9	0.005	100
0.01	1:5	17	0.01	100
0.05	1:20	107	0.06	120
0.1	1:50	200	0.1	100
0.5	1:100	906	0.5	100
1	1:500	1505	0.8	80

Table 4. Recovery of Sesame in Blank Whole Wheat Cookie Spiked with Sesame Seeds[a]

spiking level (%)	dilution of the extract	sesame protein concentration (μg/g)	sesame concentration (%)	recovery (%)
0.001	1:10	6	0.003	300
0.005	1:10	26	0.014	280
0.01	1:10	23	0.01	100
0.05	1:50	147	0.08	160
0.1	1:50	154	0.08	80
0.5	1:50	928	0.5	100
1	1:5	xxx	xxx	xxx

[a] xxx = OD value $\approx$ NSB.

Since a matrix similar to the samples to be analyzed had to be chosen the blank cracker extract was used in the analysis of all kinds of crackers and cookies, the crisp toast for all kinds of crisp toasts, and the cereal extract when analyzing cereals, mueslis, and muesli bar snacks.

Accuracy and Precision. The accuracy and precision of the ELISA were determined in recovery studies performed by spiking blank food matrices with either sesame protein or sesame seeds. In previous ELISA analysis, sesame could not be detected in these matrices (sesame protein concentration < the limit of detection of the ELISA).

Table 2 summarizes the recoveries obtained by the analysis of different blank food matrices which had been spiked with sesame protein. The extracts of the spiked foodstuffs were analyzed on either 3 or 4 subsequent days. In general, the recovery of sesame protein was in the range from 85 to 126%, independent of the spiking level, indicating the high accuracy of the ELISA. However, in the case of the multigrain crisp toast, too high recoveries (117%−160%) and in the case of the whole grain bread, too low recoveries (70%−85%) were obtained. Relative standard deviation ranged from 3% to 33%, indicating a high interday repeatability of the ELISA.

Table 3 and **Table 4** summarize the results obtained by analyzing the extracts of blank muesli and whole wheat cookies which had been spiked with sesame seeds in a concentration range from 0.001% to 1% sesame. To determine the conversion factor enabling the conversion from the sesame protein concentration per gram of food (as calculated from the calibration curve) to the sesame concentration per gram of food, sesame was extracted with the extraction procedure applied to all other food samples. The sesame protein concentration was found to be 18% which is close to the protein concentration of 20% given in literature (*3*). The sesame protein concentration in μg/g, as determined with the ELISA, was therefore multiplied by 5.5 to calculate the sesame concentration in μg/g food. **Table 3** shows that with the exception of the lowest spike level the recovery of sesame in muesli was in the range from 80% to 120%. In whole wheat cookie, considerably higher recoveries were obtained for the spiking levels 0.001%, 0.005%, and 0.05% (**Table 4**).

Table 5. Analysis of Roasted Sesame

roasting temperature (°C)	protein concentration of the extract (mg/mL)	sesame protein concentration determined by the ELISA (μg/g)
100	12.3	88.5
150	11.1	93.7
200	1.2	<LOQ
250	0.1	n.d.

Limit of Detection (LOD) and Limit of Quantification (LOQ). The limit of detection (LOD) of the ELISA was calculated by determining the mean absorbance of different blank food matrices, diluted 1:20 with PBS (n = 6). From this mean value three times the standard deviation was subtracted. The limit of quantification (LOQ) was calculated by subtracting 10 times the standard deviation.

In the case of diluting the sample extract 1:20 in crisp bread, crackers, cereals, and snacks, the LOD and LOQ were found to be 5 and 30 μg sesame protein/g of food, corresponding to 28 and 165 ppm sesame. In fresh breads and buns, the LOD and LOQ were found to be 11 and 49 μg sesame protein/g of food, corresponding to 61 and 270 ppm sesame. In oral food challenge studies the threshold dose for eliciting allergic reactions ranged from 30 mg to 10 g of sesame seed (*3*). An analytical method allowing the detection of 30 mg sesame seed in a 200 g portion of food should have a LOD of 150 ppm sesame. According to Poms et al. (*15*) the LOD of an analytical method suitable for the detection of allergenic foods should be between 1 and 100 ppm (μg allergenic protein/g of food). The ELISA developed in the present study meets these requirements.

Analysis of Roasted Sesame. To investigate the applicability of the ELISA to detect roasted sesame, white peeled sesame seeds were roasted for 10 min at four different temperatures: 100, 150, 200, and 250 °C. The roasted sesame was then subjected to extraction and the protein concentration in the extract was determined using the Bradford assay. The extracts were then diluted 1:20 with PBS and analyzed by the ELISA. **Table 5** summarizes the protein concentration obtained by the Bradford assay and the sesame protein concentration determined by the ELISA. It can be seen that the protein concentration of the extracts drastically decreased with increasing roasting temperature. The protein concentration of the extracts obtained from sesame seeds roasted at 200 and 250 °C were only 1.2 and 0.1 mg/mL, respectively. Due to this low protein concentration, sesame roasted at 250 °C could not be detected with the ELISA.

Applicability of the ELISA to Commercial Food Samples. To demonstrate the applicability of the developed ELISA, 28 commercially available samples, with different declarations, such as "may contain sesame", "contains sesame" or "does not contain sesame", were analyzed. The results are summarized in **Table 6**. In 12 of 13 sesame containing food samples sesame could be detected. Sesame could, however, not be detected in sesame oil. Only in three samples could sesame protein be quantified, for the other samples the absorbance measured were outside the quantification region of the calibration curve. In eight samples the sesame protein concentration was so high, that the resulting absorbance did not differ from the NSB value although the sample extracts were diluted up to 1:500. In sunflower crisp toast, the sesame protein concentration was below the LOQ.

With the ELISA, sesame was not detected in foodstuffs which did not have any information about containing sesame. Sesame was detected in one out of nine samples precautionary labeled with "might contain sesame".

Fifteen of the 28 food samples were also analyzed by a real-time PCR method which had recently been developed in our research

Table 6. Analysis of Commercially Available Foodstuffs

samples	declaration[a]	ELISA result[b](sesame protein concentration (μg/g))	PCR result[c]
sesame snack 1	(+)	+ (5928)	+
sesame snack 2	(+)	+ (2575)	+
sesame snack 3	(+)	+ (3404)	−
sesame balls 1	(+)	+[a]	+
sesame balls 2	(+)	+[a]	+
crisp flakes	(+)	+[a]	+
sesame flakes	(+)	+[a]	−
sesame cookies	(+)	+[a]	+
sesame peanut cookies	(+)	+[a]	−
sunflower crisp toast	(+)	<LOQ	−
peanut cookies (packed with sesame cookies)	(+)	+[a]	−
cashew nut cookies (packed with sesame cookies)	(+)	+[a]	−
sesame oil	(+)	n.d.	n.d.
whole grain crisp toast 1	(±)	n.d.	−
butter biscuits	(±)	n.d.	n.d.
crackers	(±)	n.d.	n.d.
muesli bar with lemon	(±)	n.d.	−
muesli bar	(±)	<LOQ	−
nut muesli	(±)	n.d.	n.d.
whole grain crisp toast with poppy and sunflower seeds	(±)	n.d.	−
muesli	(±)	n.d.	−
muesli bar with grapes	(±)	n.d.	n.d.
cereal 1	(−)	n.d.	n.d.
cereal 2	(−)	n.d.	−
flakes with fruits	(−)	n.d.	n.d.
whole grain crisp toast 2	(−)	n.d.	−
crisp toast	(−)	n.d.	n.d.
chocolate biscuits	(−)	n.d.	n.d.

[a] Declaration: (+), sesame listed; (−), sesame not listed; (±), may contain sesame. [b] ELISA result: +[a] = sesame was detected but could not be quantified (OD value ≈ NSB); n.d. = below the LOD. [c] PCR result: + = C_t value < 40; n.d. = no increase of the fluorescence signal was observed, (−) not analyzed.

group (*31*). **Table 6** indicates that the results obtained with the ELISA are in agreement with those obtained by PCR. Our recently published real-time PCR method did not allow the detection of sesame in sesame oil either. This limits the applicability of both methods since sesame oil is frequently unrefined, thus being able to trigger allergic reactions in sensitized patients (*3*). In oral food challenge studies the threshold dose ranged from 1 to 5 mL. The detection of food allergens in seed oils is, however, generally known to be a difficult task.

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ORIGINAL PAPER

Development and validation of a sandwich ELISA for the determination of potentially allergenic sesame (*Sesamum indicum*) in food

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Abstract This paper presents a sandwich enzyme-linked immunosorbent assay (ELISA) that allows the determination of traces of sesame in food. Chicken anti-sesame antibodies, used as coating antibodies, and rabbit anti-sesame antibodies, used as secondary antibodies, were prepared by immunization with a protein extract of white, peeled sesame. The ELISA did not show any cross-reactivity with 19 food ingredients commonly found in sesame-containing foodstuffs such as seeds, nuts, and cereals. In whole grain bread, crisp toast, and snacks, the limit of detection (S/N=3) was 0.5, 0.5, and 0.3 μg sesame protein/g, and the limit of quantification (S/N=10) was 0.6, 0.8, and 1.4 μg sesame protein/g, respectively. The analysis of blank food matrices (whole grain bread, white bread, crisp toast, and snacks) spiked with sesame protein at four spike levels generally resulted in mean recoveries from 72% to 145%. In the case of spiking blank food matrices with sesame seeds, the ELISA proved to be more accurate for whole wheat cookies than for whole wheat bread.

Keywords Sesame · *Sesamum indicum* · Allergen · Enzyme-linked immunosorbent assay (ELISA) · Food

Introduction

Sesame (*Sesamum indicum*) is cultivated for its seeds in tropical and subtropical areas of the world, with Asia (India, China, and Myanmar) and Africa (Sudan and Ethiopia) being the main producing regions. In the past, sesame was a popular food ingredient particularly in the Middle East; but nowadays, people all over the world enjoy the nutty flavor of roasted sesame seeds and sesame oil. Whole seeds are frequently sprinkled on buns, breads, biscuits, and snacks, whereas sesame oil is mainly used for cooking.

A low percentage of the human population is allergic to sesame. However, several papers indicate that the prevalence of sesame allergy is increasing [1–5]. Sesame allergy tends to appear early in life but in contrast to, e.g., cow's milk or egg allergy, most children with sesame allergy do not become tolerant to sesame over time [6]. In sensitized people, consumption of sesame can cause a variety of allergic reactions, including hives, allergic rhinitis, atopic dermatitis, oral allergy syndrome, and even life-threatening anaphylaxis [1–6]. So far, seven allergenic proteins have been identified in sesame: a sulfur-poor 2S albumin (molecular weight 10 kDa, Ses i 1), a sulfur-rich 2S albumin (7 kDa, Ses i 2), a 7S vicillin-like globulin (45 kDa, Ses i 3) [7, 8], two oleosins (17 kDa, Ses i 4; 15 kDa, Ses i 5) [9], and two 11S globulins (Ses i 6 and Ses i 7) [10]. Ses i 1 has recently been shown to be rather thermostable and highly resistant to in vitro gastric digestion [11].

Sesame allergic individuals are well advised to strictly avoid the consumption of sesame-containing food. Many countries, e.g., the member states of the European Union, legally demand that the presence of sesame has to be indicated in the list of food ingredients [12]. Analytical measurements are needed to verify if sesame-containing food products comply with the food labeling regulations and if non-labeled food products are actually free of sesame. An analytical method has to fulfill several criteria in order to be suitable for the detection of food allergens.

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The method should be specific for the allergenic food since cross-reactivity with other food components would cause systematic errors (false positive results). The limit of detection (LOD) of the method should be low enough to allow the detection of allergen amounts, which are sufficient to elicit allergic reactions in highly sensitized patients. It is generally agreed that the LOD should be in the range of 1 to 100 ppm [13]. In addition, the analytical method should be applicable to both unprocessed and processed foods to avoid false-negative results.

DNA- and protein-based methods have already been developed for the determination of allergens in food. Enzyme-linked immunosorbent assays (ELISAs) are the most frequently applied protein-based methods in food allergen analysis. In competitive ELISAs, immobilized antigen and antigen (analyte) in solution compete for specific antibodies present in limited amount. Competitive ELISAs have already been developed to determine hazelnut [14], peanut [15], or soybean [16]. Our research group recently presented a competitive ELISA that allows the determination of traces of sesame in food [17]. Immunometric assays, e.g., sandwich ELISAs, make use of two antibodies raised against different epitopes of the analyte. Sandwich ELISAs offer several advantages compared with competitive ELISAs. The LOD of sandwich ELISAs is generally lower since it is more precise to measure a large signal against a low background signal (sandwich ELISA) than to measure the difference between two large signals (competitive ELISA). In addition, sandwich ELISAs often show less interfering matrix effects than competitive ELISAs. Sandwich ELISAs have already been developed for the determination of hazelnut [18–20], peanut [21], mustard [22], or lupine [23–25].

To our knowledge, a sandwich ELISA for the detection of sesame in food has not been published in a peer-reviewed journal so far. The present study aimed at developing and validating a sandwich ELISA for the determination of sesame in food and comparing its characteristics and applicability with those of the recently published competitive ELISA.

Experimental

Reagents, buffers, and food samples

The production and isolation of polyclonal chicken anti-sesame antibodies (IgY) is described in Ref. [17]. Polyclonal rabbit anti-sesame antibodies (IgG) were produced by BioGenes (Berlin, Germany) as described below. Goat antibodies raised against rabbit IgG and rabbit antibodies raised against chicken IgY, both labeled with horseradish peroxidase, were purchased from Pierce (Thermo Fisher Scientific, Rockford, IL, USA).

Phosphate-buffered saline (PBS), pH 7.6, consisted of 21.25 g of NaCl, 31.15 g of $Na_2HPO_4 \times 2\ H_2O$, and 3.9 g of $NaH_2PO_4 \times 2\ H_2O$ in 2.5 L of water. The sample extraction buffer was prepared by dissolving 6.06 g of Tris and 11.69 g of NaCl in 1 L of water, adjusting the pH to 8.2 with 1 M HCl. The coating buffer, pH 9.6, contained 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3 in 1 L of water. The washing buffer consisted of 51.0 g of NaCl, 9.36 g of $NaH_2PO_4 \times 2\ H_2O$, 74.76 g of $Na_2HPO_4 \times 2\ H_2O$, and 30 mL of Tween 20 in 1 L of water. For blocking, a 2% (*w*/*v*) casein solution in PBS was used. The citrate buffer consisted of 46.04 g of potassium dihydrogen citrate and 0.1 g of sorbic acid in 1 L of water. The tetramethylbenzidine (TMB) solution was made by dissolving 0.375 g of TMB (Sigma, Vienna, Austria) in 5 mL of DMSO and 20 mL of methanol. The substrate solution consisted of 500 μL of TMB solution, 100 μL of 1% H_2O_2, and 25 mL of the citrate buffer; 1.0 M H_2SO_4 was used as stop solution. All solutions and buffers were prepared with bidistilled water.

White peeled, white unpeeled, and black sesame seeds were bought from different producers. Food samples were purchased from local supermarkets.

Extraction

Extraction of sesame seeds

Thirty-five gram of white peeled sesame were grinded in a kitchen mill. The sesame paste was defatted by Soxhlet extraction with 190 mL of *n*-hexane for 18 to 20 h. After drying, the defatted sesame was extracted with 300 mL of PBS under stirring for 2 h at room temperature. After centrifuging the mixture at 1,500×*g* for 30 min, the protein concentration of the supernatant was determined with the Bradford assay using bovine serum albumin (BSA) for calibration. The extract was stored in small aliquots at −18 °C.

For immunization, the sesame extract was diluted with PBS to a concentration of 1 mg protein/mL. Sesame protein standard solutions used for calibrating the ELISA were prepared by diluting the sesame extract with PBS.

Extraction of food samples

After homogenizing the food samples in a grinding mill, an aliquot of 10 g was mixed with 50 mL of the sample extraction buffer with a T25 Ultra Turrax (IKA, Staufen, Germany) for 2 min. After centrifugation at 1,500×*g* for 30 min, the supernatant was filtered through a black ribbon filter paper (Schleicher & Schuell, Dassel, Germany). The filtrate was centrifuged at 10,000 rpm for 5 min in a Model 5424 centrifuge (Eppendorf, Hamburg, Germany). After

filtering the supernatant through a black ribbon filter paper, aliquots were stored at −18 °C.

Production and isolation of rabbit anti-sesame antibodies

IgG against sesame proteins were produced by immunizing a rabbit with a protein extract (1 mg/mL) of sesame. The adjuvant was prepared by dissolving 20 mg of lipopolysaccharide (Sigma) in 1 mL of bidistilled water and mixing the solution with 15 mL of Span 80 (Sigma), 15 mL of Tween 80 (Sigma), and 400 mL of mineral oil for 5 min. Antigen and adjuvant were mixed in a ratio of 1:4. The rabbit was immunized four times in intervals of 7 days with 100 to 200 μg antigen, finishing the immunizing by a last booster injection after further 21 days. The blood was collected 7 days after the last booster. The antibody fraction was isolated using the ammonium sulfate precipitation method according to the procedure given in Ref. [26]. After removing surplus ammonium sulfate by dialysis against PBS, the protein concentration was determined with the Bradford assay using IgG (Sigma) as standard. The antibody fraction was stored in small aliquots at −18 °C.

SDS PAGE and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS PAGE) under reducing conditions, blotting of the proteins, and staining with amido black were carried out as described in Ref. [17]. Before immunostaining, the blots were blocked with a solution of Tris-buffered saline (TBS), (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and 3% (*w*/*v*) BSA at room temperature for 1 h. The blots were then incubated with anti-sesame antibodies (IgG or IgY) in 0.5% BSA/TBS solution at 4 °C overnight. After a 2 h incubation period with either goat anti-rabbit IgG or rabbit anti-chicken IgG, both labeled with horseradish peroxidase, staining was carried out with a 4-chloro-1-naphthol substrate solution. Between each step, the blots were washed thoroughly three times with TBS buffer for 10 min. Washing and incubation were carried out under gentle shaking at room temperature.

Procedure of the optimized sandwich ELISA

Microtiter plates (Maxisorp F96, Nunc, Wiesbaden, Germany) were coated with 200 μL/well of an IgY solution (protein concentration 1.2 mg/L, as determined with the Bradford assay) in coating buffer. After covering with parafilm, the plate was stored at 4 °C for 16 h. The plate was then washed three times with 300 μL/well of the washing buffer (Model 1575 immuno wash, BioRad). Non-occupied binding sites were blocked with 300 μL/well of the blocking buffer at room temperature for 60 min. After washing the plate, either 50 μL of the sesame protein standards (0.01 to 10,000 μg/L in PBS) or 50 μL of sample extracts was added in triplicates. After an incubation time of 30 min, the plate was washed again. Then, 100 μL/well of the secondary antibody (IgG) solution with a concentration of 2.6 mg/L PBS was added and incubated for 30 min. After removing unbound IgG by washing, 200 μL of the detection antibody (horseradish peroxidase labeled goat anti-rabbit IgG, diluted 1:60,000 in PBS) were added to each well and incubated for 1 h. After washing, 200 μL/well of the substrate solution was added. The enzymatic reaction was commonly stopped after 7 to 8 min by adding 100 μL/well of the stop solution. The optical density (OD) was measured at 450 nm (microtitre plate reader, Model 680 XR, BioRad). Calibration curves were obtained by plotting the OD values against the logarithm of the sesame protein concentration. A sigmoidal four parameter logistic function was used for non-linear regression.

Cross-reactivity studies

The following 19 foods and food ingredients were tested for cross-reactivity: peanut, hazelnut, walnut, Brazil nut, almond, cashew nut, pistachio, sunflower seed, poppy seed, soybean, green pea, rice, wheat, rye, oat, barley, corn, chocolate (40% *w*/*w* cacao), and honey. The protein concentration of the extracts was determined by the Bradford assay using BSA for calibration. The extracts were diluted with PBS to obtain protein concentrations between 10 μg/L and 1,000 mg/L and loaded onto the microtiter plate.

For calculating the cross-reactivity, the OD values were normalized according to the following equation:

$$\mathrm{OD}_{\mathrm{corr}} = \frac{B - \mathrm{NSB}}{B_{\max} - \mathrm{NSB}}$$

NSB (non-specific binding) was determined by carrying out the immunoassay in the absence of analyte; $B_{\max}$ (maximum binding) was the highest signal obtained. Cross-reactivity (in percent) was calculated by dividing the 50% binding concentration of sesame by the 50% concentration of the cross reactant and multiplying the value by 100.

Spiking of samples and recovery studies

Recovery studies were carried out with whole grain bread, whole wheat bread, white bread, crisp toast, snacks, and whole wheat cookies. All food samples had previously been analyzed by the ELISA and had been found to be free of sesame protein (sesame protein concentration below the LOD).

Spiking was carried out by adding either sesame proteins or sesame seeds to the blank samples.

Spiking with sesame proteins

Five milliliters of a sesame protein standard solution (containing either 50, 100, 200, or 400 mg/L) was added to 10 g of the homogenized blank food sample (whole grain bread, white bread, crisp toast, or snack) to obtain spike levels of 25, 50, 100, or 200 μg/g. After an incubation step of 15 min, the protein fraction was extracted as described above. The extracts were diluted 1:20 with PBS before being analyzed by the ELISA. The recovery was calculated from the ratio of the amount of sesame protein determined to the amount of sesame protein actually added to the sample.

Spiking with sesame seeds

Blank whole wheat cookies and whole wheat bread were used for spiking. White peeled sesame seeds were chopped in a small kitchen blender and ground with a mortar and pestle. The blank food matrices were chopped in a big kitchen blender. The following spike levels were used: 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% (*w*/*w*) sesame in food matrix. Ninety-nine grams of the chopped food sample and 1 g of homogenized sesame seeds were mixed in the big kitchen blender for 10 min to obtain the 1% spike level. After blending the mixture with a spoon, the spiked food matrix was mixed for further 10 min. The 0.5% spike level was obtained by mixing 50 g of the food matrix spiked with 1% sesame with 50 g of unspiked food matrix. Mixing 10 g of the food matrix spiked with 1% sesame with 90 g of unspiked food matrix yielded the 0.1% spike level. The other spike levels were prepared according to the same principle by "diluting" spiked food matrices with unspiked ones. In between the preparation of different batches of spiked food, the kitchen blender was washed properly with ethanol. After extracting the protein fraction as described above, the extract was diluted with PBS from 1:2 to 1:500, the dilution factor depending on the spike level. For quantification, the absorbance closest to the inflection point of the calibration curve was used.

Conversion of sesame protein concentration to sesame concentration

In order to be able to calculate the sesame concentration of food samples from the sesame protein concentration obtained by the ELISA, a conversion factor had to be determined. The protein fraction of white peeled sesame seeds was extracted three times with the procedure applied to all food samples. The mean value of the protein concentration of the extracts was found to be 36 g/L, indicating that 1.8 g protein was extracted from 10 g sesame, corresponding to a protein concentration of 18%. The sesame concentrations of the food samples were therefore calculated by multiplying the sesame protein concentration by 5.5.

Analysis of roasted sesame seeds

Thirty g of white peeled sesame seeds was roasted for 10 min at either 100, 150, 200, or 250 °C in a baking oven (Type 60/3W, Manz Backtechnik, Münster, Germany). Ten-gram aliquots were extracted as described above and subjected to ELISA analysis.

Analysis of commercial foodstuffs

Commercial foodstuffs were extracted as described above. The extracts were diluted 1:20 with PBS.

Results and discussion

Characterization of anti-sesame antibodies

For the development of the sandwich ELISA, two kinds of polyclonal anti-sesame antibodies were used: IgY which had already been applied in the development of a competitive sesame ELISA [17] and IgG. SDS PAGE under reducing conditions and immunoblotting were carried out to assess the molecular weight of the sesame proteins

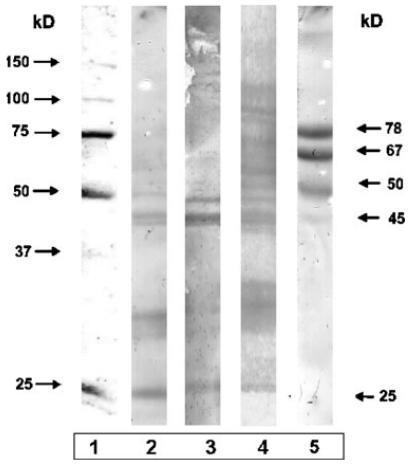

Fig. 1 Characterization of the anti-sesame antibodies by SDS PAGE under reducing conditions and immunoblotting. *Lanes 1* and *5*, blot of molecular weight markers, stained with amido black. Protein sizes (kDa) are indicated on the side of the strips (*lane 1*, precision Plus Protein Standard (10–250 kDa; BioRad); *lane 5*, protein solution containing conalbumin, BSA, ovalbumin, and IgG). *Lane 2*, blot of the sesame extract, stained with amido black. *Lane 3*, immunoblot of the sesame extract with anti-sesame IgY. *Lane 4*, immunoblot of the sesame extract with anti-sesame IgG

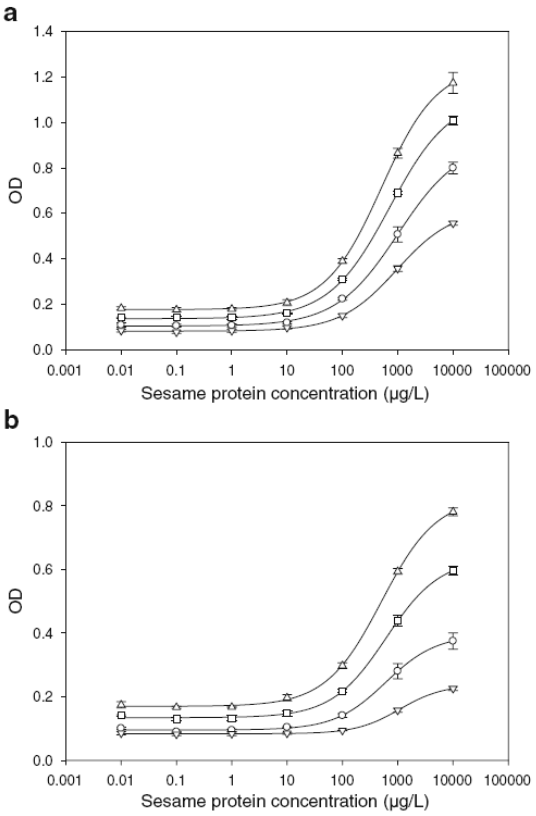

Fig. 2 Influence of the coating antibody (IgY) concentration and the coating temperature on the calibration curve. **a** Coating temperature, 4 °C. *Triangles* 1.16 mg/L, *squares* 0.48 mg/L, *circles* 0.24 mg/L, *inverted triangles* 0.12 mg/L. **b** Coating temperature, 37 °C. *Triangles* 1.16 mg/L, *squares* 0.48 mg/L, *circles* 0.24 mg/L, *inverted triangles* 0.08 mg/L. Concentration of the secondary antibody (IgG), 0.65 mg/L

being recognized by IgG and IgY. Staining the blotted sesame proteins with amido black yielded major bands at approximately 25, 29, 31, 45, and 48 kDa (Fig. 1, lane 2). The immunoblot of the sesame extract with IgY (lane 3) indicates that the antibodies produced in chicken reacted with sesame proteins of 25, 45, and 48 kDa. In addition to these bands, immunostaining with IgG (lane 4) resulted in bands at 75, 85, 95, and 121 kDa.

Development and optimization of the sandwich ELISA

In the development of the sandwich ELISA, both options of combining the two kinds of anti-sesame antibodies were applied: using IgY as coating and IgG as secondary antibody and vice versa. For each alternative, the influence of important parameters on the steepness of the sesame protein calibration curve was investigated, e.g., concentration of the coating antibody, coating temperature, type of blocking solution, blocking time, and concentration of the secondary antibody.

Coating with IgY

The coating conditions were optimized by varying the IgY concentration of the coating solution in the range from 1.16 to 0.08 mg/L, applying a coating temperature of either 4 or 37 °C. The influence of the coating parameters is shown in Fig. 2. An IgY concentration of 1.16 mg/L yielded the calibration curve with the biggest difference between the NSB value and OD_{max}. Comparison of Fig. 2a and b indicates that steeper calibration curves were obtained when the plate was coated at 4 °C. In all further experiments, coating was therefore carried out at 4 °C using an IgY solution with a concentration of 1.16 mg/L.

The concentration of the secondary antibody (IgG) was found to have a strong influence on the steepness of the calibration curve. The calibration curves obtained at IgG concentrations of 2.6, 1.3, 0.65, and 0.43 mg/L are shown in Fig. 3. Since an IgG concentration of 2.6 mg/L resulted in the steepest curve, this concentration was used in all further experiments.

In contrast to the concentration of the secondary antibody, the type of blocking solution (2% (*w*/*v*) casein in PBS or 2% (*w*/*v*) dry milk in PBS) did not have a big influence on the shape of the calibration curve. Varying the blocking time from 15 min to 1 h indicated that for both blocking solutions, an incubation time of 1 h was necessary to achieve a low extent of non-specific binding. In all further experiments, free adsorption sites of the microtiter plate were blocked with casein for 1 h.

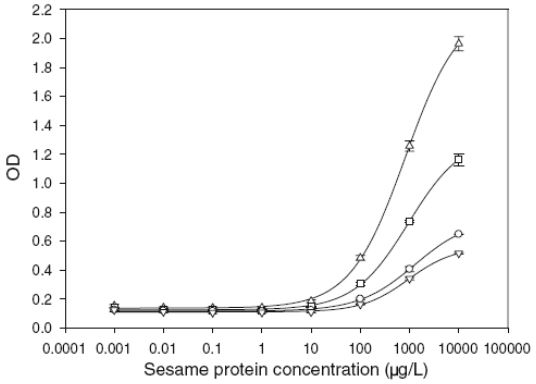

Fig. 3 Influence of the secondary antibody (IgG) concentration on the calibration curve. *Triangles* 2.6 mg/L, *squares* 1.3 mg/L, *circles* 0.65 mg/L, *inverted triangles* 0.43 mg/L. Concentration of the coating antibody (IgY), 1.16 mg/L; coating temperature, 4 °C. Blocking solution, 2% (*w*/*v*) casein in PBS

Coating with IgG

Applying different coating antibody (IgG) concentrations (0.65, 0.43, 0.33, or 0.26 mg/L) showed that steeper calibration curves were obtained at higher coating antibody concentrations (Fig. 4), which corresponds to the results described above. In contrast to coating with IgY, however, a coating temperature of 4 °C (Fig. 4a) proved to be less suitable than coating at 37 °C (Fig. 4b) since it yielded rather high NSB values. Coating was therefore carried out with an IgG concentration of 0.65 mg/L at 37 °C.

In correspondence with the results described above, the concentration of the secondary antibody (IgY) strongly influenced the steepness of the calibration curve. From the IgY concentrations applied (0.48, 0.24, 0.12, or 0.08 mg/L), a concentration of 0.48 mg/L resulted in the biggest difference between the NSB value and OD_{max}.

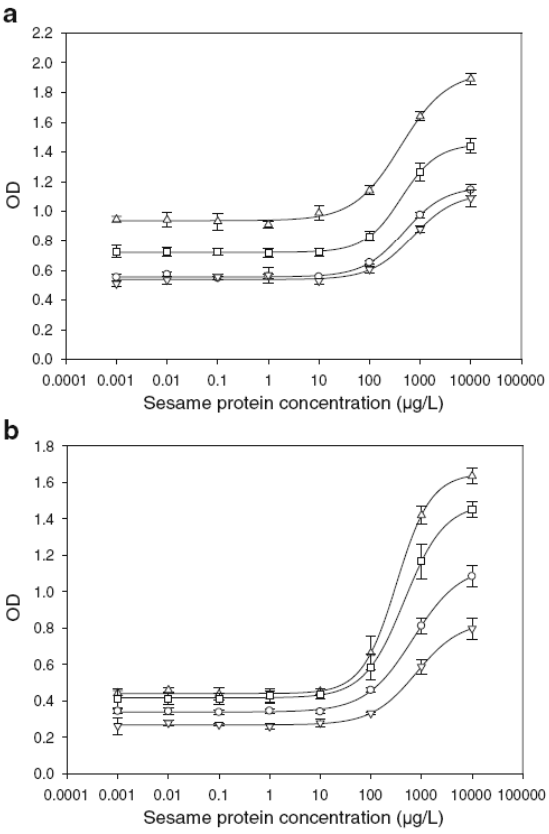

Fig. 4 Influence of the coating antibody (IgG) concentration and the coating temperature on the calibration curve. **a** Coating temperature, 4 °C; **b** coating temperature, 37 °C. *Triangles* 0.65 mg/L, *squares* 0.43 mg/L, *circles* 0.33 mg/L, *inverted triangles* 0.26 mg/L. Concentration of the secondary antibody (IgY), 0.48 mg/L

In order to optimize the blocking conditions, microtiter plates were blocked with either 2% (*w*/*v*) casein or 2% (*w*/*v*) powdered milk by varying the blocking time from 15 min to 1 h. In contrast to the type of blocking solution, the blocking time had an influence on the extent of non-specific binding, which is in agreement with the results described above. The lowest NSB values were obtained after a blocking time of 1 h.

In principle, both alternatives—using IgY as coating and IgG as secondary antibody or vice versa—yielded steep calibration curves. Since under optimized conditions coating with IgY resulted in a lower extent of non-specific binding and a steeper calibration curve (Fig. 3, data represented as triangles) than coating with IgG (Fig. 4b, data represented as triangles), further experiments were carried out with IgY as coating and IgG as secondary antibody.

Cross-reactivity of the sandwich ELISA

Cross-reactivity of the ELISA was determined by analyzing food ingredients commonly found in sesame-containing foodstuffs. Protein extracts of 19 foodstuffs, e.g., seeds, nuts, and cereals, were prepared as described above and diluted with PBS to obtain protein concentrations between 10 µg/L and 1,000 mg/L. Table 1 indicates that the ELISA

Table 1 Cross-reactivity of selected foods and food ingredients in the sandwich ELISA

Food/food ingredient	Protein concentration (g/L)	Cross-reactivity (%)
Peanut	12.3	n.d.
Hazelnut	13.4	n.d.
Walnut	2.5	n.d.
Brazil nut	48.5	n.d.
Cashew nut	34.9	n.d.
Almond	42.3	n.d.
Pistachio	22.5	n.d.
Sunflower seed	16.6	n.d.
Poppy seed	13.5	n.d.
Soybean	15.2	n.d.
Green pea	2.6	n.d.
Rice	2.0	n.d.
Wheat	13.1	n.d.
Rye	17.0	n.d.
Oat	14.4	n.d.
Barley	1.6	n.d.
Corn	0.6	n.d.
Chocolate	7.2	n.d.
Honey	0.05	n.d.

n.d. <0.1%

did not show cross-reactivity with any of the 19 food ingredients tested. In contrast, in the competitive sesame ELISA, a low cross-reactivity of 0.7% was determined for chocolate [17]. We assume, however, that the decrease in the OD value was not caused by specific interactions with the IgY but by non-specific interference of the chocolate matrix.

Matrix influence

In the development of the competitive sesame ELISA, matrix effects were observed [17]. Different food matrices were found to influence the calibration curve to a different degree. Among the matrices tested, crisp toast showed the biggest influence. In order to take matrix effects into account, sesame protein standard solutions were not prepared in PBS but by dilution with extracts from blank food matrices similar to the samples to be analyzed.

To test for any matrix effects in the sandwich ELISA, calibration curves obtained with sesame protein standards in PBS were compared with those prepared by diluting the sesame extract with extracts of blank foodstuffs. The protein extracts of a snack, crisp toast, and whole grain bread were prepared as described above, diluted either 1:5 or 1:20 with PBS and used to prepare the sesame protein standard solutions. Figure 5 shows that the calibration curves obtained by diluting the sesame extract with blank food matrix almost overlapped with those obtained by preparing the sesame protein standards in PBS. This holds for each of the food matrices, snack, crisp toast, and whole grain bread. In all further experiments, the sesame protein standard solutions were therefore prepared by diluting the sesame extract with PBS. The sandwich ELISA thus shows superior practicability to the competitive sesame ELISA recently presented [17].

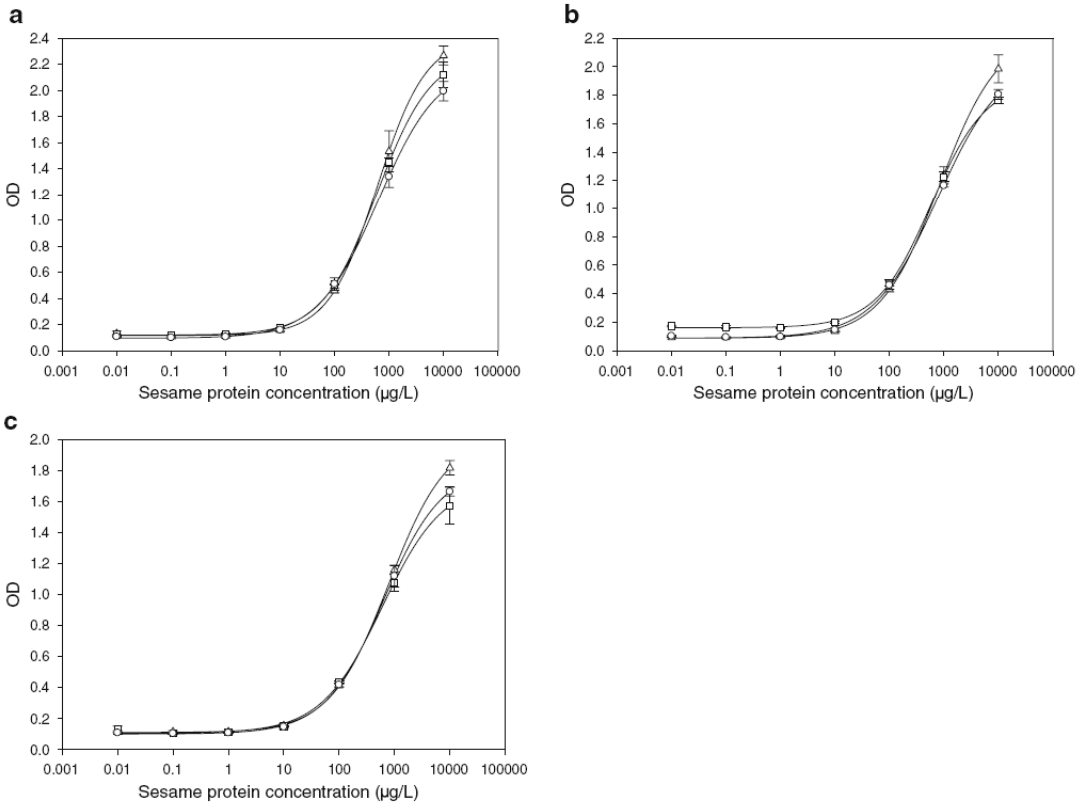

Fig. 5 Influence of various food matrices on the calibration curve. **a** Snack, **b** crisp toast, **c** whole grain bread. Sesame protein standards were prepared in PBS (*triangles*), with the extract of blank matrix, which had been diluted 1:5 with PBS (*squares*), or with the extract of blank matrix, which had been diluted 1:20 with PBS (*circles*)

Table 2 Recovery of sesame protein in commercially available blank foodstuffs

Food sample	Spiking concentration (μg/g)	Recovery (%)					Mean recovery (%)	Interassay relative standard deviation (%)
		Day 1	Day 2	Day 3	Day 4	Day 5		
Whole grain bread	25	145	72	103	86	61	93	35
	50	96	89	110	95	83	95	11
	100	64	82	102	97	79	85	18
	200	39	73	86	86	73	72	26
White bread	25	116	161	147	78	101	120	28
	50	89	145	129	77	128	114	25
	100	77	109	94	50	95	85	26
	200	79	105	104	65	71	85	22
Crisp toast 1	25	134	146	154	–	–	145	7
	50	135	111	146	–	–	130	14
	100	93	89	88	–	–	90	3
	200	100	64	102	–	–	89	24
Crisp toast 2	25	71	162	109	57	–	100	47
	50	63	137	101	115	–	104	30
	100	88	105	93	28	–	78	44
	200	58	94	90	67	–	77	23
Snack	25	109	86	130	–	–	108	20
	50	116	80	97	–	–	98	19
	100	84	62	99	–	–	81	23
	200	47	39	59	–	–	48	21

(–) not determined

Limit of detection and limit of quantification

The LOD (S/N=3) and the limit of quantification (LOQ, S/N=10) in buffer were determined from the NSB values obtained by analyzing PBS in triplicates on 16 microtiter plates. The LOD was calculated by adding three times, and the LOQ by adding ten times, the standard deviation of the obtained absorbance to the mean absorbance. The corresponding concentration was calculated by using the equation of the calibration curve established with sesame protein standard solutions. The LOD was found to be 4 μg sesame protein/L; the LOQ, 13 μg sesame protein/L.

LOD and LOQ in food were determined from the NSB values obtained by repeatedly (n=6) analyzing the extracts of blank food matrices (whole grain bread, crisp toast, and snack), diluted 1:20 with PBS. The LOD was found to be 5 μg sesame protein/L in whole grain bread and crisp toast and 3 μg sesame protein/L in snack. In the case of diluting the sample extract 1:20, the values obtained correspond to 0.5 μg sesame protein/g and 0.3 μg sesame protein/g, respectively. The LOQ in whole grain bread, crisp toast, and snack was found to be 6, 8, and 14 μg sesame protein/L, corresponding to 0.6, 0.8, and 1.4 μg sesame protein/g, respectively. According to Poms et al., the LOD of an analytical method suitable for the detection of allergenic foods should be lower than 100 ppm (μg allergenic protein/g food) [27]. The ELISA developed in the present study meets this requirement.

Table 3 Recovery of sesame in blank whole wheat cookies spiked with sesame seeds

Spiking level (%)	Sesame protein concentration (μg/g)	Sesame concentration (%)	Recovery (%)
0.001	1.6	0.0009	90
0.005	8.5	0.0047	94
0.01	18.2	0.01	100
0.05	72.7	0.04	80
0.10	363.6	0.20	200
0.50	727.2	0.40	80
1.00	–	–	–

(–) OD value >3

Table 4 Recovery of sesame in blank whole wheat bread spiked with sesame seeds

Spiking level (%)	Sesame protein concentration (μg/g)	Sesame concentration (%)	Recovery (%)
0.001	2.7	0.0015	150
0.005	20.0	0.011	220
0.01	36.4	0.02	200
0.05	163.6	0.09	180
0.50	454.5	0.25	50
1.00	3,454.5	1.90	190

In order to obtain the LOD and LOQ for sesame (instead of sesame protein), the values given above were multiplied by 5.5. The LOD of the sandwich ELISA was found to be 2.8 μg sesame/g in whole grain bread and crisp toast and 1.7 μg sesame/g in snack, the LOQ 3.3, 4.4, and 7.7 μg sesame/g, respectively, presuming that the extract is diluted 1:20.

The sandwich ELISA showed significantly lower LOD and LOQ than the competitive ELISA recently developed [17]. In crisp toast and snacks, LOD and LOQ of the competitive ELISA were found to be 5 and 30 μg sesame protein/g, corresponding to 28 and 165 μg sesame/g, respectively.

Accuracy and precision

Blank food matrices were spiked with either sesame protein or sesame seeds to determine the accuracy and precision of the ELISA. Table 2 gives the recoveries obtained by analyzing the extracts of whole grain bread, white bread, crisp toast, and snack, which had been spiked with sesame protein at four spike levels. The protein extracts were analyzed on three, four, or five subsequent days. Mean recoveries were in the range from 72% to 145%, with the exception of the snack sample spiked with 200 μg sesame protein/g (mean recovery 48%). In general, lower recoveries were found at higher spike levels. Spiking with 25 or 50 μg sesame protein/g yielded recoveries ≥93%, whereas spiking with 100 or 200 μg/g resulted in recoveries ≤90%. Relative standard deviation was in the range from 3% to 47%, indicating acceptable repeatability of the sandwich ELISA.

Tables 3 and 4 show the results obtained by analyzing the extracts of blank whole wheat cookies and whole wheat bread, which had been spiked with sesame in a concentration range from 0.001% to 1%. For whole wheat cookies, the ELISA proved to be more accurate than for whole wheat bread. In whole wheat cookies, the recovery of sesame was in the range from 80% to 100%, with the exception of the 0.1% spike level. In whole wheat bread, at almost all spike levels, the recovery was >100%.

Analysis of sesame seeds from different producers

To investigate the applicability of the ELISA to sesame seeds from different producers, two white peeled, two white unpeeled, and two black sesame samples were analyzed. Figure 6 indicates that the ELISA allows the detection of white peeled, white unpeeled, and black sesame seeds with comparable sensitivity.

Analysis of roasted sesame

Figure 7 shows the results obtained by investigating the detectability of white peeled sesame seeds roasted for 10 min at either 100, 150, 200, or 250 °C. Compared with unroasted sesame, the curves obtained for roasted sesame were generally shifted towards higher concentrations, indicating that the ELISA is less sensitive to roasted than to unroasted sesame. Sesame roasted at 250 °C for 10 min could not be detected at all. From these results, it can be concluded that calibrating the ELISA with a protein extract of unroasted sesame will result in some underestimation of the sesame concentration in food samples containing roasted sesame.

Analysis of commercial food products

The sandwich ELISA was applied to verify if food products commercially available in Austria are labeled in accordance with the regulations. Twenty-five commercial food products

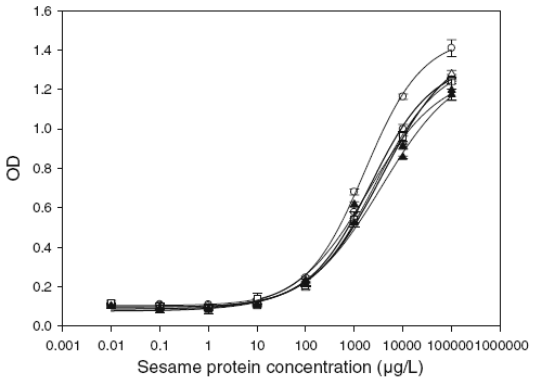

Fig. 6 Analysis of sesame seeds from different producers. *Circles* white unpeeled sesame, *squares* white peeled sesame, *triangles* black sesame

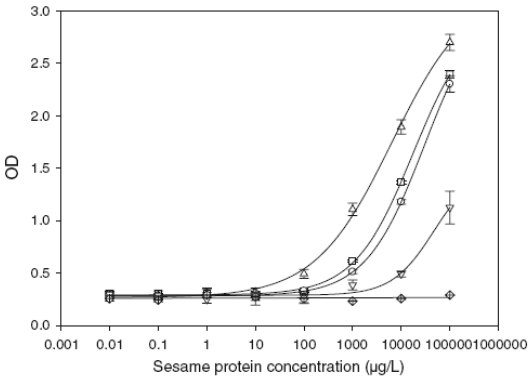

Fig. 7 Analysis of unroasted and roasted white sesame seeds. *Triangles* unroasted, *squares* 100 °C, *circles* 150 °C, *inverted triangles* 200 °C, *diamonds* 250 °C. Roasting time, 10 min

were analyzed. Seven products contained sesame, 12 were labeled with "may contain sesame" and six did not show any information on sesame. Table 5 indicates that sesame was detected in each of the sesame-containing food products. However, the concentration of sesame protein could not be determined since the 1:20 diluted extracts resulted in OD values >3. Lower OD values would have been obtained by applying higher dilution factors. Higher dilution factors were, however, not applied in the present study. Sesame was not detected in foodstuffs without any information on sesame. Sesame was detected in 75% of the samples precautionary labeled with "may contain sesame".

Conclusions

The sandwich ELISA developed in the present study allows the determination of traces of sesame in complex matrices. It detects white peeled, white unpeeled, and black sesame as well as sesame seeds roasted for 10 min up to a temperature of 200 °C. However, the ELISA was found to be less sensitive to roasted than to unroasted sesame. The sandwich ELISA has several advantages compared with the competitive ELISA published recently. First of all, the sandwich ELISA showed significantly lower LOD and LOQ than the competitive ELISA. In addition, the sandwich ELISA offers superior practicability with regard to calibration. Due to the lack of matrix effects, calibration

Table 5 Analysis of commercially available foodstuffs

Sample	Declaration	ELISA result (sesame protein concentration (µg/g))
Sesame dessert 1	(+)	+
Sesame dessert 2	(+)	+
Sesame cookies	(+)	+
Sesame flakes	(+)	+
Vegetarian schnitzel unroasted	(+)	+
Vegetarian schnitzel roasted	(+)	+
Cookies with black sesame	(+)	+
Muesli bar 1	(±)	<LOD
Muesli bar 2	(±)	<LOD
Muesli bar 3	(±)	<LOQ
Cookies with spelt	(±)	<LOD
Nut muesli	(±)	<LOQ
Crisp toast 1	(±)	1.1
Crisp toast 2	(±)	34.7
Crisp toast 3	(±)	14.4
Crisp toast with poppy and sunflower seeds	(±)	<LOQ
Bread with sunflower seeds	(±)	4.6
Cookies packed with sesame cookies	(±)	168.1
Snack 1	(±)	2.1
Snack 2	(−)	<LOD
Snack 3	(−)	<LOD
Flakes 1	(−)	<LOD
Flakes 2	(−)	<LOD
Flakes with fruits	(−)	<LOD
Cookies with quinoa	(−)	<LOD

Declaration: (+) sesame listed, (−) sesame not listed, and (±) may contain sesame. ELISA result: + sesame was detected but could not be quantified (OD>3). LOD 0.5 µg/g, LOQ 0.8 µg/g

could be carried out with sesame protein standard solutions prepared in PBS. In contrast, in the competitive ELISA, sesame protein standard solutions had to be prepared by diluting the sesame protein extract with extracts from blank food matrices similar to the samples to be analyzed. However, spiking experiments revealed that the accuracy and repeatability of both ELISA formats are quite similar.

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Antibodies and Their Replicae in Microfluidic Sensor Systems—Labelfree Quality Assessment in Food Chemistry and Medicine

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Sesame protein is one of the most potent food allergens making it an interesting topic for analytical chemistry and sensor approaches. Within this paper, we compare different strategies to obtain sensitive layers for this purpose: immobilizing natural anti-sesame IgY on the gold electrodes of a quartz crystal microbalance (QCM) leads to appreciable sensor responses with selectivity factors of about four towards brazil nut protein, which shows cross reactions in sesame allergy patients. Molecularly imprinted polymers (MIP) generated directly from sesame protein yield the same selectivity, but sensitivity is increased by a factor of three as compared to the natural antibodies. Synthesizing anti-sesame IgY MIP nanoparticles and utilizing these as templates in a surface imprinting procedure yields cavities exposing "copies" of the initial immunoglobulin molecules on their surfaces. On QCM, these materials again show the same selectivity as the natural one, but sensitivity is increased by a factor of ten. Therefore, the templating process does not only yield rugged, robust materials but also gives way to substantially increased sensor responses due to the higher surface density of selective recognition sites on the respective sensor surface.

Keywords: Bioanalyte Sensing, Immunoglobulin, Sesame, Surface Imprinting, Nanoparticles, Artificial Antibody Replica.

1. INTRODUCTION

Especially in industrialized countries food allergies become an increasing problem currently affecting up to 2% of the adult population and 8% of children.[1] Therefore, reliable analysis of potentially allergenic compounds is of substantial interest in both food industry and diagnosis, e.g., for quality control of raw materials and processed food and the respective production processes. Sesame protein is a suitable model compound for implementing novel measuring strategies, because it is one of the most important food allergens[2] and contains at least 10 different allergenic proteins. Furthermore, the symptoms of sesame allergy appear already at protein concentrations in the ppm range. Generally speaking, for analyzing/detecting bioanalytes, such as e.g., proteins or smaller biogeneous compounds,[3] natural antibodies are highly suitable recognition elements in terms of affinity and selectivity (sometimes almost reaching specificity). This fact for instance lead to immunoassays[4] for detecting sesame proteins; furthermore, ELISA kits are commercially available (e.g., from ElisaSystems) for the same purpose. Both methods reach lower detection limits of about 1 ppm. The very appreciable recognition properties of natural antibodies towards their respective antigens result from optimum chemical fit between the respective binding site and the antigen structure. This is achieved in nature by tailoring the variable regions of the respective immunoglobulin molecule and optimizing both the shape and the chemical properties via clonal selection. During this process, B-cells are created carrying randomly mutated receptors on their respective surfaces. As mutations are much more frequent in the variable region of the immunoglobulin than in the constant region, most substantial chemical variability can be expected there. In the next step, only B-cells interacting with a fitting antigen are proliferated thus leading to a selection of the suitable antibody structure.

However, in analysis, natural immunoglobulins frequently are not the optimal analytical technique, because synthesizing an exactly fitting species for a defined

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analyte compound usually requires considerable effort both in terms of synthesis time and costs making them comparably expensive. Furthermore, proteins can easily be denatured by oxidation, micro-organisms or harsh environmental conditions and hence have limited life times.

For gaining deeper insight into the underlying sensor properties of the materials and for comparing natural systems with different artificial ones, we present different analytical strategies for developing chemical sensors towards sesame protein. This includes immobilized immunoglobulins as well as different rationally templated structures based on various molecularly imprinted polymers[5] (MIP). The latter includes template-directed synthesis of a highly cross-linked polymeric matrix ideally adapted to the respective analyte of interest. Such molecularly imprinted polymers have already proven successful in various applications, such as stationary phases in chromatography,[6,7] catalysts[8] or sensor materials for a variety of analytes among others including e.g., drugs,[9] proteins[10] or cells.[11] The rather straightforward synthetic approach and the high flexibility of molecular imprinting also makes it a highly promising tool for detecting sesame allergens.

2. EXPERIMENTAL DETAILS

We purchased all monomers, reagents and solvents from Fluka, Sigma-Aldrich and Merck in analytical grade or the highest available purity for synthetic purposes.

2.1. Preparing Allergen Samples

We obtained the allergenic proteins by Soxhlet extraction of food samples. For this purpose, we ground 10 g of the respective food sample and extracted under reflux with 50 ml of n-hexane for 18–20 hours. The samples were dried over night, diluted in PBS (phosphate buffered saline) and centrifuged. The respective supernatant solutions served as analytes. In each case, we photometrically determined the overall protein concentration in the respective extract according to Bradford.

2.2. Natural Antibodies

Natural antibodies originated from eggs of immunised hens purified according to the procedure by McKinney and Parkinson.[12] For immobilizing the antibodies on the quartz crystal microbalance (QCM) surface, we first washed the device with N-methylpyrrolidone to remove organic residues from QCM productions. Then, we injected antibody solution containing 1 mg/ml of IgY. After reacting for ten to fifteen minutes to obtain S-Au binding, we washed with de-ionized water.

2.3. Sesame Protein MIP

For the direct printing process, we adsorbed 5 μl of sesame protein extract on a suitable quartz or glass carrier at 4 °C for half an hour. Then, we spun off the excess solvent to prevent formation of buffer crystals on the respective surface. This resulted in stamps for generating a specific surface by molecular imprinting.

In parallel, we prepared the pre-polymer by mixing 50 mg methacrylic acid, 20 mg vinylpyrrolidone and 30 mg dihydroxyethylenebisacrylamide (DHEBA) with 800 μl water and stirring at 70 °C until the DHEBA is dissolved. Then we neutralized the solution to pH = 7 with 1 M KOH and added 1.5 mg sodiumperoxydisulfate. Finally, we pre-polymerized under UV (254 nm) for half an hour. Then, we spin-coated QCM with 5 μl of this oligomer at 2000 rpm and pressed the sesame protein stamps into the respective thin films.

2.4. Antibody Replicas via Nanoparticle MIP

For synthesizing nanoparticles, we applied the same polymer mixture as for direct imprint, but with 60 mg DHEBA instead of thirty. After pre-polymerizing in the reaction vessel, we dispersed the solution in acetonitrile (20 ml pre-polymer per liter acetonitrile) and stirred rapidly to precipitate nanoparticles. These, we then obtained by centrifugation, washing two times by re-suspending in 3 mL water and subsequently centrifuging again. Finally, we sedimented the nanaoparticles on a stamp substrate (as above, 5x5 mm). Non-imprinted particles for reference were produced by the same procedure but without templating. We then pressed these stamps in thin films of the polymer also applied for direct imprinting of sesame protein.

2.5. Measurements

As transducers, we applied QCM based on quartz blanks with a resonance frequency of 10 MHz and a diameter of 15.5 mm with dual electrode geometries screen-printed on them with brilliant gold paste from Heraeus and burnt in for 2 hours at 400 °C. To ensure that burning entirely removed the organic parts, we soaked the final devices in N-methylpyrrolidone over night. Then, we mounted the QCM in a measuring cell with a cell volume of 150 μl and connected it to custom-made oscillators. Electrodes facing the aqueous phase (diameter 5 mm) were electrically grounded, whereas the respective counter electrodes have a diameter of only 4 mm. Agilent 53131A frequency counter read out the respective oscillator frequencies and transferred them to a computer via a GP-IB interface.[13] A LabView routine recorded all the frequency responses.

AFM measurements of nanoparticles took place on a Veeco Nanoscope IVa system in contact mode.

3. RESULTS AND DISCUSSION

3.1. Natural Antibodies as Recognition Elements

As already mentioned, immune reactions protecting organisms from pathogens are among the most selective reactions in nature.[14] Therefore, it should be possible to directly apply immunoglobulins as selective recognition elements in chemical sensing. For this purpose, we injected a solution containing 1 mg/ml antibodies obtained from eggs of immunized hens into our measuring cell for bioanalyte detection equipped with a QCM device having unmodified gold electrodes. Upon doing this, sulphur-containing functional groups within the individual protein molecules react with the gold surface of the electrode leading to covalent bonds between the two partners. Thus, the antibodies become immobilized on the respective sensor surface and can be applied for chemical sensing without the risk of washing them off the device again. Such immobilized antibodies should then undergo reactions with sesame protein they are exposed to according to the sketch depicted in Figure 1. The results of such an experiment can be seen in Figure 2. Here, the device is first exposed to water to reach its equilibrium frequency and thus a constant signal. When adding the abovementioned IgY solution, the frequency signal drops by around 500 Hz indicating binding of substantial mass to the sensor surface. This shows that the antibodies are indeed bound to the respective sensor surface. In the next step, we then exposed the device to a sesame extract, which of course contains sesame allergens, the antigen for the immobilized antibodies. After one minute following exposure of the sensor to the protein, we washed the device with water to eliminate electrostatic effects due the difference in ionic strengths between water and buffer. The sesame protein again leads to substantial mass effects in the kHz range, which is a strong indication for antibody–antigen interactions to occur. However, the effect can be fully reversed by washing with guanidinium hydrochloride solution and adding water again. The full reversibility of the second

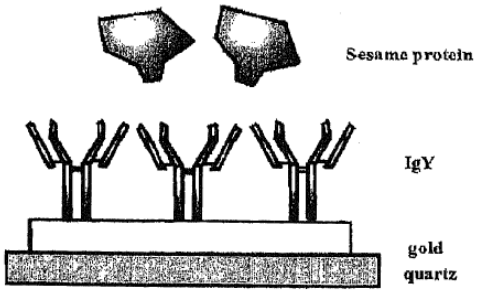

Fig. 1. Measuring principle with natural antibodies immobilised on a gold surface.

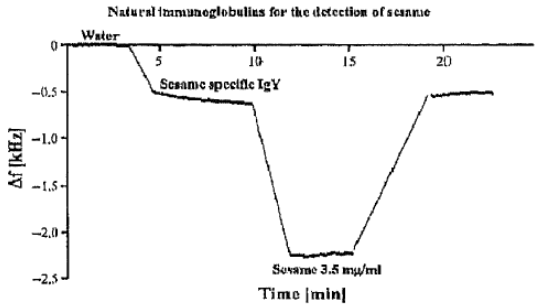

Fig. 2. Measurement of sesame based on immobilised immunoglobulins (1 mg/ml) on the surface of a 10 MHz QCM. 3.5 mg/ml sesame were added and washed with guanidinium hydrochloride. Finally, water was added to show the reversibility of the effect. Straight lines indicate that the measurement was stopped during washing steps to prevent spikes in the curve.

sensing step indicates both selective sesame protein detection and covalent binding of the antibody on the surface. It has to be noted that the straight lines in Figure 2 show the washing steps undertaken during measurements. At those intervals, we stopped recording the respective sensor responses. The results show that natural antibodies immobilised via thiol groups bound to the gold electrode are indeed suitable tools for chemical sensing and allow implementing a label-free detection strategy with appreciably sensitivity and selectivity. However, generating selective immunoglobulins is a rather time-consuming task and comes at substantial costs. For this reason, our next step was to replace the immunoglobulins with synthetic materials made from commercially available monomers leading to cost-effective, robust materials.

3.2. Sesame Protein MIP

Combining molecularly imprinted polymers (MIP) with QCM devices has already proven to yield appreciable results.[15, 16] This fact together with the results obtained with natural antibodies strongly suggest that this is also true for the detection of allergenic protein. Thus, we prepared surface MIP with sesame protein and exposed them to both sesame and brazil nut extracts that show immunological cross-reactivity. These measurements indeed reveal mass-effects on the respective devices for both analytes (data summarized in Fig. 7), however, the sesame extracts lead to ten times higher sensor signals than the ones from brazil nut. This does not only underpin the quality of MIP in terms of selectivity, but also indicates that MIP interact with the entire polymer surface rather than the specific antigenic site and therefore take into account more structural information of the respective analyte than an Ig molecule during recognition. However, in order to exclude high non-specific effects between sesame protein and the respective polymers, we also prepared a MIP with the

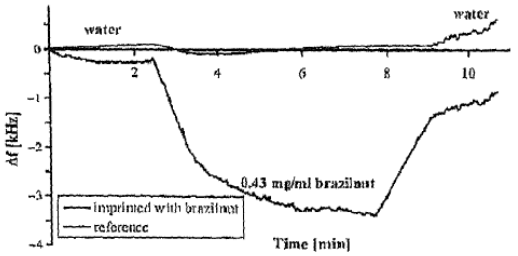

Fig. 3. Measurement of brazilnut with a directly imprinted layer placed on the surface of a 10 MHz QCM. The dark curve represents the printed electrode and the light one the reference.

brazil nut protein extract as the template. Figure 3 shows the results of this approach: in the beginning of the measurement, the sensor is exposed to water. After reaching equilibrium, the layer is then exposed to the brazil-nut extract. Obviously, the MIP channel takes up substantial amounts of this analyte, as can be seen from the frequency effect in the range of almost 3 kHz. In contrast to this, the non-specific effect on the non-imprinted electrode is substantially smaller, namely only some 250 Hz. Sesame extract showed similar sensor responses with both the reference and the brazil nut MIP thus further underpinning the outstandingly appreciable selectivity that can be obtained by molecular imprinting. Selectivity of all sensor layers presented will be discussed in Figure 7. In contrast to the immobilized IgY, the MIP layer shows almost fully reversible behaviour of the sensor signal even without washing with guanidinium hydrochloride. This also strongly supports MIP for sensing: whereas nature optimizes antibodies to reach more or less irreversible binding between antibody and substrate, the MIP materials retain reversibility thus underpinning their high potential in functioning as "artificial antibodies."

3.3. Plastic Antibody Replicas for Sensing

As both natural antibodies and MIP lead to very appreciable sensor results, it would be of interest to develop fully artificial antibodies to investigate whether polymeric "copies" of IgY are accessible and, if yes, whether they also lead to appreciable sensor responses. The best way for achieving this is first generating an antibody MIP and using this as a template for another surface imprinting or a casting process finally leading to a plastic copy of the initial antibody on the QCM surface. The basic strategy underlying this approach is given in Figure 4: first, we synthesized Ig-MIP in the form of imprinted nanoparticles by precipitation polymerization from acetonitrile. We then assembled the particles on a stamp surface and pressed them into an oligomeric film on the QCM surface and hardening the latter. This approach in principle is the stamping technique we developed for detecting other

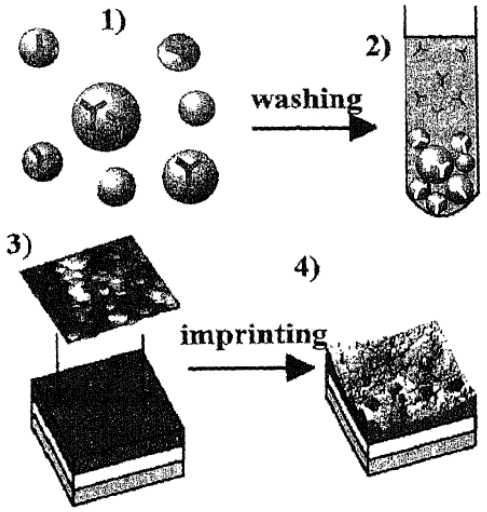

Fig. 4. Preparation of antibody replica: (1) Particles are precipitated in acetonitrile while stirring in the presence of natural antibodies. (2) Beads are washed with water to remove the template. (3) Particles adhered on a glass plate are used for the printing process of the polymer placed on one electrode of a QCM. (the AFM images are 3×3 μm in size) (4) After removing the stamp, holes including structures of antibodies, the replicas, are left behind.

biospecies in the size region of 10–1000 nm. In principle, the cavities generated in the polymer this way should not only reproduce the geometrical features of the particles, but also details in their surface structures including the surface chemistry. Thus, the imprints left behind by the particles should also contain "plastic copies" of the initial IgY molecules having been used as templates during particle synthesis.

Particle synthesis can be strongly influenced by formulation: their dimensions can be tuned by the amount of cross linker used during synthesis, as can be seen in Figure 5(a) showing the average particle size for different formulations of the respective polymer. Interestingly, particle size increases with the amount of cross-linker. Furthermore, it is also positively correlated with pre-polymerization time and the amount of pre-polymer injected into acetonitrile. For imprinting, we chose particle diameters of a few hundred nanometres, as depicted in the AFM image in Figure 5(b). Evidently, the size of the individual particles is in the range of 200–500 nm. The reason for choosing comparably small particles is the high imprinting density that can be achieved this way. In addition to that, their small dimensions make them accessible for a solvent when removing the templating stamp which favours separating the two polymers (i.e., the particles in the stamp and the imprint formed) from each other.

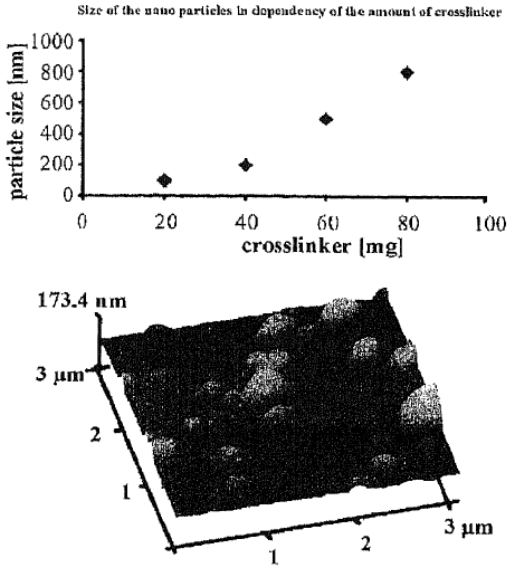

Fig. 5. (a) particle size as a function of cross-linker concentration; (b)AFM images of the particles selected for sensitivity/selectivity experiments.

Figure 6 shows the outcome of this approach: it gives the frequency responses of a dual channel QCM, one channel coated with a layer containing the "plastic antibodies" and the other one as a reference. In a first step, we exposed the sensor to rye extract that should not show allergenic cross-activity with sesame. Indeed, the frequency shift obtained at both channels is the same thus proving the absence of a cross reaction. The overall sensor responses in this case are due to the fact that switching from a buffer to a protein extract substantially changes the environment of the respective sensor. After the rye extract, we exposed the device to a mixture doped with sesame. In this case, the imprinted channel yields substantial effects: the frequency decreases by about 600 Hz! Hence, substantial recognition

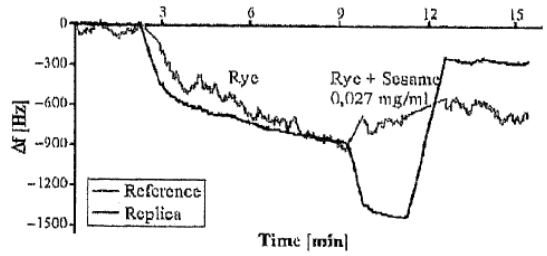

Fig. 6. QCM-Measurement of sesame with antibody replica in rye extract. The light curve shows the sensor response of the replica (printed with imprinted particles) whereas the dark curve shows the reference (printed with reference particles).

of sesame protein takes place on the MIP. However, it has to be emphasized that this MIP does not contain cavities optimized to interact with the sesame protein, but "plastic copies" of the antibody on its surface interacting with the target analyte in the same way as a natural antibody. Thus, the imprinting process makes it possible to generate such replicas of IgY and also transferring the biological activity into an artificial system.

Figure 7 summarizes the experiments on the different recognition approaches: evidently, the selectivity between sesame and brazil nut extract of the natural antibody, the direct imprint with sesame protein and the antibody replica cast from nanoparticles, respectively, is the same. The brazil nut imprint also shows similar recognition ability by preferably incorporating its own template. However, the main differences between the different sensors can be found in sensitivity: here, the natural antibodies immobilized on the device surface show the lowest effects, followed by the sesame protein MIP and the artificial IgY replica. This can be explained by the different surface properties of the respective materials: in the case of the immobilized immunoglobulins, the individual molecules are attached to the gold electrode of the QCM by covalent gold-sulphur bonds. This binding process is statistically governed, which means that only a limited amount of Ig molecules are immobilized in a way that their recognition sites are sterically accessible. In the case of the sesame protein MIP, one can in the ideal case assume most densely packed recognition sites on the MIP surface. This increases the mass effects by a factor of three as compared to the natural system. Compared to this, the layer containing replicated IgY on its surface leads to more than threefold increase in sensitivity yielding responses being a factor of ten higher than the ones obtained with the natural antibodies. The reason for this of course is that imprinting with nanoparticles substantially increases the active

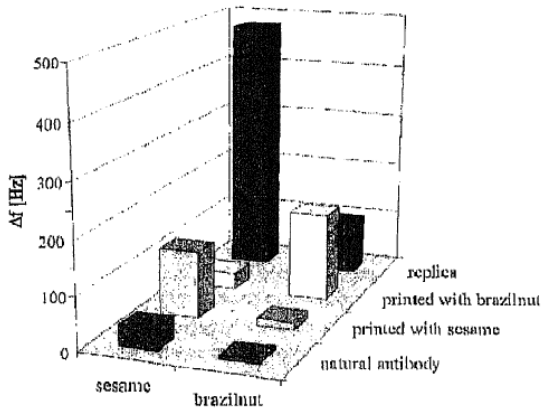

Fig. 7. Comparison of sensor responses with 10 MHz quartz for different measuring strategie.

surface of the respective sensor layer thus offering substantially larger numbers of interaction sites on the surface.

4. CONCLUSION

Molecular imprinting can not only be applied for generating selective recognition sites for a defined species (e.g., sesame protein), but it also gives way to generating polymer-based replicas of natural antibodies. When coated onto the electrodes of a QCM, anti-sesame IgY, sesame protein imprints and IgY replicas lead to the same selectivity, respectively. Therefore, the templating procedures very appreciably retain the functional properties of their respective template without substantially losing quality even if two casting steps take place, as it is the case with the IgY replicas. Directly comparing the three sensor layers underpins this claim by the similar selectivity obtained. Sensitivity of the artificial systems, however, is substantially higher than in the case of immobilized natural antibodies due to higher amount of binding sites on the surface. Consequently, it is possible to synthesize plastic copies of IgY retaining the initial selectivity despite the fact that the chemical composition of the artificial system is of course substantially different from the original protein.

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