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Comparison of two ELISAs and one real-time PCR method for the detection of potentially allergenic sesame in food

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Contents

1 Introduction **1**

1.1 Food allergy . . . 1

1.1.1 Disease definition . . . 1

1.1.2 Symptoms of food allergy . . . 2

1.1.3 What establishes a protein as an allergen? . . . 3

1.1.4 Influence of food processing on the allergenicity . . . 4

1.1.5 Epidemiology of food allergies . . . 5

1.1.6 Diagnosis of food allergy . . . 6

1.1.7 Treatment . . . 6

1.1.8 Food labeling . . . 7

1.2 Sesame (*Sesamum indicum*) . . . 8

1.2.1 Background . . . 8

1.2.2 Sesame allergy . . . 9

1.2.3 Sesame allergens . . . 10

1.3 Methods for the detection of food allergens . . . 11

1.3.1 Requirements for analytical methods . . . 11

1.3.2 Enzyme linked immunosorbent assays (ELISAs) . . . 12

1.3.3 Polymerase chain reaction (PCR) . . . 12

1.3.4 Biosensors . . . 13

1.3.5 Immuno blotting . . . 14

1.4 Overview of published methods for the detection of allergens in food . . . 14

2 Aim **19**

3 Theoretical background **21**

3.1 Antibodies . . . 21

3.1.1 Classification and structure . . . 21

3.1.2 Production of polyclonal antibodies . . . 21

3.1.3 Production of monoclonal antibodies . . . 22
3.1.4 Polyclonal versus monoclonal antibodies . . . 23
3.2 Enzyme linked immunosorbent assay (ELISA) . . . 23
3.2.1 Competitive ELISA . . . 24
3.2.2 Sandwich ELISA . . . 24
3.3 Enzymes . . . 25
3.4 Photometry . . . 27
3.5 DNA extraction . . . 28
3.5.1 Steps of DNA extraction . . . 28
3.6 Polymerase chain reaction (PCR) . . . 29
3.6.1 Principle . . . 29
3.6.2 Real-time PCR . . . 31
3.6.3 Data analysis . . . 33

4 Results and discussion **35**

4.1 ELISAs . . . 35
4.1.1 Protein extraction from sesame . . . 36
4.1.2 Determination of the cross reactivity of the competitive ELISA . . . 37
4.1.3 Optimization of the sandwich ELISA . . . 41
4.2 Spiking of food samples . . . 42
4.2.1 Determination of the recovery of sesame . . . 42
4.3 Limit of detection (LOD) . . . 58
4.3.1 Sandwich ELISA . . . 58
4.3.2 Competitive ELISA . . . 58
4.4 Polymerase chain reaction . . . 61
4.4.1 DNA extraction . . . 62
4.5 PCR analysis . . . 66
4.5.1 PCR optimization . . . 66
4.5.2 PCR analysis of sesame spiked samples . . . 66

4.6 Comparison of the sandwich ELISA, the competitive ELISA and the PCR method . . . 70

5 Experimental part **73**

5.1 Protein extraction from sesame seeds . . . 73
5.2 Protein extraction from food samples . . . 73
5.3 Spiking of food matrices . . . 74
5.4 Determination of the protein concentration . . . 74
5.4.1 Bradford assay . . . 74
5.4.2 Method by Warburg and Christian . . . 75
5.5 Production and isolation of rabbit anti-sesame antibodies (IgG) . . . 76
5.5.1 Isolation of the IgG fraction . . . 77
5.5.2 Determination of the concentration of the isolated antibodies . . . 77
5.6 Production and isolation of chicken anti-sesame antibodies (IgY) . . . 77
5.6.1 Isolation of the IgY fraction from the egg yolk . . . 78
5.7 Enzyme linked immunosorbent assay (ELISA) . . . 78
5.7.1 Reagents . . . 78
5.7.2 Instruments . . . 79
5.7.3 Sandwich ELISA . . . 79
5.7.4 Competitive ELISA . . . 80
5.7.5 Determination of the recovery of sesame . . . 82
5.8 DNA extraction . . . 82
5.8.1 DNA extraction with the CTAB method . . . 82
5.8.2 DNA extraction with the QIAmp Stool Kit . . . 84
5.8.3 Determination of the yield and quality of DNA extracts . . . 86
5.9 Real-time PCR method . . . 86
5.9.1 Reagents: . . . 87
5.9.2 Protocol . . . 88
5.9.3 Analysis of spiked samples . . . 88

5.9.4 Inhibition control 89
5.10 Chemicals 89
5.11 Equipment 91

6 Conclusion **93**

Appendix **95**

List of abbreviations 95
List of Tables 97
List of Figures 98
References 99
German abstract 107
Curriculum Vitae 109

1 Introduction

1.1 Food allergy

1.1.1 Disease definition

Food allergies pose a major problem in our society. About 6 to 8% of young children and 3% of adults suffer from allergic reactions to food [1]. However, not all adverse reactions to food are food allergies. Adverse reactions to food can be divided into toxic and non-toxic reactions [2]. The non-toxic reactions can be sub-divided into immune mediated reactions (food allergies) and non-immune mediated reactions (food intolerances) [1] [2].

- Toxic adverse reactions result from toxins present in food. These reactions affect all individuals that consume the toxic substances in sufficient amounts [2].
- Non-toxic adverse reactions:
  - Food intolerance: This type of adverse reaction is an abnormal physiologic response caused by
    1. an enzymatic defect
    2. pharmacological intolerance or
    3. non-classifiable reactions.
  - Food allergy: is an immunological reaction elicited by the ingestion of food constituents. A difference exists between:
    1. IgE-mediated and
    2. non-IgE-mediated reactions [1] [2] [3]

IgE-mediated food allergy

This hypersensitive reaction to food occurs after ingestion of a constituent to which oral tolerance has not been developed. The immunological reaction leads to the excessive production of immunoglobulin E (IgE). IgE is normally produced from the immune system

as a reaction to parasitic infections. However, in some individuals it is synthesized when they are exposed to agents such as pollen, dust and/or food components [2] [4].

The mechanism of the development of food allergy is a complex process which has not yet been completely understood [5]. IgE mediated food allergy can be divided into two phases. In the first phase, the sensitization, IgE are produced against a certain food constituent. The first contact with the allergen does not provoke any symptoms. IgE bind to specific Fcϵl receptors on mast cells and basophils [1] [2].

The second phase occurs when the allergen is ingested again. IgE linked to mast cells bind the absorbed target molecule. Conjugation of the IgE with the allergen triggers degranulation and the release of histamine and other inflammatory mediators like prostaglandins, leukotriens and cytokines. These mediators cause vasodilatation, smooth muscle contraction and mucus secretion. The physiological changes are expressed as allergic reactions [1] [2] [4].

1.1.2 Symptoms of food allergy

The clinical manifestations of food allergies can range from local itching to anaphylaxis. Different organ systems can also be involved.

Anaphylaxis is the most dramatic reaction, it is defined as "severe, life-threatening, generalized or systemic hypersensitivity reaction". It is caused by an abrupt overproduction of mediators released from the mast cells. Anaphylaxis can affect the gastrointestinal system, the skin, the respiratory system and in some cases the cardiovascular system leading to hypotension, collapse and dysrhythmia [6].

Table 1: Symptoms of food allergy [2] [6] [7]

Organic system	Clinical aspect
Skin	Urticaria
	Atopic dermatitis
	Angiodema
Gastrointestinal tract	Nausea
	Vomiting
	Gastric retention
	Abdominal pain
	Cramps
	Eosinophilic oesophagytis
	Gastroenteritis
Respiratory system	Rhinoconjunctivitis
	Bronchospasm
	Asthma
Generalized symptoms	Anaphylaxis

1.1.3 What establishes a protein as an allergen?

Only a few reasons are known why proteins react as food allergens. In general, food allergens are foreign proteins with a molecular weight of 5000-70000 Da. The individual has to be exposed to the allergen in substantial amounts over a long period. The protein has to be stable enough to pass the gastric transit without degradation. Proteins with intact epitope structures can trigger allergic reactions as represented in Figure 1.
IgE binding epitopes can either be linear (continuous) or conformational (discontinuous). Linear IgE binding sites could be identified for allergenic proteins from cow's milk, soybean, peanuts and other sources. It is also possible that identified linear epitopes are part of large conformational epitopes. Their three-dimensional structural integrity is considered as a major characteristic of food allergens. On the other hand no conformational

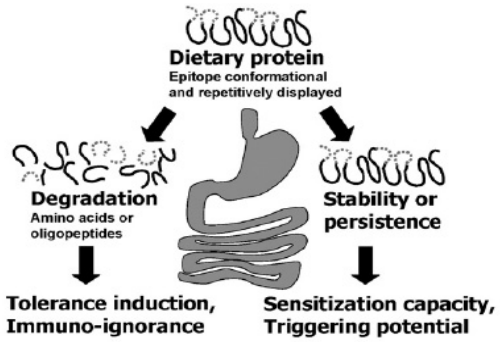

Figure 1: Stability and allergenicity of proteins [8]

sequence could be identified which is responsible for allergic reactions.

Many food allergens are glycoproteins consisting of carbohydrate structures which represent important IgE binding sites. However, glycosylation is not a prerequisite for the allergenicity of a protein [5] [8].

1.1.4 Influence of food processing on the allergenicity

In general, food undergoes a number of processing steps, including thermal and non-thermal treatments. However, the influence of food processing on the allergenicity of proteins is poorly understood.

It is known that food processing can inactivate conformational epitops, but can also increase allergenicity by generating new ones [9].

It has been shown that the Maillard reaction increases the allergenicity of the two major peanut allergens, Ara h 1 and Ara h 2. After roasting, both allergens showed a 90-fold higher affinity to IgE and were better soluble and less digestible than the allergens from unroasted peanuts [10].

In contrast, the major allergen from hazelnut, Cor a 1, is highly sensitive to thermal treatment such as roasting. Roasting has been shown to decrease the allergenicity of hazelnuts [10].

Investigations on the major sesame allergen Ses i 1 indicated that the allergen is not affected by temperatures up to 100°C at neutral or acid pH. Conformation changes were found to be reversible [11].

Moreno et al. (2006) [12] simulated the transport of Ses i 1 through human intestinal epithelial cell monolayers and demonstrated that the allergen could pass the monolayer without any loss of activity. Even after 2 hours of gastric digestion the allergen was largely intact. Small subunits broke down after 15 min of duodenal digestion [12].

1.1.5 Epidemiology of food allergies

Food allergy is a matter of geography, prevalence and age. Some food allergies, such as egg and cow milk allergy, occur particularly in young children. Since in many cases egg and cow milk allergies are outgrown rapidly their prevalence in adults is much lower. In contrast, some food allergies such as shellfish allergy are only common in adults. On the other hand, the prevalence of peanut allergy is high in adults and children [7] [13]. In addition, food allergies show geographical variations.

These differences in the prevalence of allergies can be explained by exposure factors. Milk consumption is much higher in children's age while adults consume more often shellfish than infants [7]. Mustard allergy is very seldom in countries where the consumption of mustard is low. However, due the high consumption of mustard in France many French people suffer from mustard allergy. Due to the high consumption of celery, this type of allergy is very common in Germany, Switzerland and France [7].

Dalal et al. (2002) reported that sesame allergy is the third frequent IgE mediated food allergy in Israel, whereas peanut allergy is particularly common in Europe and North America. Another example which demonstrates the influence of geographical factors on the prevalence of food allergies is fish allergy which appears mostly in countries with high fish consumption, such as Norway, Japan and Portugal. [13]

The number of diagnosed food allergies also depend on the panel of foods recommended for allergy screening tests. The children which were screened for food allergy live mostly in western countries and have similar nutrition [13].

Many patients with hazelnut allergy are first sensitized to birch pollen. IgE raised against birch pollen allergens can cross-react with the homologous allergens in hazelnut [14].

1.1.6 Diagnosis of food allergy

The diagnosis of food allergy is based on medical history, skin prick tests (SPT), IgE determination and provocation tests. The diagnosis begins with the medical history of the patient; it involves documentation of the symptoms and the consumed food that may have caused the allergic reaction. The recollection is followed by a clinical examination to exclude food intolerances such as lactase deficiency. A diet diary can be helpful to detect hidden allergens. In many cases an elimination diet is recommended.

Patients with suspected IgE-mediated food allergy are often screened by SPT with commercial allergen extracts. Glycerinated food extracts are applied simultaneously with histamine and saline as positive and negative controls, respectively, by the prick or puncture technique. As an alternative, fresh food can also be used, resulting in an increased sensitivity of the SPT. The skin prick test is considered as an excellent test to exclude IgE-mediated food allergies but it can also lead to false positive results due to cross reactivities.

The double blind placebo controlled food challenge (DBPCFC) is the only validated test for diagnosis of adverse reactions. However, it is time consuming and complex. Psychogenic factors and systematic errors due to subjective observations can be eliminated. False negative results can be obtained when the food antigen has altered the allergenic epitop due to lyophilization of the food [1] [2] [3].

1.1.7 Treatment

By now, the only way to manage food allergies is the strict avoidance of the allergenic food. However, elimination diets can cause malnutrition or eating disorder. Nutritional deficiency can be eliminated by the use of vitamin and mineral supplements. Medications have been used to treat allergic symptoms but they suffered from minimal efficiency and/or unacceptable side effects. In acute anaphylaxis the use of epinephrine is vitally important.

Immunotherapy

A promising approach is immunotherapy which includes the development of humanized anti-IgE monoclonal antibodies, plasmid-DNA-therapy and "engineered" recombinant protein immunotherapy [1] [2] [3].

1.1.8 Food labeling

For an allergic individual, complete elimination of the certain allergenic food is the only way to avoid allergic reactions. Correct food labeling is therefore inevitable to protect these persons. In November 2007 the European Commission published a list with 14 different food constituents which have to be declared on the food package. The Directive 2007/68/EG [15] is based on the previous Directives (2000/13/EG), (2003/89/EG) and (2006/142/EG). The 14 ingredients and their products which are listed in the Council Annex IIIa are:

- Cereals containing gluten (i.e. wheat, rye, barley, oat, spelt, kamut or their hybridized strains)
- Crustaceans
- Eggs
- Fish
- Peanuts
- Soybeans
- Milk (including lactose)
- Nuts, i.e. almonds (*Amygdalus communis L.*), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anarcadium occidentale*), pecan nuts (*Carya ilinoiesis* (Wagenh.) K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia verde*), macadamia nuts and Queensland nuts (*Macadamia temifolia*)

- Celery
- Mustard
- Sesame
- Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/L expressed as SO_2

1.2 Sesame (*Sesamum indicum*)

1.2.1 Background

Sesamum indicum is the most important species of the *Sesamum* genus which belongs to the familiy of *Pedialaceae*. Its origins are in Africa, now it is cultivated in big parts of Asia (China, India and Burma), Africa (Sudan) and Mexico which are the major producers of sesame seeds. 70% of the sesame is used for the production of oil and flour.

Sesame is, especially in Israel, a very popular ingredient of a variety of products. It is used for tehina, a sesame paste, and halva which contains ground sesame seeds. In addition, bakery products, fast-food, "health foods", ethnic dishes and vegetarian food are common products which contain sesame, mainly because of the nutty taste of roasted sesame. Sesame oil is often used as a substitute for olive oil in European foods and routinely used for cooking in the Middle East, Asia and South America [7].

Nowadays, sesame is known as a functional food ingredient because of its high content of phytochemicals. Cardiovascular diseases, osteoporosis and oxidative stress can be prevented, controlled and managed by the use of sesame seeds [16]. It is often recommended for infant nutrition, especially in eastern countries, because of its high calcium, iron and protein content [17].

However, sesame seeds are known to contain several allergenic proteins. In sensitized persons the consumption of small amounts of sesame can lead to life threatening anaphylaxis [7] [13] [16] [17] [18].

1.2.2 Sesame allergy

In the last years, the prevalence of sesame allergy increased in many countries. In the past, it only appeared in eastern countries such as Israel but with the higher popularity of sesame in western foods the number of people which are allergic to sesame has increased in Europe, Australia and the USA [13] [19] [20] [21].

Only 30 mg to 10 g sesame seed and 1 to 5 ml of sesame oil can provoke an allergic reaction [7]. The clinical symptoms involve skin reactions (angiodema of lips, tongue or uvula) or respiratory symptoms such as asthma or nausea. Allergic reactions to sesame can also negatively affect the gastrointestinal system [7] [20] [21].

Sesame allergy is often accompanied with atopic dermatitis as it was shown by Levy et al. [17]. The diagnosis of sesame allergy can cause false-negative results if only skin prick tests or IgE measurements are carried out. Pajno et al. (1999) reported anaphylaxis to sesame in an 18-year-old woman, suffering from celiac disease, who was negatively tested with SPT and had no specific IgE antibodies against sesame [19]. The woman had to be treated medically after the food challenge which was carried out with 5 g sesame masked in bread.

Dalal et al. carried out investigations of young children suffering from sesame allergy. They found out that all their patients had IgE-mediated sesame allergy while 3 of them were tested negatively with SPT. During their investigations 6 sesame induced anaphylactic shocks occurred [13].

Cohen et al. (2007) [21] and Zavalkoff et al. (2008) [22] recommended a combination of at least SPT and patient history for sesame allergy confirmation. Zavalkoff et al. showed that IgE levels of sesame tolerant children (73 probands) were much lower than IgE levels of allergic children (28 probands). Children with IgE levels < 0.35 kU_A/L (kilounits of antibody per liter) did not suffer from sesame allergy.

Cohen et al. (2007) further reported that most of the sesame allergic patients were younger than 2 years old. 80% of the children continue suffering from sesame allergy while children allergic to egg and cow's milk can develop tolerance [21].

In agreement to Cohen et al. and Levy and Danon, Dalal et al. defined sesame allergy as a major problem for infants, especially in Israel [13] because of the early exposure of infants to sesame containing food. Consumption of sesame is high because healthcare professionals recommend parents to add tehina and halva to their infants' diet since it is a good source of protein and iron [13] [17] [21].

Due to the frequent occurrence of sesame allergy among children Levy and Danon [17] and Perkins [23] propose sesame free products for children and infants. Perkins also recommends declaration of sesame for medicinal and pharmaceutical products. Unintended ingestion of sesame poses a high risk for allergic persons. Perkins therefore suggested that food labelling should be clear and differ between "may contain sesame allergens" and "free from sesame allergens" [23].

1.2.3 Sesame allergens

Sesame contains about 20% proteins. 25% of the proteins are soluble, these are mainly 2S albumins. Until now, several sesame allergens have been identified.

Ses i 1 was identified by Pastorello et al. (2001) [24] and belongs to the group of the 2S albumins. The protein has a molecular weight of 9 kDa and is not glycosylated. It shows 47% homology to allergens of Brazil nut, 41% to allergens of castor beans and 43% to allergens of sunflower. Ses i 1 was the first identified and sequenced sesame allergen.

Ses i 2 is the smallest of the allergenic proteins of sesame with a molecular weight of 7 kDa and belongs to the group of the 2S albumins. The allergen shows 38% homology to the walnut allergen Jug r 1, 40% to the Brazil nut allergen Ber e 1 and 34% to the peanut allergen Ara h 2 [25].

Ses i 3 is a 7-vicilin-type globulin which belongs to the 2S albumins and has a molecular weight of 45 kDa. Homologies are shown to the peanut allergen Ara h 1 (36%) and to the walnut allergen Jug r 2 (41%). The IgE binding epitop of Ara h 1 shows 80% homology

to the comparable region of Ses i 3 [25].

Ses i 4 and **Ses i 5** are oleosins occurring in sesame oil and pose a risk for sesame allergic persons. Leduc et al. (2006) [26] identified the two allergens with a molecular weight of 17 kDa (Ses i 4) and 15 kDa (Ses i 5). The allergens show homology to oleosins of Chinese spice shiso (Perilla frutescens) with 75% and a 56% homology to carrot oleosin.

In 2007 Beyer et al. [27] identified two further allergens of sesame, Ses i 6 and Ses i 7. Wolff et al. (2003) [28] reported that most of their tested sera reacted to a 14 kDa protein and suggested that this protein is a major allergen of sesame. The identified protein could be associated with the 7 kDa 2S albumin which was identified by Beyer et al. (2002) [25] because of the identical amino acid sequence of the 7 kDa protein to large subunits of the 14 kDa protein.

Further investigations on the 14 kDa β-globulin were carried out by Wolff et al. (2004) [29] to identify the linear B-cell epitops. Nine different epitops could be detected by using the sera of 20 subjects. Three of the 9 epitops appear to be immunodominant.

1.3 Methods for the detection of food allergens

1.3.1 Requirements for analytical methods

Analytical methods should fulfill the following criteria to be applicable to the detection of allergens in foods. The method has to be specific for a particular food and should not show cross reactivity to other food or other food ingredients. The method should allow the detection of traces of the allergen not only in raw but also in highly processed foods. There is a general agreement that the detection limit of the method should not be higher than 1 to 100 mg/kg food (1 to 100 ppm) [30]. In addition, the method should allow the analysis of a high number of samples within a short period of time. It is advantageous when the method can be automated.

1.3.2 Enzyme linked immunosorbent assays (ELISAs)

Immunoanalytical methods are preferred for the detection of allergenic proteins in food. ELISAs are part of these methods and are often the method of choice of the food industry or official food agencies because of their high sensitivity and selectivity. Moreover ELISAs can be carried out rapidly and can be automated to reduce human errors. Furthermore ELISAs are cost-effective and can be used as (semi-)quantitative methods [31] [32] [33].

Many ELISAs have been published for the detection of allergens in food. An overview is given in section 1.4. ELISAs are based on the binding of an antigen to an antibody. The detection of the antibody-antigen complex is carried out by an enzyme labeled antibody. The enzyme changes a colorless substrate in a colored product which can be measured by a photometer [31]. ELISAs often use polyclonal antibodies raised in animals such as chicken or rabbit. The antibodies can be raised against the allergen itself which requires highly purified protein extracts. This assay is called "allergen assay". Due to the fact that allergic patients often react to more than one allergenic protein (e.g. in milk all proteins can cause allergic reactions), antibodies are often raised against an extract of proteins, called "protein assay" [33]. In the present work the antibodies were raised against a sesame protein extract.

1.3.3 Polymerase chain reaction (PCR)

PCR techniques are already routinely used for the identification of pathogens, genetically modified organisms and food related plant species. Due to the specificity of the PCR method it has attracted attention to be used for the detection of allergenic foods. In contrast to immunoanalytical techniques, the PCR does not detect the allergen itself but the presence of DNA coding the protein. This poses an advantage for allergenic ingredients with low protein content, e.g. celery. But it is not well suited for food with high protein and low DNA content such as eggs. Therefore ELISA methods are more suitable.

Advantages of the PCR method are the reproducibility of the synthesis of primers,

probes and polymerase in unlimited amounts and constant quality [32] [34].

In the last few years many PCR methods for the detection of allergenic food components have been developed. Schöringhumer and Cichna-Markl (2007) [35] published a real-time PCR method for the detection of Ses i 1, a major allergen of sesame. Piknová et al. (2008) [36] presented a real-time PCR method for the detection of the hsp 1 gene from hazelnut. Other real-time PCR methods were published by Hird et al (2008) [37] for Ara h 2, a major allergen of peanut and for lupin flour by Scarafoni et al. (2009) [38]. Mustorp et al. (2008) [39] developed a real-time PCR method for the detection of celery and mustard. A real-time PCR method for the quantitative detection of Secale cereale was published by Terzi et al. (2004) [40].

PCR methods offer the possibility of multi-analyte detection which means that several food components can be detected simultaneously. A duplex PCR method for the simultaneous detection of hazelnut and sesame was published by Schöringhumer et al. in 2009 [41].

1.3.4 Biosensors

Only a few biosensors have been developed for the detection of allergens; although they offer high specificity and sensitivity for the detection of analytes in complex food matrices. In biosensors the target molecule, an antibody, a single-stranded DNA sequence or an enzyme is immobilized on a sensor chip surface and used for biological recognition. If antibodies are immobilized the sensor is called "immmunosensor". The immobilized molecule is in intimate contact with a transducer. Quartz crystal mircrobalance (QCM) and surface plasmon resonance (SPR) sensors allow the label-free detection of the target analyte. For QCM thin layers are deposited on the crystal surface and their mass changes and physical properties can be measured. SPR biosensors belong to optical-based sensors. A change in the refractive index is induced by either the adsorption of a biomolecule on the metallic film (often a gold film) or by molecular interactions. The change in the refractive index leads to a shift in the refractive angle which is directly proportional to the

concentration of the analyte [30] [42].

1.3.5 Immuno blotting

In protein blotting or Western blotting, proteins are separated according to their molecular mass in a sodium dodecyl sulphate (SDS) polyacryl amide gel by gel electrophoresis. The separated proteins are transferred and immobilized onto a membrane by capillary transfer or electro transfer. Immune detection can be carried out with radio- or enzyme labeled antibodies.

The method allows the identification of new allergens by the use of human IgE antibodies. However, immuno blotting is a very time-consuming method. In addition, the limit of detection (5 ppm) is usually higher than that of ELISAs [30] [43].

1.4 Overview of published methods for the detection of allergens in food

Numerous methods, mainly PCR methods and ELISAs, have already been developed for the detection of allergenic foods such as hazelnut, peanut, lupin or celery. Until now, no ELISA has been published allowing the detection of sesame. However, three PCR methods for the detection of sesame have been published. In addition, 2 sesame test kits are commercially available - one real-time PCR method and a protein test kit based on a sandwich ELISA. Next, some of the papers are presented.

Holzhauser and Vieths (1999) [44] published a sandwich ELISA for the detection of traces of hazelnut protein. Polyclonal anti-hazelnut antiserum from rabbit was used for the detection of native and toasted hazelnuts. 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 recoveries were in the range from 67 to 132%. The limit of detection was found to be 60 $\pm$ 50 ppb hazelnut protein in the sample.

Akkerdaas et al. (2004) [14] developed a sandwich enzyme-linked immunosorbent assay to detect pepsin resistant hazelnut proteins in food products. Rabbits were immunized with either pepsin-digested hazelnut or untreated hazelnut. Less than 1 μg hazelnut in 1 g food matrix could be detected. The recovery of hazelnut in spiked chocolate (0.5-100 μg hazelnut/g chocolate) was 97.3%. Ten plant foods were tested for cross reativity, the highest cross reactivity was found for peanut (0.034%).

Drs et al. (2004) [45] developed an indirect competitive enzyme-immunoassay for the detection of hazelnut in food. Hens were immunized with roasted hazelnut and polyclonal antibodies were isolated from the egg yolk and purified. The immunoaffinity purified antibodies showed cross reactivity with beans (8.09%), sunflower seeds (6.26%), poppy seeds (5.36%) and chickpea (3.42%). Chestnut, brazil nut, green pea, sesame, barley, wheat, yeast, rolled oats and corn showed cross reactivity below 3%. 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 hazelnut protein/L with an average recovery of 128%.

Piknová et al. (2008) [36] developed a real-time PCR method for the detection of the hsp 1 gene from hazelnuts in food. Detection was carried out with a TaqMan probe. No cross reactivity was found with plants such as peanuts, walnuts, almonds, pistachio nuts and cashews. The limit of detection was 13 pg hazelnut DNA. In spiked pastry samples hazelnut could be detected at concentrations $\geq 0.01\%$.

Holzhauser and Vieths (1999) [46] published an indirect competitive ELISA for the detection of hidden peanut proteins. 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. The assay tolerates 20% of walnut

or pinto in the food matrix without resulting in a false positive signal. The LOD was 2 ppm peanut protein (μg/g food), at this concentration the revovery of peanut protein was 143%. At peanut protein concentrations > 13 ppm the recovery was in the range from 84 to 126%.

Hird et al. (2003) [37] presented a real-time PCR method for the detection of peanut. The primers and the probe were designed for the Ara h 2 gene. Amplification of the peanut specific DNA sequence was not observed in 33 tested samples tested for cross reactivity such as pistachio nut, walnut and almonds. 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.

Scarafoni et al. (2009) [38] developed a real-time PCR method based on SYBRgreen for the detection of lupin flour in food products. The primers did not show any cross reactivity to other foods. The amplification efficiency, determined by serial dilution of purified genomic leaf, was between 96% and 100%. Genomic lupin flour showed an amplification efficiency between 95% and 101%. The limit of detection was found to be 7 pg of lupin DNA which corresponds to 0.1% lupin flour in food.

Schöringhumer and Cichna-Markl (2007) [41] published a real-time PCR method for the detection of the gene coding for the Ses i 1 protein which presents a major allergen of sesame. A molecular beacon probe labeled with the reporter dye FAM and the quencher DABCYL was used. 17 samples, such as Brazil nut, sunflower seed, peanuts or black sesame, did not show any cross reactivity. Amplification efficiency of 100.3% was shown up to a serial dilution of 1:10000 of sesame DNA extracts. For the determination of the limit of detection in food matrix 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%. Ct values over 35 were obtained for sesame concentrations below 0.05%.

Brzezinski (2006) [47] presented a real-time PCR method for the detection of sesame 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. No cross reactivity was found for seeds which are commonly used in bakery products, such as pumpkin, poppy and sunflower seeds. The limit of detection was determined to be 5 pg DNA which corresponds to 50 mg/kg sesame DNA in food products.

Mustorp et al. (2008) [39] developed a sesame specific PCR method for the gene encoding 2S albumin - a major allergen of sesame. No cross reactivity was shown with a variety of plant materials. The limit of detection was 0.5 pg sesame DNA, the amplification efficiency 96%. The method allowed the detection of 0.005% (w/w) sesame in barbecue spice and wheat flour.

Schöringhumer et al. (2009) [41] developed a duplex real-time PCR method for the simultaneous detection of sesame and hazelnut in food. Primers and probes were chosen 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.

A **"sesame protein test kit"** is commercially available from BioKits, Tepnel Biosystems (Flintshire, UK). The test kit is based on a sandwich ELISA and can be used for the qualitative and quantitative determination of sesame in food products. However, no information is given on the applicability in different food matrices. The test is specific for sesame and does not show cross reactivity with nuts, seeds, pulses, grains, fruits and other foods.

The **"SureFood® allergy sesame real-time PCR kit"** is available from r-biopharm AG and Congen Biotechnology GmbH (Germany). It allows the qualitative detection of sesame DNA in food matrices such as instant soup, Hamburger bread, sesame sticks and bread. The test does not show any cross reactivity with 12 common food ingredients. The limit of detection is 10 mg sesame in 1 kg food (10 ppm).

2 Aim

Food allergens pose an important health problem to our society; 6-8% of young children and about 3% of adults suffer from allergic reactions to food. Small amounts of allergens are known to induce an allergic response which can be as severe as life threatening anaphylaxis. Correct food labelling is the only way to help sensitized persons to avoid consumption of the allergenic food. Sesame is one of 14 potentially allergenic foods which have to be declared according to the EU directive 2006/142/EC.

Numerous analytical methods have already been developed allowing the detection of allergens in food. So far, immunoassays and PCR methods play the most important role. It is generally known that both methods have advantages and disadvantages. However, by now only few studies directly compare the applicability of ELISAs and PCR methods in the analysis of food allergens.

Recently, in our group three analytical methods have been developed to detect traces of sesame in a variety of food matrices: a competitive ELISA (using anti-sesame antibodies from a chicken), a sandwich ELISA (using anti-sesame antibodies from a chicken and a rabbit) and a real-time PCR method. In the real-time PCR method, the target DNA sequence was a fragment of the gene coding for Ses i 1, a major allergen of sesame.

The aim of the present work was to spike several complex food matrices with known amounts of peeled, non-roasted sesame seeds (1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.001% and 0.005%), to analyse the spiked samples with each of the three methods and to compare the results, in particular the limit of detection (LOD) and the recoveries for sesame.

3 Theoretical background

3.1 Antibodies

Many methods make use of the specific interactions between antibodies and antigens.

3.1.1 Classification and structure

Immunoglobulins are glycoproteins which are synthesized and secreted from plasma cells [48] as response to the exposure to an antigen [31].

Five different classes of immunoglobulins exist: IgM, IgG, IgA, IgD and IgE. The classification is based on their immune function and their structure [31] [48]. IgG are the most common antibodies in human serum [48]. They can be described as Y-shaped units of 4 polypeptide chains, two identical copies of a heavy chain (H) and two identical copies of a light chain (L). The chains are linked together noncovalently and by inter-chain disulfide bonds. Both the heavy and the light chains can be further divided into constant and variable regions. There are three constant regions in the heavy chains (C_H) and one constant region in the light chains (C_L). In addition all chains contain one variable region (V_H and V_L). The variable regions contain three hypervariable regions which are characterized by a high variability of the amino-acid sequence. The three hypervariable regions of the light chains and the three hypervariable regions of the heavy chains form two identical antigen binding sites, so-called paratops [49]. The epitop is the part of the antigen which binds to the paratop. The complex between antigen and antibody is formed by hydrogen bonds, electrostatic, van der Waals and hydrophobic interactions. Antibodies are highly selective but often show cross reactivity to similar epitops [49].

3.1.2 Production of polyclonal antibodies

Polyclonal antibodies are produced by immunizing a host animal with the antigen. Frequently used animals are rabbits, goats, mice and sheep. Immunization is usually carried out according to a certain immunization schedule. The first injection of the antigen leads to the production of IgM. The following injections, called booster immunizations, result

in the production of IgG. In many cases, adjuvants are used to enhance the immune response to the antigen. The molecular weight of the antigen can dramatically affect the quality and quantity of the antibodies produced. Small substances (< 3000 Da), called haptens, have to be conjugated to carrier proteins to become immunogenic. The antigen used for immunization should be pure. However, sometimes time-consuming purification steps have to be applied to remove impurities. In general, μg to mg quantities of the immunogen are necessary to elicit a high immune response.

The serum which is collected after immunization is called antiserum. The antiserum contains a variety of different antibodies, only a small fraction of the antibodies is directed against the antigen. In ELISAs the antiserum can often be used in unpurified form. However, some applications make it necessary to isolate the antibodies. The following methods can be used for isolating the IgG fraction from an antiserum [49]:

- Salting out
- Fractionate precipitation with ammonium sulphate
- Ultrafiltration
- Dialysis
- Ion exchange chromatography
- Size exclusion chromatography
- Hydrophobic chromatography
- Affinity chromatography

3.1.3 Production of monoclonal antibodies

Monoclonal antibodies are produced by the hybridoma technology. The aim of this method is to produce a pure antibody in large amounts. In the first step, a mouse is immunized with the antigen. After the first booster the B-cells are removed from the spleen of the mouse. B-cells are fused with myelome tumor cells, which can grow like

cancer cells and produce antibodies. The fused hybrid cells are called hybridomas. The desired antibody has to be selected by a screening test which involves an antigen-antibody interaction. This cell will be cloned on macrophage layers in microtitre plates. To obtain a large scale of monoclonal antibodies they can be further cloned in vivo or in vitro. In vivo antibody production is called "ascetic method". The hybridoma cells are injected into the peritoneal cavity of the mouse where a tumor is developed. The tumor produces ascetic fluid which contains monoclonal antibodies. The solution has to be purified from a wide range of mouse proteins. The method is not allowed in Austria and Germany. In vitro production of monoclonal antibodies can be carried out in two different ways. One possibility is the immobilization of hybrioma cells onto a solid matrix, the other one is to suspend the cells homogenously. The two processes are highly reproducible and the amount of contaminants is much lower compared to the in vivo method. The produced monoclonal antibodies have to be purified like polyclonal antibodies (purification methods are mentioned in 3.1.2) [49] [50].

3.1.4 Polyclonal versus monoclonal antibodies

The production of polyclonal antibodies is less time intensive and less expensive than the production of monoclonal antibodies. However, one does not obtain a pure antibody but a mixture of different antibodies. In addition, the antibody amount which can be obtained from one animal is limited. Moreover, the production of polyclonal antibodies is not reproducible since different animals react differently. Monoclonal antibodies are pure reagents which can be obtained in unlimited amounts. However, the production of monoclonal antibodies takes much time and is therefore rather expensive.

3.2 Enzyme linked immunosorbent assay (ELISA)

There are two commonly used ELISA formats, the sandwich ELISA and the competitive ELISA.

3.2.1 Competitive ELISA

The competitive ELISA is mainly used for the detection of small substances, called haptens. In the first step, the antigen is immobilized onto the well of a microtiter plate (see Figure 2). Coating is usually carried out at temperatures from 4 to 37°C. Between all steps unbound components have to be removed by washing the plate with a buffer solution containing a detergent. After a blocking step the sample and the antibody raised against the analyte are added. Since the primary antibody is added in limited amount, the antigen in the sample and the antigen immobilized on the well of the microtiter plate compete for the binding of the antibody. A secondary antibody labeled with an enzyme is used to detect the antigen-antibody complex bound to the wells. In the next step the substrate is added. The enzyme catalyzes the conversion of the substrate into a colored product. The reaction can be stopped by denaturing the enzyme. The optical density which can be measured by a photometer is indirectly proportional to the analyte concentration in the sample, i.e. 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 [30] [31] [33] [49] [51] [52].

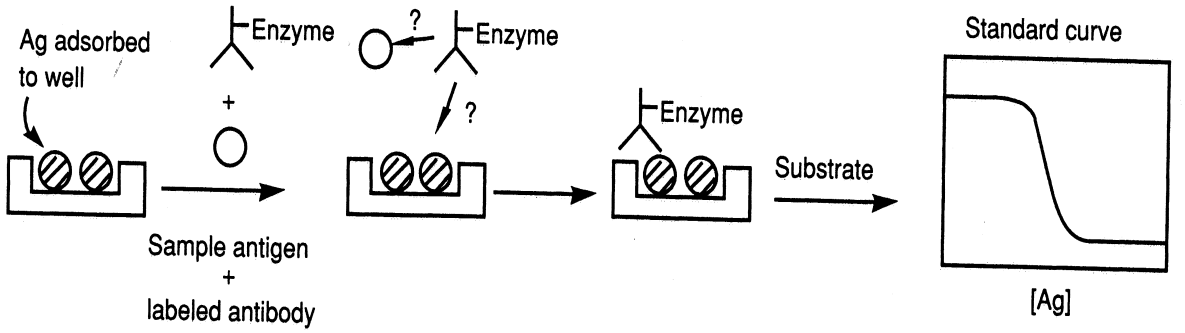

Figure 2: Principle of the competitive ELISA [51]

3.2.2 Sandwich ELISA

The sandwich ELISA is the most commonly used ELISA format in the analysis of proteins [30]. In this assay type, antibodies raised against the analyte are immobilized on the well of

the microtiter plate (see Figure 3). In the next step, the analyte is added and an antigen-antibody complex is formed. Then a secondary antibody is added which recognizes a different epitope on the antigen. Since the antigen is in between two antibodies this ELISA type is called sandwich ELISA. A tertiary antibody which is enzyme labeled is added for detection. In the next step a chromogenic substrate is added which is transformed to a colored product by the enzyme. The OD value is directly proportional to the concentration of the analyte in the sample [30] [31] [33] [49] [51] [52].

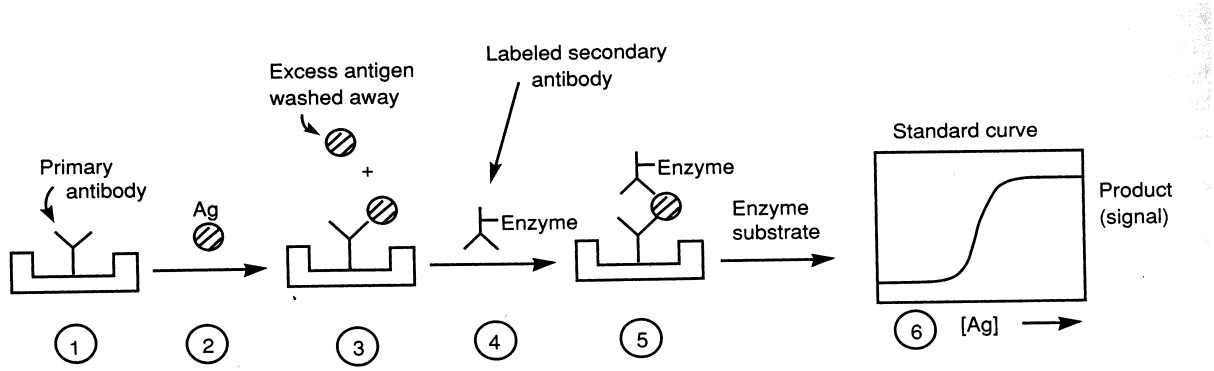

Figure 3: Principle of the sandwich ELISA [51]

3.3 Enzymes

In the ELISA, enzyme labeled antibodies are used to detect the antigen-antibody complex. The most common enzymes are horseradish peroxidase, alkaline phosphatase and β-galactosidase since they fullfill the following criteria:

- Availability in high amounts
- Low cost
- High specificity and big turnover rates
- Precise detection of the product
- Easy conjugation to the antibody without loss of activity
- Low non-specific binding

- Minimal influence of interferences from the sample media or assay conditions (pH, ionic strength, detergents, buffers, enzyme cofactors)

Horseradish peroxidase is a glycoprotein with a low molecular weight of 44 kDa. The enzyme can be conjugated to the antibody without loss of activity via four lysin residues. Also the carbohydrate residues can be used for conjugation which is carried out by the "periodat-method" [53] [54]. H_2O_2 is used as substrate to form an oxidized "compound I", that is further reduced by "compound II" via a hydrogen donor (also called substrate) in two single electron steps to the initial state. The final product is the oxidized hydrogen donor, the OD value of which can be measured by a photometer.

Common substrates are:

- ABTS, (2,2'-azino-di-(3-ethylbenzothiazoline-6-sulphonate), absorbance measured at 415 nm
- OPD, (*O*-phenylenediamine), absorbance measured at 492 nm
- TMB, (3,3',5,5'-tetramethylbenzidine), absorbance measured at 450 nm

Among these substances TMB is preferred because it gives the highest absorbance values. Moreover, it is not mutagenic [53] [54].

Alkaline phosphatase is a homodimer isolated from calf intestine. The molecular weight of 140 kDa is much higher than that of the horseradish peroxidase. The enzyme can be coupled covalently to antibodies via the free amino groups by the reaction with glutardialdehyde. Zinc and magnesium are important cofactors. EDTA inhibits the enzyme because of the formation of chelates. Alkaline phosphatase catalyzes the hydrolysis of phosphate esters of primary alcohols. A commonly used substrate solution is: 5-15 mM para-nitrophenyl phosphate in 1 M diethanolamine, 0,5 mM $MgCl_2$, pH 9.8

Compared to horseradish peroxidase the incubation time of alkaline phosphatase is longer, it is less detectable and more expensive. On the other hand it is more stable, less

sensitive to inhibition by bacteria, matrix influences and laboratory conditions [53] [54].

β-Galactosidase belongs to the group of holoenzymes. It is a tetramer with a molecular weight of 465 kDa. The enzyme has several amino and sulphydrol groups to be conjugated to the antibody. Conjugation is carried out by the introduction of a maleinimid group via a spacer. β-Galactosidase catalyzes the hydrolysis of *o*-nitrophenylgalactoside. The catalysis is activated by mercaptoethanol, which acts as an acceptor alcohol. Chelating agents, heavy metals and organo mercuric-compounds can inhibit the reaction. The enzyme offers no further advantages compared to horseradish peroxidase and alkaline phosphatase [53] [54].

3.4 Photometry

Photometric measurements are based on the Lambert-Beer law, which forms a relationship between the attenuation of a beam of radiation and the analyte concentration in a sample. The attenuation of the beam of radiation depends on the path length of the cuvette (d), the concentration of the analyte (c in mol/L) and the molar absorptivity ϵ (λ) (in L $\mathrm{mol^{-1}cm^{-1}}$). The molar absorptivity is characteristic for a certain substance and is influenced by the wave length of the beam of radiation. A monochromatic beam has therefore to be used for photometric measurement.

Equation of Lambert-Beer law:

$$I = I_0 \cdot e^{-\epsilon c d} \tag{1}$$

Absorbance:

$$A = \epsilon(\lambda) \cdot c \cdot d \tag{2}$$

The absorbance is directly proportional to the concentration of the analyte in the sample. A standard curve can be obtained by plotting the absorbance of standard solutions against their concentrations. Due to molecular interactions at high concentrations, deviations

from the Lambert-Beer law usually arise at concentrations > 0.01 M. [55]

3.5 DNA extraction

The successful isolation of DNA is a fundamental requirement for carrying out further experiments such as the amplification by polymerase chain reaction.

Three important factors determine the suitability of isolated DNA for being amplified:

1. **Amount or concentration:** The amount and concentration of the isolated DNA must be sufficient for carrying out the experiments.
2. **Purity:** The purity of the isolated DNA is a critical point, since matrix components may inhibit the DNA polymerase, leading to false negative results.
3. **Integrity:** The DNA extraction method should not result in degradation of the DNA in order to allow its amplification.

3.5.1 Steps of DNA extraction

Sample preparation: Heterogenous samples have to be homogenized either in a blender or with mortar and pestle.

Cell or membrane lysis: The cell wall/membrane is disrupted and the DNA is released from cellular and organelle membranes. In the same step a detergent or denaturant is used to form a complex with proteins and soluble membranes. A commonly used enzyme for cell and membrane lysis is proteinase K, that releases nucleic acids from adhering proteins. In order to increase the stability of the DNA, substances (for example EDTA) forming a chelate complex with magnesium ions, which act as co-factor for DNase, are added to the extraction buffer.

Separation of nucleic acids from cell debris or sample matrix: The separation of nucleic acids is usually performed by phenol-chloroform and chloroform extraction. The

remaining proteins are soluble in chloroform while the DNA is soluble in the aqueous phase. An alternative to the phenol-chloroform method is the application of commercial DNA affinity columns (based on silica particles) or anion exchange resins (based on cellulose or dextran). In the presence of high concentrations of chaotropic agents silica efficiently binds DNA. Anionic exchange resins bind the strongly anionic DNA whereas proteins and RNA are removed. RNA can also be removed by adding RNase.

Precipitation of DNA: The most common way is the precipitation by adding alcohol and a monovalent salt. After centrifugation the pellet has to be washed with 70% (v/v) ethanol to purify the extracted DNA from salt and alcohol.

Concentration of DNA: The concentration of the DNA extract is determined by a photometric measurement. The absorbance is usually measured at wavelengths of 260 and 280 nm. The purity should be in the range from 1.8 to 2.0 to guarantee efficient amplification of the DNA [56] [57]. The equations for calculating the results are shown in section 5.8.3.

3.6 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is based on the detection of a specific DNA-sequence by its amplification. It is frequently applied in different fields of research such as molecular biology, cell biology, medicine, immunology, forensic sciences and paternity testing.

3.6.1 Principle

PCR analysis consists of a number of cycles. Each PCR cycle is divided into three steps:

1. **Denaturation:** The double-stranded template DNA is melted (denatured) at temperatures of 92-95°C. The hydrogen bonds are broken and two single strands are obtained (see Figure 4)

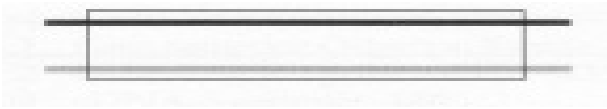

Figure 4: Denaturation of double-stranded DNA in two single strands [58]

2. **Annealing:** Two specific oligonucleotides called primers which are complementary to the target DNA hybridize to their complementary sequence of the 3' and 5' end of the single stranded DNA. The annealing temperature is between 45 and 65°C (see Figure 5).

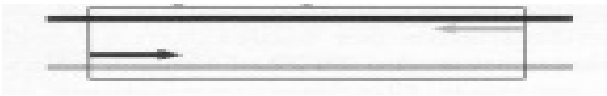

Figure 5: Annealing of primers on the single strands [58]

3. **Elongation:** The Taq DNA polymerase isolated from the bacterium *Thermus aquaticus* is thermostable. It binds on the 3' end of the primers and synthesizes a new DNA strand complementary to the template. The dNTP's (desoxynucleotide-triphosphates) required for the synthesis are contained in the PCR reaction mix. Elongation (Figure 6) is commonly carried out at 72°C, the temperature optimum of the Taq DNA polymerase.

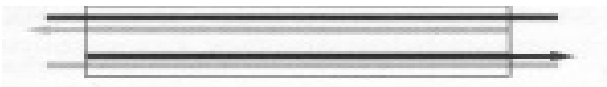

Figure 6: Elongation of the DNA templates by Taq DNA polymerase [58]

One PCR cycle consists of the three steps explained above and is usually repeated 25 to 45 times. In theory, the amount of the amplified PCR product is doubled in each cycle resulting in an exponential amplification [34] [58] [59].

The amplified PCR-product can be detected either after the PCR run by agarose-gel-electrophoresis or in "real-time" with the help of a fluorescently labeled probe.

Visualization by agarose-gel-electrophoresis

Agarose-gel-electrophoresis is the classical method for qualitative DNA detection. Due to its negative charge DNA migrates to the positive pole in an electric field. In agarose-gel-electrophoresis DNA molecules can be separated according to their size: small DNA fragments migrate faster than larger ones. The size of the amplicon can be determined with the help of a marker DNA containing DNA fragments of known sizes. DNA can be visualized with ethidium bromide which intercalates between double stranded DNA and shows fluorescence when the gel is put on a UV transilluminator [34] [59].

3.6.2 Real-time PCR

Real-time PCR allows the detection of the amplified PCR product in "real-time" by measuring the increase of a fluorescence signal. The fluorescence signal can be obtained by the use of SYBRGreen^{TM} or a target specific oligonucleotide probe which is attached with a fluorescence reporter and a quencher.

SYBRGreen^{TM} detection is based on the intercalation of SYBRGreen between double stranded DNA. The advantages of using SYBRGreen are: no gene specific probe is needed and a melt-curve analysis can be carried out after the PCR run to check the specificity of the DNA amplification reaction [58] [60].

The target specific probes are based on a process called fluorescence resonance energy transfer (FRET). The probe is labeled with a reporter and a quencher. In the annealing

phase, the probe binds to the single stranded segment between the two hybridized primers. Until the 5'exonuclease activity of the polymerase cleaves the probe and separates the reporter from the quencher no fluorescence signal can be measured. The main advantage of real-time PCR is the possibility to obtain a signal without loading the amplicons onto a gel. The elimination of postamplification manipulations drastically minimizes the risk of contamination. A disadvantage of real-time PCR is the necessity to apply a thermal cycler equipped with a fluorescence detection module.

Probes

For real-time detection several probes are used. The probes differ in the mechanism by which the reporter and the quencher are separated after the probe has bound to the sequence of the target DNA template [60]. The most commonly used probes are: TaqMan™ probes, Molecular-Beacons, Scorpions™. FAM and Cy5 are often used as fluorophors, DABCYL and methylred as quenchers [34] [58] [60].

TaqMan™-Probe: A TaqMan probe is labeled with a fluorophor at the 5'end and a quencher on the 3'end. During the elongation phase the Taq DNA polymerase cleaves off the reporter from the quencher by its 5' 3' exonuclease activity. The fluorophor is now free and emits fluorescence [58].

Molecular-Beacons are labeled with a fluorophor on the 5'end and a quencher on the 3'end. Additional complementary nucleotides are on the 5' and on the 3' end causing a hairpin-structure with a stream and a loop. The fluorophor and the quencher are in direct contact which means that no signal can be detected when the probe is not bound to the DNA template. After binding to the target sequence the fluorophor and the quencher are separated rsulting in fluorescence. During the elongation phase the Taq DNA polymerase displaces the probe of the DNA template and the hairpin-structure can be built up again.

Scorpions™ primer assays consist of 2 primers. One of the primers serves as a probe,

which contains of a loop-structure with a 5'fluorophor and the 3'quencher. The fluorophor and the quencher are in direct contact. The loop sequence contains a gene specific sequence and a blocker molecule, that prevents read-through during extension of the opposite strand. The end of the 3'end of the scorpion probe represents the 5'end of the gene specific sequence. After the elongation and binding of the probe to the template, the scorpion probe gets bound covalently to the new synthesized fragment. In the next annealing phase the sequence of the loop-structure binds to the complementary nucleotides and the quencher is separated from the fluorophor. After the scorpion probe is cleaved off, the loop-structure is built up again [58] [60].

3.6.3 Data analysis

The real-time PCR run can be divided into two phases: the exponential phase (each cycle results in duplication of the template amount) and the non exponential plateau phase (several factors, for example the limited availability of reaction components, prevent further amplification). A PCR curve is obtained by plotting the fluorescence signal against the number of cycles (see Figure 7). The Ct (threshold cycle)-value is defined as the cycle number at which the fluorescence signal exceeds the background noise. The Ct-value can be used for quantification of the amplified DNA segment [30] [34]. The amplification

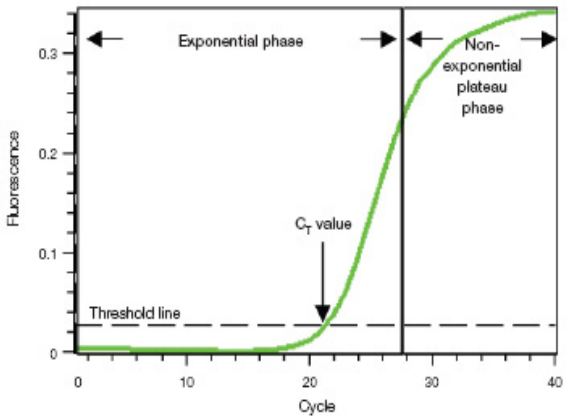

Figure 7: Two phases of real-time PCR [60]

efficiency of a real-time PCR method can be assessed by serially diluting the DNA sample,

running the PCR and making a standard curve by plotting the Ct-values against the log of the starting DNA concentration for each dilution. The efficiency can be calculated from the slope of the standard curve using the following equation:

$$E = 10^{-1/slope} \tag{3}$$

$$E[\%] = (E - 1) \cdot 100 \tag{4}$$

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% [60].

4 Results and discussion

The aim of the present diploma thesis was to spike food samples with known amounts of sesame and to analyse the spiked food samples with three analytical methods - a competitive ELISA, a sandwich ELISA and a real-time PCR method. This was done in order to compare the applicability and performance of the three methods.

4.1 ELISAs

The **sandwich ELISA** was developed by Gerda Redl during her diploma thesis [61]. Polyclonal chicken and polyclonal rabbit antibodies, both raised against sesame proteins, were used as coating and secondary antibodies, respectively. The procedure of the sandwich ELISA is described in 5.7.3. The sandwich ELISA was found to be specific for sesame and did not show cross reactivity with any of the following 14 food ingredients: peanut, oat, hazelnut, honey, almonds, poppy seed, rice, rye, chocolate, sunflower seed, soybean, Brazil nut, walnut and wheat. The recovery of the sandwich ELISA was determined by spiking food samples with known amounts of sesame proteins. At spike levels of 25 and 50 μg sesame proteins/g sample the recoveries were 113 $\pm$ 18% and 108 $\pm$ 13%, respectively. The limit of detection (LOD, S/N=3) was found to be 0.4 μg/g and the limit of quantification (LOQ, S/N=10) was 1.3 μg sesame protein/g sample.

The **competitive ELISA** was developed by Fatima Tazeen Husain during her doctoral thesis. The antibodies raised against sesame proteins were produced in a chicken. The details of the competitive ELISA are described in 5.7.4. The competitive ELISA was found to be specific for sesame. Cross reactivity tests were negative for peanut, hazelnut, walnut, Brazil nut, poppy seed, wheat, rye, oat, chocolate, honey and other several food ingredients. A matrix effect was found for matrices such as crackers, crisp toast, and cereals (diluted 1:20 with PBS). The LOD (S/N=3) was found to be 5 μg protein/g and the LOQ (S/N=10) was 50 μg/g for sesame protein in crackers, cereals and snacks. For sesame in fresh bread and buns the LOD was 11 μg/g and the LOQ was 49 μg/g. Samples

such as crisp toast, salty snack, fresh bread and fresh whole grain bread were spiked with sesame proteins between 25 and 200 μg/g. The recovery was between 78 and 150%.

4.1.1 Protein extraction from sesame

At the beginning of the research, the protein fraction of sesame had to be extracted in order to be able to establish calibration curves for both the sandwich and the competitive ELISA. Proteins were extracted from unpeeled, non-roasted white sesame seeds. The sesame seeds were grinded, defatted by Soxhlet extraction, dried and extracted with PBS for 2 h. The details of the sesame protein extraction are described in 5.1. The concentration of the protein extract was determined by the Bradford assay using BSA as standard protein (see 5.4.1). During the lab work sesame was extracted twice. The protein concentrations were found to be 7.7 and 12.0 mg/mL.

Determination of the protein concentration in sesame

Previous validation experiments by Gerda Redl and Fatima Tazeen Husain were carried out by analyzing food samples which had been spiked with known amounts of sesame proteins. In contrast to the previous studies, in the present diploma thesis sesame seeds were used for spiking. It was therefore necessary to determine a factor enabling the conversion from the sesame protein concentration per g food (as calculated from the standard curves) to the sesame concentration per g food. For this reason, sesame was extracted with the extraction procedure which was applied to all other food samples (for details see section 5.2). The protein concentration of the extract was determined with both the Bradford assay (5.4.1) and the UV-extinction method by Warburg and Christian (see 5.4.2). With the Bradford assay the protein concentration in sesame was found to be 10.7% (mean value from two determinations). The method by Warburg and Christian resulted in a sesame protein concentration of 18% (mean value from three determinations). An advantage of the latter method is that the protein concentration can be determined without calibration. The sesame protein concentration of 18% is close to the protein concentration of 20% which can be found in the literature [7]. For calculating the sesame

concentration in a food sample, the sesame protein concentration determined with the ELISA had therefore to be multiplied by 5.5.

4.1.2 Determination of the cross reactivity of the competitive ELISA

As already mentioned above, cross reactivity tests had already been carried out for both the sandwich ELISA and the competitive assay. In both cases, none of the tested food ingredients showed any cross reactivity. However, previous cross reactivity tests were only carried out at protein concentrations from 0.001 ng/mL to 10000 ng/mL. In the present diploma thesis, cross reactivity tests of the competitive ELISA were therefore extended up to a protein concentration of 1 mg/mL. However, cross reactivity tests with the sandwich ELISA were not repeated, since sandwich ELISAs are generally more specific than competitive ELISAs due to the fact that two antibodies raised against two different epitopes are used. The protein fraction of common food ingredients, such as nuts and seeds, was extracted as described in 5.2. The protein concentration was determined by using the Bradford Assay as described in 5.4.1. In Table 2 the concentrations of the protein extracts are listed. Cross reactivity tests were carried out as described in 5.7.4. In order to be able to compare the results obtained with different microtiter plates, on each plate, the NSB (non-specific binding) value and the B_0 value were determined. The signals (OD values) were normalized by subtracting the NSB value and dividing the corrected OD value by B_0. For calculating cross reactivity (CR) in %, the concentration of sesame (c_{Ses}) at 50% of $\frac{B-NSB}{B_0-NSB} \cdot 100$ was divided through the concentration of the cross reactivity sample (c_{Cr}) at 50% of $\frac{B-NSB}{B_0-NSB} \cdot 100$ and multiplied by 100.

$$\frac{c_{Ses}(50\%)}{c_{Cr}(50\%)} \cdot 100 = CR(\%) \tag{5}$$

The following Figures (8, 9, 10 and 11) show the results of the cross reactivity tests.

Table 2: Protein concentration of food extracts tested for cross reactivity

Food	Protein concentration (μg/mL)
Almonds	51900
Honey	53
Chocolate (40% cacao)	9181
Peanut	12300
Poppy seed	18000
Sunflower	16640
Rye	17040
Brazil nut	48500
Walnut	2477
Wheat	13070
Rice	1996
Oat	14403
Hazelnut	13400

The Figures 8, 9, 10 and 11 indicate that the competitive ELISA does not show cross reactivity with sunflower, almond, honey, rye, Brazil nut, wheat, peanut, poppy seed, oat and hazelnut. However, for chocolate cross reactivity was found to be 0.7%.

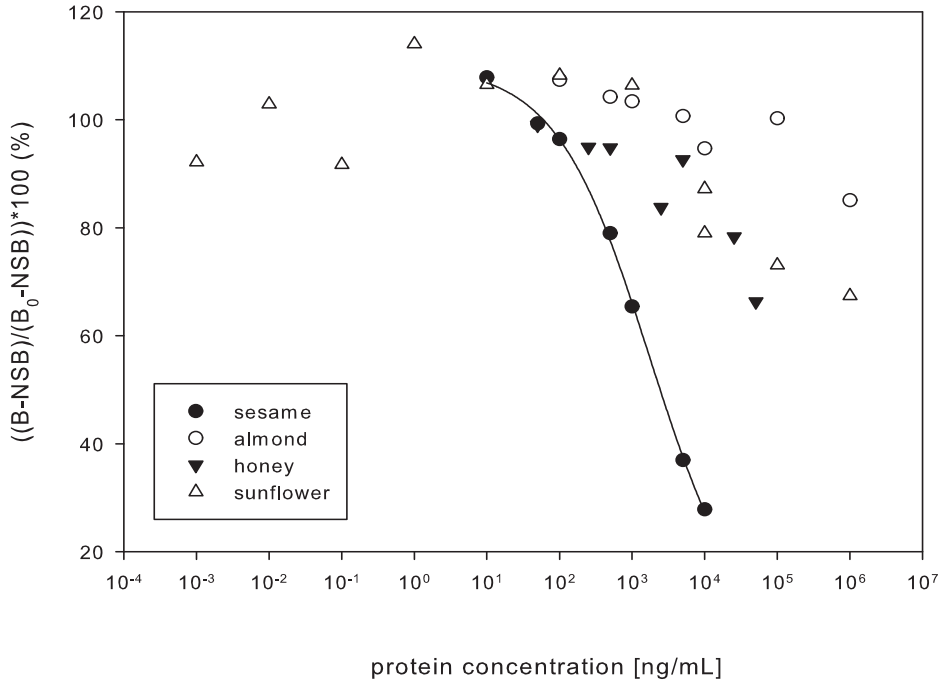

Figure 8: Results of cross reactivity tested for almond, honey and sunflower

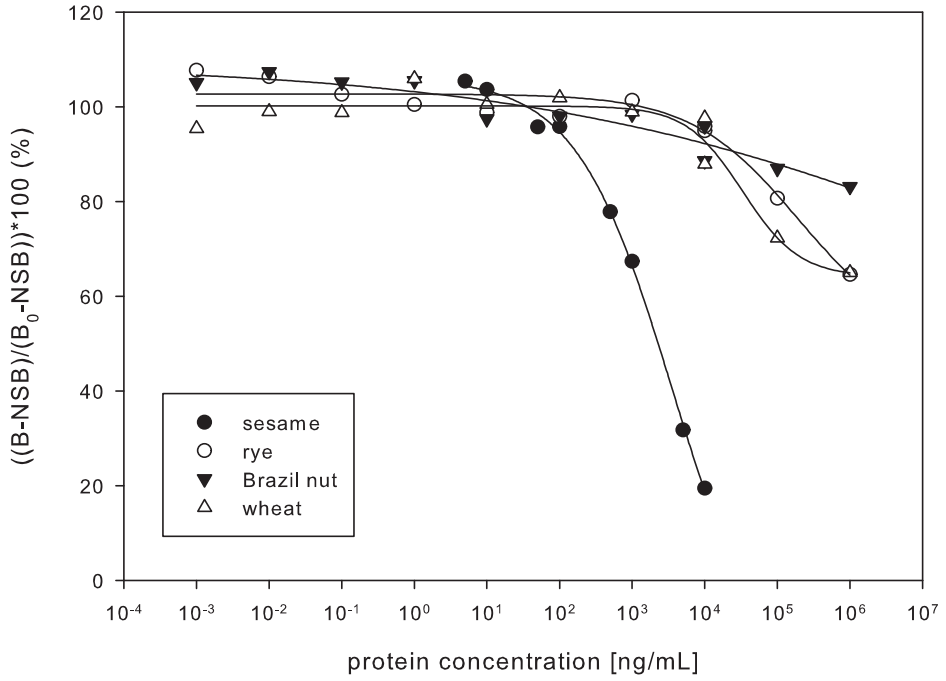

Figure 9: Results of cross reactivity tested for rye, Brazil nut and wheat

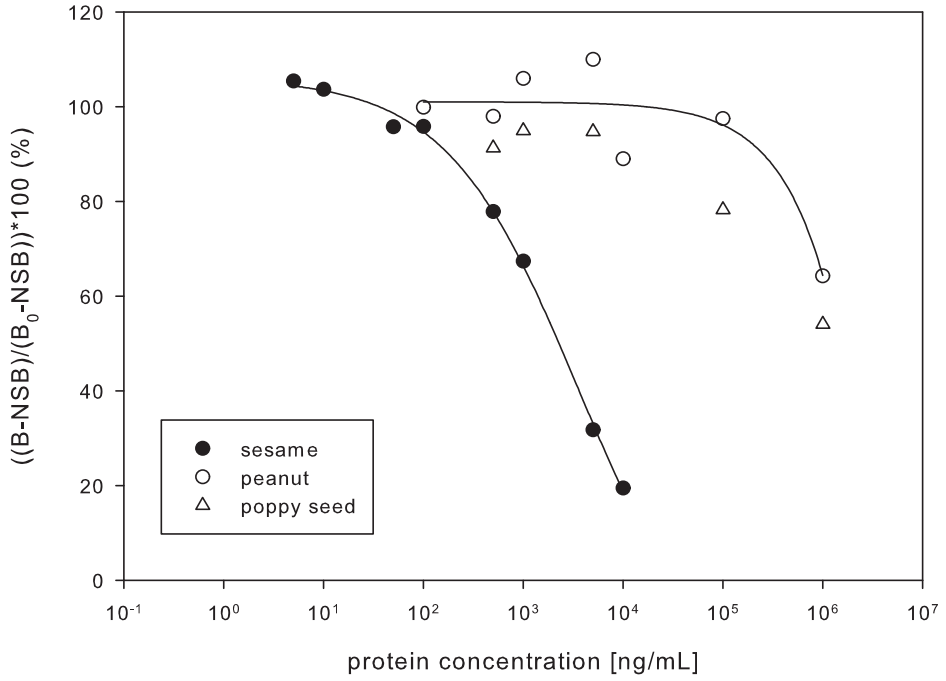

Figure 10: Results of cross reactivity tested for peanut and poppy seed

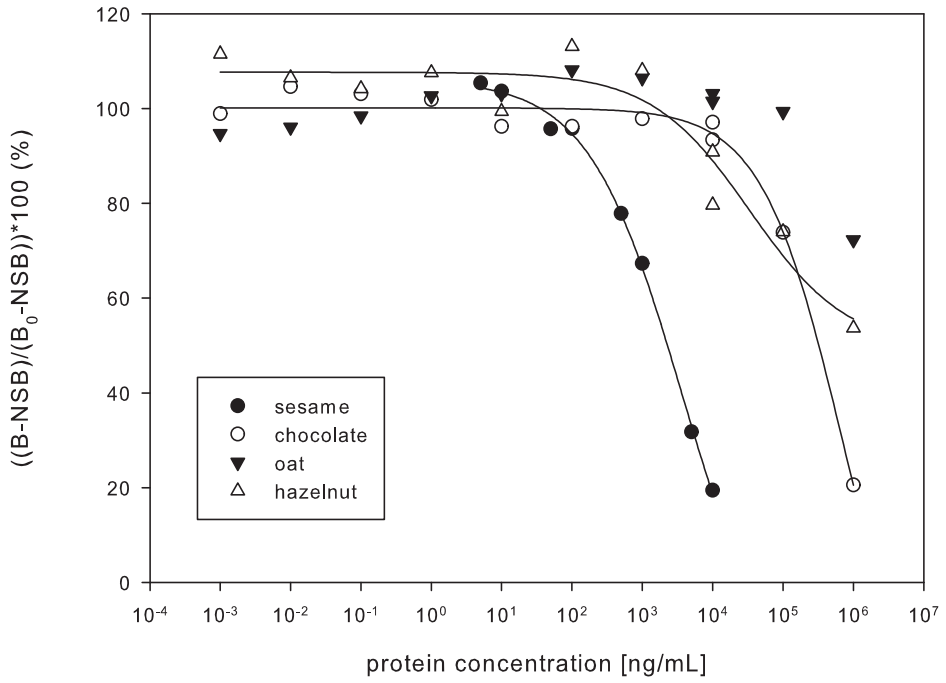

Figure 11: Results of cross reactivity tested for chocolate, oat and hazelnut

4.1.3 Optimization of the sandwich ELISA

In the sandwich ELISA developed by Gerda Redl [61] 50 μL of the antigen containing solution (sesame protein standard solution or food extract) were loaded onto the microtiter plate. In previously published sandwich ELISA methods, however, higher volumes of the antigen containing solutions are used. In the present diploma thesis, the influence of the volume of the sesame protein standard solution on the calibration curve was therefore investigated by varying the volume from 50 to 200 μL. Simultaneously, the volume of the capture antibody solution was increased from 100 μL to 200 μL.

Figure 12 shows that a volume of 200 μL yielded the steepest calibration curve. Due to this result, all following experiments were carried out under these conditions.

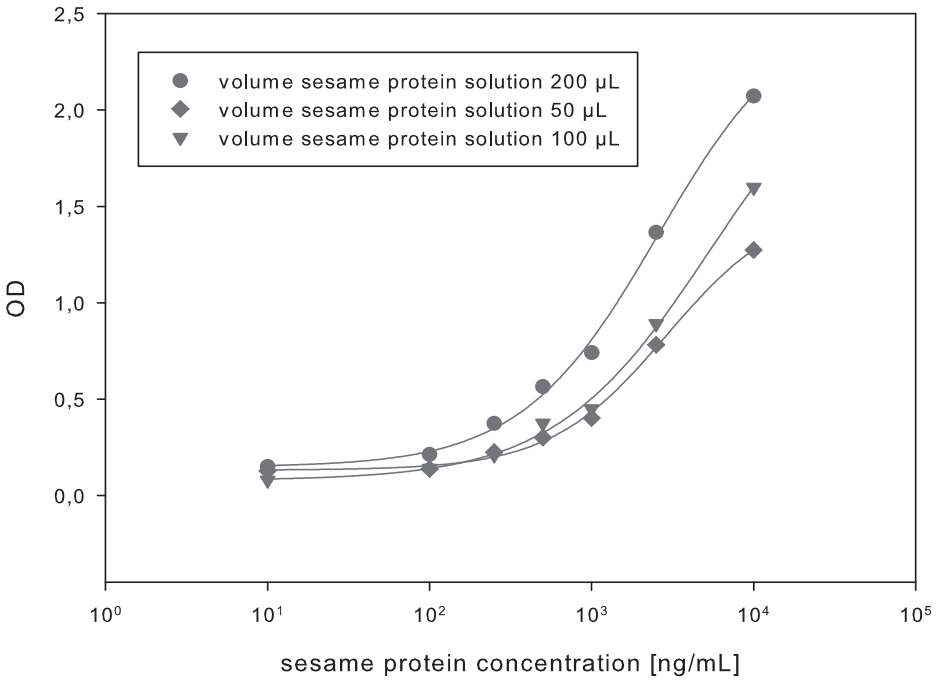

Figure 12: Optimization of the sesame protein solution volume. The sesame protein standards were diluted in PBS. The ELISA protocol was the same as described in 5.7.3

4.2 Spiking of food samples

Four food matrices (rusk, whole wheat bread, whole wheat cookies and muesli) were spiked with known amounts of sesame seeds. Each of the four samples was previously analysed with both ELISA methods. Sesame was not detected in any of the samples. Rusk was selected because it is a popular bakery product and often consumed by children. Whole wheat bread was chosen because sesame is a common ingredient in bakery products. In bakeries and supermarkets, sesame containing breads are often stored close to sesame free bread, increasing the risk of contamination. The whole wheat bread was home made in order to prevent contamination with sesame. Many whole wheat cookies have a note that they "may contain sesame", because they are produced by a manufacturer that produces sesame cookies or other sesame containing products. Numerous mueslis or cereal bars contain sesame. Even though production facilities are always cleaned carefully, possible contamination of sesame free mueslis can not completely be excluded. Mueslis are rather complex matrices since they often contain many different ingredients such as dried fruits, cereals and seeds.

Each of the four food matrices was spiked with sesame seeds in the following concentrations: 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005% and 0.001%. The details of the spiking procedure are given in section 5.3.

4.2.1 Determination of the recovery of sesame

The protein fractions of the spiked food samples were extracted according to the procedure given in 5.2. The extracts were analysed with both the sandwich and the competitive ELISA. All protein extracts were loaded in different dilutions: 1:1 (1 mL + 1 mL), 1:5 (1 mL + 4 mL), 1:10 (1 mL + 9 mL) and 1:20 (1 mL + 19 mL) with PBS.

For the sandwich ELISA, sesame protein standard solutions were prepared by diluting the sesame extract (see 5.7.3) with PBS. The following standard solutions were used: 2500 ng/mL, 1000 ng/mL, 750 ng/mL, 500 ng/mL, 250 ng/mL, 100 ng/mL and 10 ng/mL.

For the competitive ELISA, sesame protein standard solutions had to be prepared

by diluting the sesame extract with the certain food matrix in order to avoid any matrix effects. Dilution was carried out with the food matrix which was previously diluted 1:20 with PBS. The sesame protein concentrations were as follows: 10000 ng/mL, 5000 ng/mL, 1000 ng/mL, 500 ng/mL, 100 ng/mL, 50 ng/mL and 10 ng/mL. The procedures of the sandwich and the competitive ELISA are described in detail in 5.7.3 and 5.7.4, respectively. The OD values of the standard solutions were plotted against the logarithmic protein concentration and a non-linear regression was done to get a sigmoid calibration curve. The dilution which yielded the OD value closest to the inflection point of the calibration curve was used for calculating the sesame protein concentration. In order to obtain the sesame concentration (%), a conversion factor of 5.5 was applied (see 4.1.1). In the following tables the results of the recovery tests are listed. The values are the mean values of triplicate determinations.

Matrix 1: whole wheat cookies

Table 3 and 4 show the OD values, the sesame protein concentrations, the sesame concentrations [%] and the recoveries obtained with the sandwich ELISA and the competitive ELISA, respectively.

Table 3: Recovery of sesame in spiked whole wheat cookies obtained with the sandwich ELISA. xxx ... OD value > 3

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	xxx	1:100	-	-	-
0.5	1.233	1:50	148095	0.4	80
0.1	1.036	1:50	72587	0.2	200
0.05	0.868	1:20	15995	0.04	80
0.01	0.678	1:10	3831	0.01	100
0.005	0.887	1:1	1713	0.0047	94
0.001	0.510	1:1	340	0.0009	90

As it is shown in Table 3 the recovery was in the range from 80 to 100%, only at a spike level of 0.1% a too high recovery of 200% was obtained.

Table 4: Recovery of sesame in spiked whole wheat cookies obtained with the competitive ELISA. xxx ... OD value > 3

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	xxx	1:5	-	-	-
0.5	0.301	1:50	185647	0.5	100
0.1	0.399	1:50	30957	0.08	80
0.05	0.576	1:50	29459	0.08	160
0.01	0.435	1:10	4735	0.01	100
0.005	0.592	1:10	5351	0.014	280
0.001	0.597	1:10	1328	0.003	300

Table 4 shows that at low spike levels particularly at 0.005 and 0.001% too high recoveries were obtained. However, sesame could be detected down to a spike level of 0.001%.

In Table 5, the recoveries obtained with both ELISA methods are compared.

Table 5: Recovery of sesame in spiked whole wheat cookies obtained with sandwich and competitive ELISA

	Recovery of sesame [%]	
Spike level [%]	Sandwich ELISA	Competitive ELISA
1	-	-
0.5	80	100
0.1	200	80
0.05	80	160
0.01	100	100
0.005	94	280
0.001	90	300

Table 5 indicates that sesame could be detected with both ELISA methods down to a spiking level of 0.001% (10 ppm). At lower concentrations, the recoveries obtained with the sandwich ELISA were more accurate than those obtained with the competitive ELISA.

Matrix 2: whole wheat bread

The following tables show the recovery of sesame in spiked whole wheat bread.

Table 6: Recovery of sesame in spiked whole wheat bread obtained with the sandwich ELISA

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	0.936	1:500	696618	1.9	190
0.5	0.771	1:100	92522	0.25	50
0.05	0.666	1:50	34688	0.09	180
0.01	0.707	1:10	7790	0.02	200
0.005	0.748	1:5	4361	0.011	220
0.001	0.413	1:1	568	0.0015	150

Table 6 indicates that in whole wheat bread the recoveries were generally too high. In order to check if the results were caused by matrix effects, sesame protein standards diluted with PBS and sesame protein standards diluted with matrix (blank whole wheat bread, diluted 1:20 with PBS) were loaded onto one and the same microtiter plate. The two calibration curves are shown in Figure 13. The two standard curves are completely overlapping at low sesame protein concentrations. The Figure 13 indicates that the high recoveries are not caused by matrix effects.

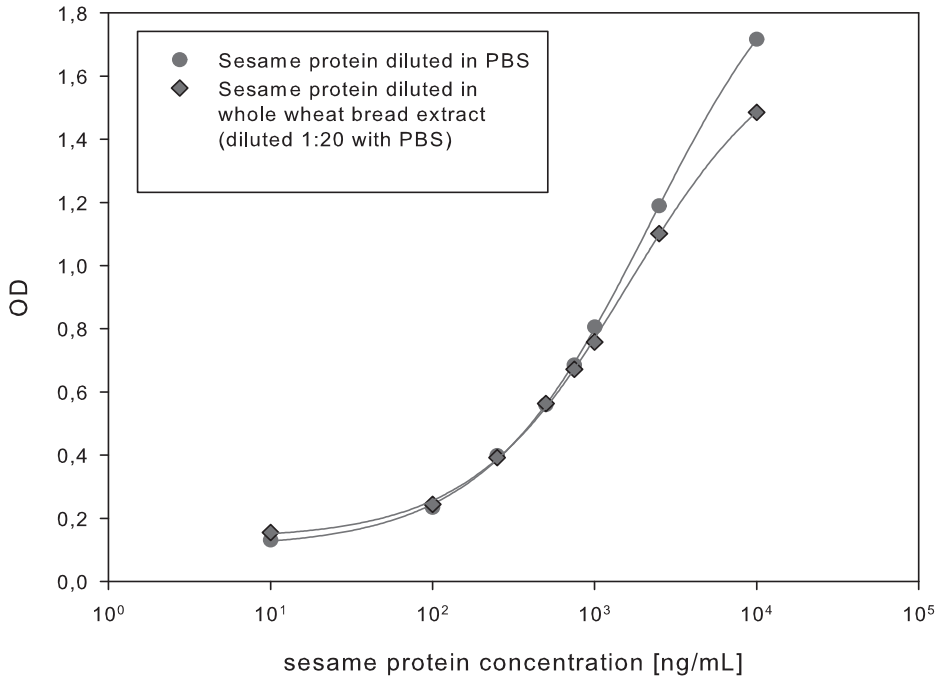

Figure 13: Influence of the whole wheat bread matrix on the calibration curve

In Table 7 the recoveries for sesame in spiked whole wheat bread obtained with the two standard curves are summarized.

Table 7: Influence of the whole wheat bread matrix on the recovery of sesame

	Recovery of sesame [%]	
Spike level [%]	Sesame protein diluted with PBS	Sesame protein diluted in whole wheat bread extract
1	190	210
0.5	50	54
0.05	180	200
0.01	200	200
0.005	220	200
0.001	150	100

It can be seen that the recoveries obtained with the two different calibration curves were very similar. The data indicate that calibration with sesame proteins in whole wheat bread matrix does not improve the recovery.

Next, the spiked whole wheat bread was analyzed with the competitive ELISA. The results are shown in Table 8.

Table 8: Recovery of sesame in spiked whole wheat bread obtained with the competitive ELISA

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	0.382	1:500	156184	0.43	43
0.5	0.356	1:100	42664	0.12	24
0.05	0.334	1:20	11024	0.03	61
0.01	0.405	1:10	2311	0.006	60
0.005	0.286	1:1	1994	0.005	100
0.001	0.424	1:1	351	0.001	100

Table 8 shows that in the competitive ELISA recoveries of 100% were obtained at spike

levels of 0.005% and 0.001%, whereas at higher spike levels too low recovery ratios were obtained. In Table 9 the results obtained with both ELISA methods are summarized.

Table 9: Recovery of sesame in spiked whole wheat bread obtained with sandwich and competitive ELISA

	Recovery of sesame [%]	
Spike level [%]	Sandwich ELISA	Competitive ELISA
1	190	43
0.5	50	24
0.05	180	61
0.01	200	60
0.005	220	100
0.001	150	100

The Table 9 indicates that both methods allow the detection of sesame down to a sesame concentration of 0.001%. However, quite different recoveries were obtained with the two methods. These data indicate that the deviations from the theoretical recovery (100%) cannot be caused by errors in the preparation of the spiked samples.

Matrix 3: rusk

The following Tables (Table 10 and 11) show the recoveries obtained in the analysis of rusk which had previously been spiked with sesame seeds.

Table 10: Recovery of sesame in spiked rusk obtained with the sandwich ELISA

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	0.501	1:500	3560551	9	900
0.5	0.320	1:100	94448	0.26	52
0.1	0.286	1:50	30181	0.08	80
0.05	0.304	1:50	38652	0.1	200
0.01	0.328	1:5	5208	0.014	140
0.005	0.353	1:1	2752	0.008	150
0.001	0.345	1:1	2525	0.007	700

Table 11: Recovery of sesame in spiked rusk obtained with the competitive ELISA

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	0.250	1:500	-	-	-
0.5	0.171	1:100	153119	0.4	80
0.1	0.204	1:50	32963	0.09	90
0.05	0.183	1:20	22167	0.06	120
0.01	0.184	1:10	10805	0.03	300
0.005	0.177	1:5	6495	0.018	360
0.001	0.171	1:1	3031	0.008	800

Table 12: Recovery of sesame in spiked rusk obtained with sandwich and competitive ELISA

	Recovery of sesame [%]	
Spike level [%]	Sandwich ELISA	Competitive ELISA
1	900	-
0.5	52	80
0.1	80	90
0.05	200	120
0.01	140	300
0.005	150	360
0.001	700	800

The Tables (10, 11) indicate that particularly at lower spike levels too high recoveries were obtained. In contrast to matrix 2 (whole wheat bread), both ELISA methods yielded too high recoveries. It is probable, that the results are caused by an heterogeneity of the spiked sample.

Matrix 4: muesli

The recoveries for sesame in the fourth food matrix, muesli, are shown in Table 13 and 14, the results obtained with both ELISA methods are compared in Table 15.

Table 13: Recovery of sesame in spiked muesli obtained with the sandwich ELISA

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	0.793	1:500	1130306	3	300
0.5	1.251	1:100	971210	2.6	520
0.1	0.789	1:100	223307	0.6	600
0.05	0.763	1:50	104272	0.3	600
0.01	0.729	1:10	19093	0.05	500
0.005	0.667	1:5	102488	0.03	600
0.001	0.646	1:1	3043	0.008	800

Table 14: Recovery of sesame in spiked muesli obtained with the competitive ELISA. Calibration was carried out with sesame proteins diluted with muesli matrix

Spike level [%]	OD	Dilution of the extract	Sesame protein concentration [ng/mL]	Sesame concentration [%]	Recovery of sesame [%]
1	0.236	1:500	301034	0.8	80
0.5	0.166	1:100	181303	0.5	100
0.1	0.215	1:50	40028	0.1	100
0.05	0.194	1:20	21456	0.06	120
0.01	0.223	1:5	3595	0.01	100
0.005	0.202	1:1	1894	0.005	100
0.001	0.239	1:1	1150	0.003	300

Table 15: Recovery of sesame in spiked muesli obtained with sandwich and competitive ELISA. Calibration of the competitive ELISA was carried out with sesame proteins diluted with muesli matrix

	Recovery of sesame [%]	
Spike level [%]	Sandwich ELISA	Competitive ELISA
1	300	80
0.5	520	100
0.1	600	100
0.05	600	120
0.01	500	100
0.005	600	100
0.001	800	300

Table 15 indicates that too high recoveries (>300%) were obtained with the sandwich ELISA. It can be seen that significantly better recoveries were obtained with the competitive ELISA. However, in the sandwich ELISA calibration was carried out with sesame protein standard solutions in PBS. In contrast, the competitive ELISA was calibrated with sesame proteins diluted with muesli matrix. It can therefore be assumed that matrix effects were responsible for the too high recoveries obtained with the sandwich ELISA.

In the following Tables (16 and 17) the recoveries for sesame in the four matrices are summarized.

Table 16: Recovery of sesame in four spiked food matrices obtained with the sandwich ELISA

	Recovery of sesame [%]			
Spike level [%]	Matrix 1 (whole wheat cookies)	Matrix 2 (whole wheat bread)	Matrix 3 (rusk)	Matrix 4 (muesli)
1	-	190	900	300
0.5	80	50	52	520
0.1	200	-	80	600
0.05	80	180	200	600
0.01	100	200	140	500
0.005	94	220	150	600
0.001	90	150	700	800

The Table 16 indicates that the sandwich ELISA allows the detection of sesame in all four matrices down to a sesame concentration of 0.001%. The best results were obtained with whole wheat cookies, indicating that quantification of sesame in this matrix is possible. The worst recoveries were obtained with muesli. Since with the same matrix significantly better results were obtained with the competitive ELISA which was calibrated with sesame proteins in matrix, matrix effects presumably caused the too high recoveries.

For the Sandwich ELISA, Gerda Redl [61] did not observe matrix effects when extracts where diluted 1:20. In the present diploma thesis, lower dilution factors were used. However, loading higher concentrated extracts onto the plate led to OD values lower than the B_0 value, indicating that matrix effects do not occur.

Table 17: Recovery of sesame in four spiked food matrices obtained with the competitive ELISA

	Recovery of sesame [%]			
Spike level [%]	Matrix 1 (whole wheat cookies)	Matrix 2 (whole wheat bread)	Matrix 3 (rusk)	Matrix 4 (muesli)
1	-	43	-	80
0.5	100	24	80	100
0.1	80	-	90	100
0.05	160	61	120	120
0.01	100	60	300	100
0.005	280	100	360	100
0.001	300	100	800	300

The competitive ELISA allows the detection of sesame in all four matrices down to a sesame concentration of 0.001%. However, the Table 17 indicates that at very low concentrations very high recoveries were obtained, which is similar to the sandwich ELISA.

Further investigations should have been carried out and more matrices should have been tested but at the end of the experiments described above the sensitivity (steepness of the calibration curve) of both ELISAs was significantly decreased compared to results obtained at the beginning of the diploma thesis. Experiments were therefore carried out to increase the sensitivity of both ELISA methods.

Sandwich ELISA

In the experiments described above the enzyme labeled antibody (anti-IgG*) was diluted 1:60000. In the following experiments, the dilution factor of the enzyme labeled antibody was varied from 1:10000 to 1:30000. In addition, two different concentrations of IgG were applied. The results are shown in Figure 14. The Figure 14 indicates that diluting the enzyme labeled antibody to 10000 resulted in the steepest calibration curve.

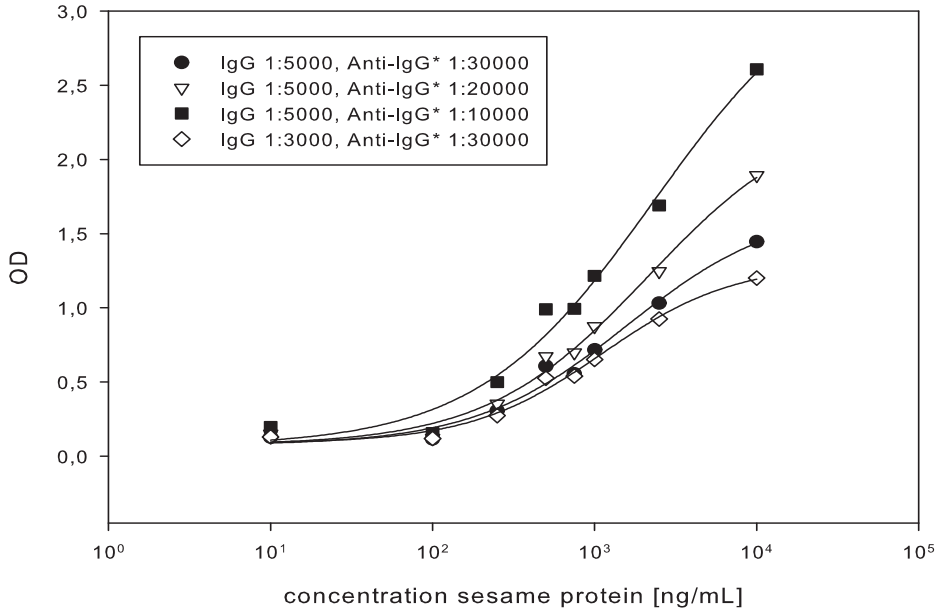

Figure 14: Influence of the concentration of the enzyme labeled antibody (anti-IgG*) on the calibration curve. Coating-antibody (IgY): diluted 1:25000.

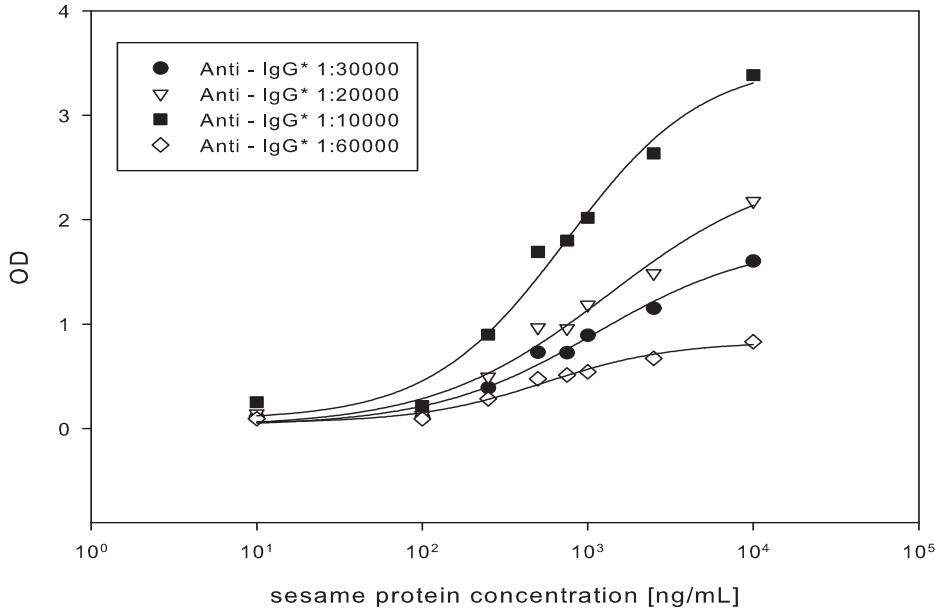

Figure 15: Influence of the concentration of the enzyme labeled antibody (anti-IgG*). IgY: diluted 1:12500, IgG: diluted 1:5000.

On another plate, the influence of the concentration of the enzyme labeled antibody on the calibration curve was investigated again. In this case, the coating antibody was diluted 1:12500, the secondary antibody 1:5000. The results are shown in Figure 15.

Figure 15 shows that the steepest calibration curve was obtained when the enzyme labeled antibody was diluted only 1:10000. However, under this condition the production of the colored product was so fast that the reaction had already to be stopped after 3 min. In addition, at lower dilution factors (1:10000 and 1:20000) the results were more scattering than at higher dilution factors.

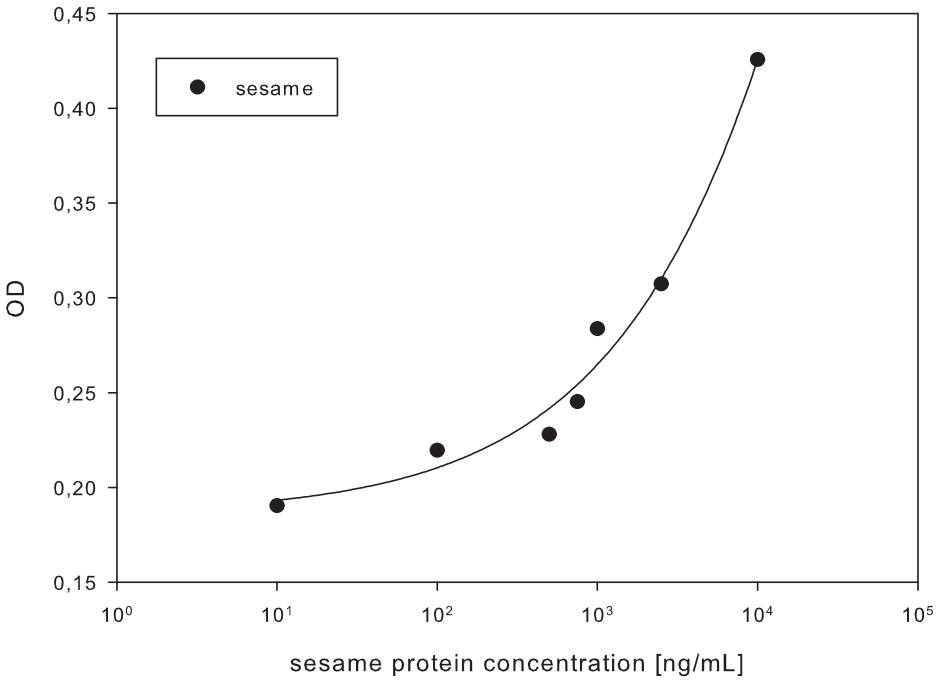

Figure 16: Calibration curve for sesame obtained after optimization of the sandwich ELISA. The IgY were diluted 1:12500, IgG 1:5000 and enzyme labeled anti IgG 1:30000.

As it is shown in Figure 16 even though the sandwich ELISA was optimized and some good calibration curves for sesame were obtained, after a few experiments the sensitivity of the ELISA decreased again. Additionally many outliers were found and the limit of detection was not as good as obtained in the beginning of the investigations.

Competitive ELISA

In order to increase the sensitivity of the competitive ELISA, several parameters were modified. Blocking was extended from 15 to 60 min. Instead of diluting the coating AG 1:6000 it was diluted only 1:1000. The concentration of the IgY was varied by applying dilution factors from 1:5000 to 1:10000. In addition, a new charge of secondary antibodies (enzyme labeled anti IgY) was used (diluted 1:50000).

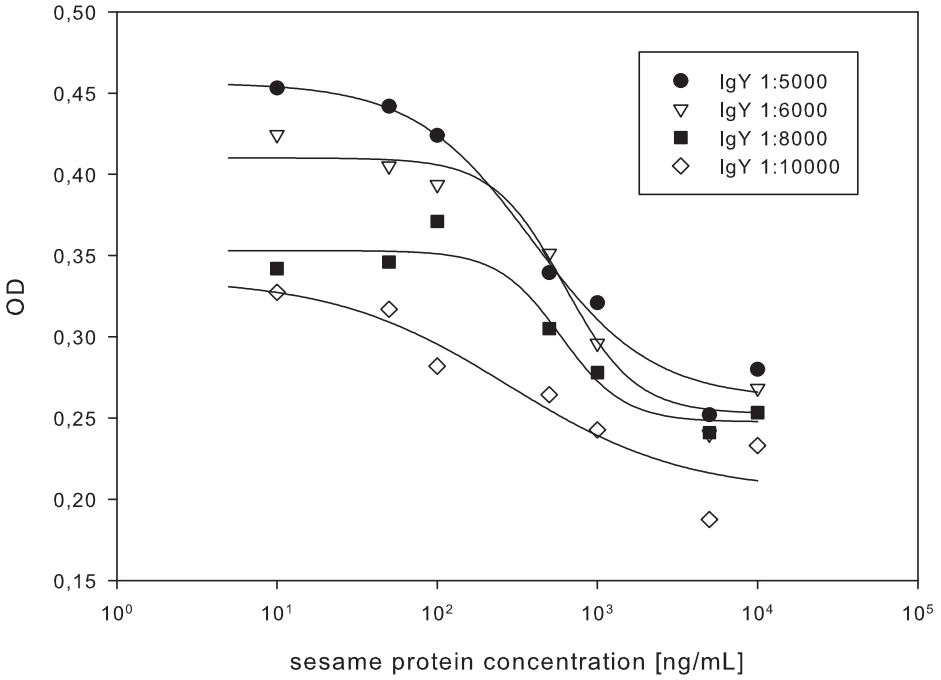

Figure 17: Influence of the concentration of the IgY on the calibration curve. Coating antigen: diluted 1:1000, enzyme labeled antibody: diluted 1:50000.

As Figure 17 indicates, diluting the IgY 1:5000 resulted in the steepest calibration curve. However, the difference between the highest and the lowest OD value is only about 0.15, indicating that the sensitivity of the ELISA was very poor.

4.3 Limit of detection (LOD)

4.3.1 Sandwich ELISA

The limit of detection (LOD) was calculated by taking the mean of the B_0 values of three different ELISA plates and adding 3 times of the standard deviation. In the present diploma thesis, food extracts were diluted from 1:1 to 1:20 before being loaded onto the microtiter plate. In Table 18, the influence of the dilution factor on the LOD of the sandwich ELISA is shown.

Table 18: Limit of detection of the sandwich ELISA

	Limit of detection (S/N=3)		
Dilution of the food extract with PBS	Sesame protein concentration [μg/g]	Sesame concentration [%]	Sesame concentration [ppm]
1:20	7.9	0.0044	44
1:10	4	0.0022	22
1:5	3	0.0011	11
1:1	0.8	0.0004	4

4.3.2 Competitive ELISA

The LOD was calculated using the OD values of triplicates of the blank food sample diluted 1:20 with PBS. To the mean of the OD values (B_0) three times of the standard deviation was added. Food extracts were diluted from 1:1 to 1:20 before being loaded onto the microtiter plate. In Tables 19, 20, 21 and 22, the influence of the dilution factor on the LOD of the competitive ELISA for each of the 4 matrices is shown.

Table 19: Limit of detection of the competitive ELISA for sesame in whole wheat cookies

	Limit of detection (S/N=3)		
Dilution of the whole wheat cookie extract with PBS	Sesame protein concentration [μg/g]	Sesame concentration [%]	Sesame concentration [ppm]
1:20	6.7	0.0032	32
1:10	3.3	0.0018	18
1:5	1.67	0.0009	9
1:1	0.67	0.0003	3

Table 20: Limit of detection of the competitive ELISA for sesame in whole wheat bread

	Limit of detection (S/N=3)		
Dilution of the whole wheat bread extract with PBS	Sesame protein concentration [μg/g]	Sesame concentration [%]	Sesame concentration [ppm]
1:20	20	0.011	110
1:10	10	0.0055	55
1:5	5	0.0028	28
1:1	2	0.0011	11

Table 21: Limit of detection of the competitive ELISA for sesame in muesli

	Limit of detection (S/N=3)		
Dilution of muesli extract with PBS	Sesame protein concentration[μg/g]	Sesame [%]	Sesame [ppm]
1:20	25.4	0.014	140
1:10	12.7	0.007	70
1:5	6.3	0.0035	35
1:1	2.5	0.0014	14

Table 22: Limit of detection of the competitive ELISA for sesame in rusk

	Limit of detection (S/N=3)		
Dilution of rusk extract with PBS	Sesame protein concentration[μg/g]	Sesame concentration [%]	Sesame concentration [ppm]
1:20	13.9	0.0076	76
1:10	6.9	0.0038	38
1:5	3.5	0.0019	19
1:1	5.7	0.0008	8

Table 23: Comparison of the LOD for sesame of both ELISA methods

	Limit of detection for sesame in food samples [ppm]	
Dilution of sample extract with PBS	Sandwich ELISA	Competitive ELISA
1:20	44	90
1:10	22	45
1:5	11	22
1:1	4	9

Table 23 indicates that the limit of detection of the sandwich ELISA is lower than that of the competitive ELISA (LOD was determined by the mean of the LOD of all 4 matrices). There is a general agreement that the LOD of analytical methods applicable in the analysis of allergenic components in foods should be between 1 and 100 ppm. Table 23 indicates that both ELISA methods fulfill this criterion.

4.4 Polymerase chain reaction

Three of the four spiked food samples (rusk, whole wheat bread and muesli) which were analysed with the two ELISA methods were also analysed with a real-time PCR method. Whole wheat cookies were already tested by Schöringhumer et al. [41]. The real-time PCR method applied in the diploma thesis was developed by Kerstin Schöringhumer as part of a duplex assay during her doctoral thesis. The method has already been published [41].

Primers and probes were chosen 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.

No cross reactivity was shown with 25 tested common food ingredients which included several nuts, seeds and grains. The LOD was determined as 0.005% sesame in spiked

whole wheat cookies.

4.4.1 DNA extraction

DNA was extracted from the spiked samples which were already used for ELISA measurements. For DNA extraction either the CTAB method or the QIAmp Stool Kit was used, depending on the sample matrix. The concentration of the extracted DNA was determined by measuring the absorbance at 260 nm. The purity of DNA was calculated by dividing the absorbance at 260 nm by the absorbance measured at 280 nm. The details of DNA extraction are given in 5.8.

Matrix 2: whole wheat bread

In the case of whole wheat bread, at the beginning the DNA was extracted with the CTAB method in triplicates. Since for some spike levels, DNA could not be isolated in sufficient amounts (see Table 24), all spiked samples were purified with a commercial DNA extraction kit (QIAmp Stool Kit) in duplicates. However, only for the spike levels 0.01% and 0.05% the concentration of the DNA extracted with the QIAmp Stool Kit was high enough for being analysed with the PCR method.

Matrix 3: rusk

Table 25 summarizes the purity and concentration of DNA extracted with the CTAB method from nonspiked and spiked rusk. The table shows that both the purity and the concentration of the DNA were high enough for carrying out PCR analysis.

Matrix 4: muesli

Due to the complexitiy of the matrix, the DNA from muesli was extracted with both the CTAB method and the commercial extraction kit. The results are summarized in Table 26.

Table 24: Concentration and purity of DNA extracted from spiked whole wheat bread by the CTAB method and by QIAmp Stool Kit. xxx ... no DNA could be extracted; - ... was not determined.

Spike level [%]	c DNA [ng/μL]		Purity (A_{260}/A_{280})		Amount [mg]		Extracted DNA [mg/g sample]	
	CTAB	Stool Kit	CTAB	Stool Kit	CTAB	Stool Kit	CTAB	Stool Kit
Blank	0.25	-	0.50	-	107	-	-	-
	11.75	-	1.74	-	100	-	0.12	-
	10.75	-	1.79	-	105	-	0.10	-
1	xxx	4.25	xxx	1.41	102	184	xxx	0.02
	xxx	4.50	xxx	3.60	101	208	xxx	0.02
	xxx	-	xxx	-	103	-	xxx	-
0.5	13.75	10.00	1.52	1.73	101	203	0.14	0.05
	4.75	8.00	2.37	1.45	109	195	0.04	0.04
	24.75	-	1.73	-	113	-	0.22	-
0.1	7.25	1.25	1.70	1.25	101	186	0.07	0.01
	11.00	81.25	1.91	1.54	106	186	0.10	0.44
	23.50	-	1.95	-	102	-	0.23	-
0.05	39.50	88.50	1.90	2.54	102	200	0.39	0.44
	26.00	164.75	1.92	7.48	99	231	0.26	0.71
	14.00	-	1.75	-	106	-	0.13	-
0.01	5.00	12.00	2.50	1.33	100	212	0.05	0.06
	9.25	42.00	2.17	10.50	105	198	0.09	0.21
	13.00	-	2.00	-	100	-	0.13	-
0.005	10.25	1.75	2.15	1.00	103	180	0.10	0.01
	17.00	8.25	1.94	1.50	105	194	0.16	0.01
	xxx	-	xxx	-	104	-	xxx	-
0.001	21.00	4.25	1.86	1.54	102	182	0.21	0.02
	20.50	3.50	1.82	1.55	105	229	0.20	0.02
	17.25	-	1.77	-	104	-	0.17	-

Table 25: Concentration and purity of the DNA extracted from spiked rusk. xxx ... no DNA could be extracted

Spike level [%]	Purity (A_{260}/A_{280})	c DNA [ng/μL]	Amount [mg]	Extracted DNA [mg/g sample]
Blank	1.80	378.25	105	3.60
	1.74	237.75	104	2.28
	1.71	135.75	95	1.43
1	1.03	10.5	104	0.10
	xxx	xxx	119	xxx
	1.89	02.87	106	4.74
0.5	1.92	556.87	101	5.51
	1.89	323.25	101	3.20
	1.82	509.62	98	5.20
0.1	1.89	515.62	96	5.37
	1.83	265.50	97	2.74
	1.85	523.87	109	4.81
0.05	1.88	512.25	91	5.63
	1.87	504.37	116	4.35
	1.88	549.00	115	4.77
0.01	1.89	251.62	113	2.23
	xxx	xxx	101	xxx
	1.83	921.00	107	8.61
0.005	1.92	705.75	114	6.16
	1.88	461.25	109	4.23
	1.87	504.37	112	4.50
0.001	1.90	492.00	96	5.13
	1.88	495.37	102	4.86
	1.75	429.37	113	3.80

Table 26: Concentration and purity of the DNA extracted from spiked muesli by the CTAB method and by QIAmp Stool Kit. - . . . DNA extraction was not carried out

Spike level [%]	c DNA [ng/μL]		Purity (A_{260}/A_{280})		Amount [mg]		Extracted DNA [mg/g sample]	
	CTAB	Stool Kit	CTAB	Stool Kit	CTAB	Stool Kit	CTAB	Stool Kit
Blank	164.25	-	1.91	-	103	-	1.59	-
	398.25	-	1.93	-	104	-	3.83	-
	270.25	-	1.97	-	103	-	2.62	-
1	744.25	96.50	1.67	3.57	109	222	6.83	0.43
	385.50	64.5	1.96	2.06	109	188	3.54	0.34
	418.50	-	1.93	-	106	-	3.95	-
0.5	620.00	59.25	1.82	1.89	107	195	5.79	0.30
	484.50	46.25	1.94	1.92	108	206	4.49	0.22
	465.75	-	1.96	-	110	-	4.23	-
0.1	382.25	53.00	1.92	1.85	110	216	3.48	0.25
	400.00	45.00	1.92	1.89	104	196	3.85	0.23
	336.50	-	1.97	-	102	-	3.30	-
0.05	529.75	105.50	1.88	1.52	107	204	4.95	0.52
	607.25	81.75	1.93	1.42	105	212	5.75	0.39
	489.50	-	1.91	-	104	-	4.71	-
0.01	426.50	54.25	1.92	2.00	108	201	3.95	0.27
	246.75	25.75	1.88	1.90	110	194	2.24	0.13
	208.50	-	1.84	-	104	-	2.00	-
0.005	127.75	25.75	1.63	1.32	111	214	1.15	0.12
	197.25	44	1.83	2.02	104	185	1.90	0.24
	138.00	-	1.87	-	108	-	1.28	-
0.001	289.25	61.50	1.84	2.08	101	206	2.86	0.30
	261.50	54.25	1.87	1.92	109	211	2.40	0.26
	137.75	-	1.95	-	105	-	1.31	-

The Table 26 indicates that the purity of the extracts obtained with both methods was between the recommended range of 1.8 and 2.0 with both methods

4.5 PCR analysis

4.5.1 PCR optimization

The influence of the primer concentration on the amplification was investigated. Three different primer concentrations were tested: 50 nM, 250 nM and 500 nM. The PCR protocol was the same as described in 5.9.2.

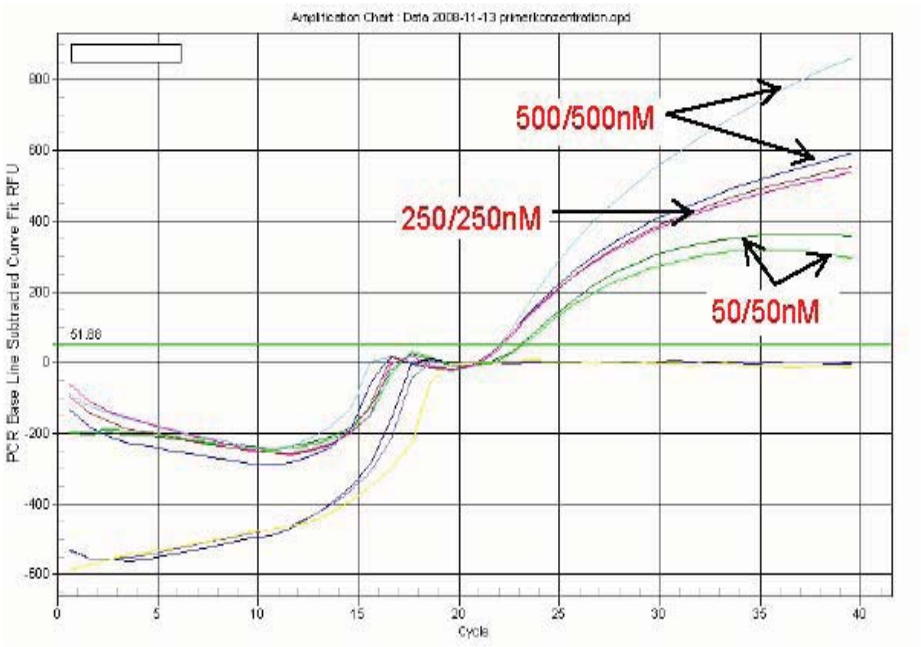

Figure 18: Amplification of sesame DNA with different primer concentrations

The Figure 18 shows that the highest signal and the lowest Ct-value were obtained with primer concentrations of 500 nM.

4.5.2 PCR analysis of sesame spiked samples

All samples, from which DNA could be extracted in sufficient concentration and purity, were analysed by PCR.

Matrix 1: whole wheat cookies

Whole wheat cookies had already been extracted and analyzed with the real-time PCR method by Schöringhumer et al. [41]. It was shown that sesame could be amplified down to a concentration of 0.005% sesame. The slope of the standard curve was -3.867, indicating an amplification efficiency of 81.4%. The efficiency was calculated according to the equation described in 3.6.3.

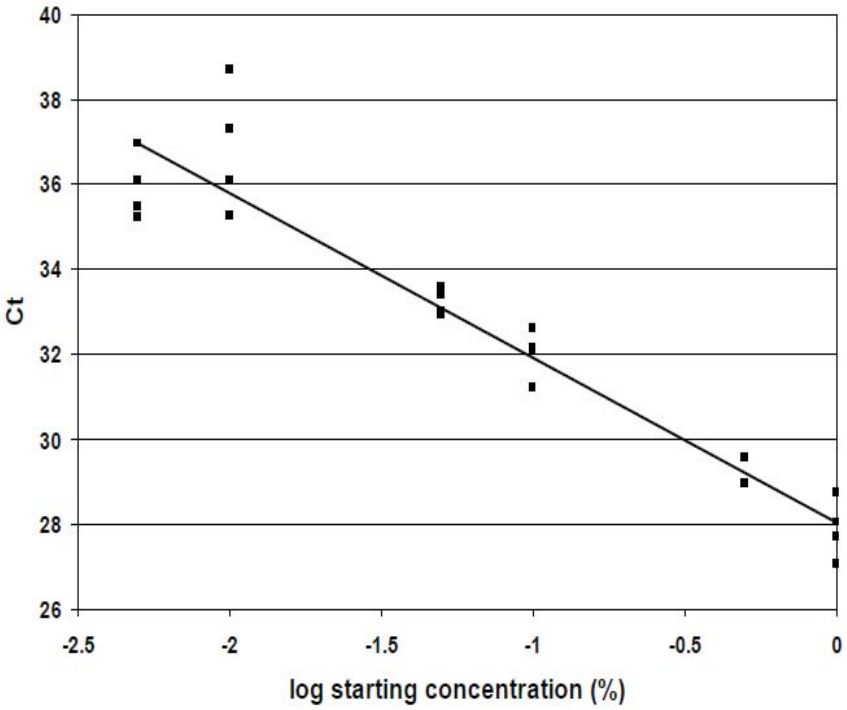

Figure 19: Standard curve obtained by amplifying DNA extracted from spiked whole wheat cookies [41]

Matrix 2: whole wheat bread

As already mentioned (section 4.4.1) DNA from nonspiked and spiked whole wheat bread was extracted with both the CTAB method and the QIAmp Stool Kit. In the case of the spike levels 0.5%, 0.05%, 0.001% and 0.005% the amount of extracted DNA with the CTAB method was sufficient for PCR analysis. Figure 20 shows the obtained amplification curves. In Table 27 the Ct values are summarized.

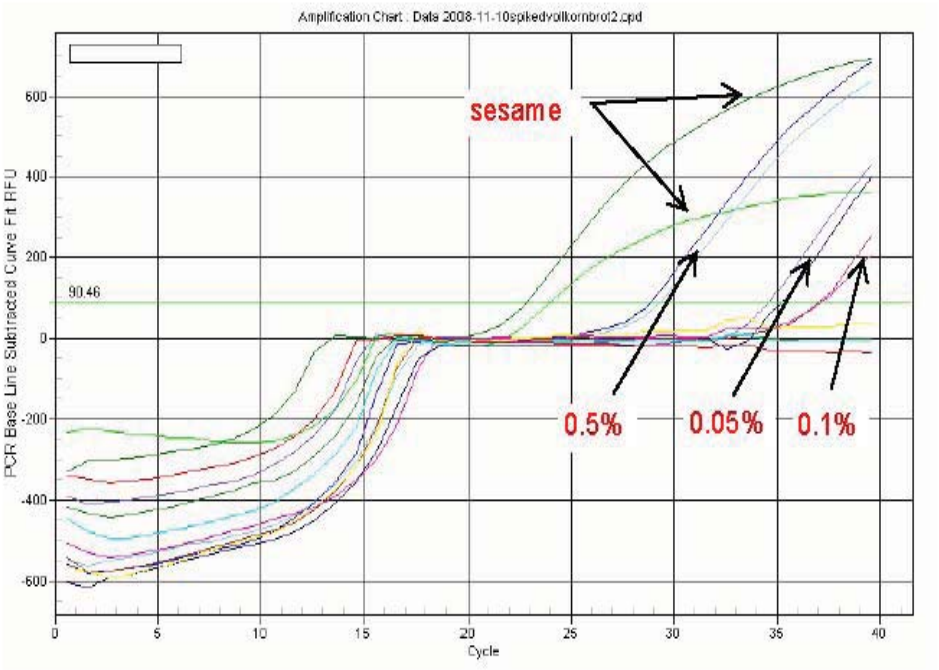

Figure 20: Amplification of sesame DNA and DNA extracted from spiked whole wheat bread

The Figure 20 indicates that amplification was possible down to a sesame concentration of 0.05%.

However, the Table 27 shows that for the sample spiked with 0.05% sesame a lower Ct value was obtained than for the sample which had been spiked with 0.1%.

Table 27: Ct values obtained for the spiked whole wheat bread. - ... no Ct value was obtained for the blank

Spike level [%]	Ct value	Mean Ct value
Blank	- -	-
0.5	29.32 28.88	29.10
0.1	37.22 37.04	37.13
0.05	35.27 34.60	34.93

Matrix 3: rusk

DNA was extracted with the CTAB method. Figure 21 represents the PCR standard curve obtained with DNA extracted from spiked rusk. Sesame could be detected down to a concentration of 0.005% sesame. The slope of the calibration curve was -4.0693, corresponding to an amplification efficiency of 76%.

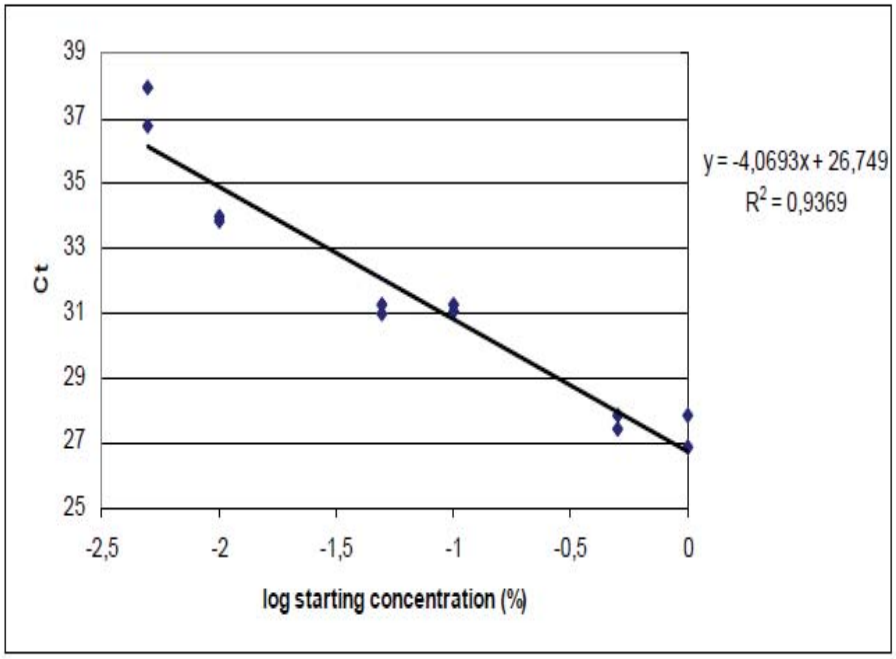

Figure 21: Standard curve obtained by amplifying DNA extracted from spiked rusk

Matrix 4: muesli

The first PCR run was carried out with DNA extracted from muesli by the CTAB method. The template could not be amplified in any of the spiked samples. Therefore an inhibition control as described in 5.9.4 was carried out. The inhibition control was negative, indicating that the negative results were not caused by inhibition of the DNA polymerase. Next, DNA was extracted by using the QIAmp Stool Kit. The DNA concentrations and purities are shown in Table 26. Figure 22 shows that DNA extracted from muesli with the stool kit could be amplified. However, amplification was only possible down to a sesame concentration of 0.5% (see Table 28). In the case of lower spike levels,

only one of two determinations led to a positive signal.

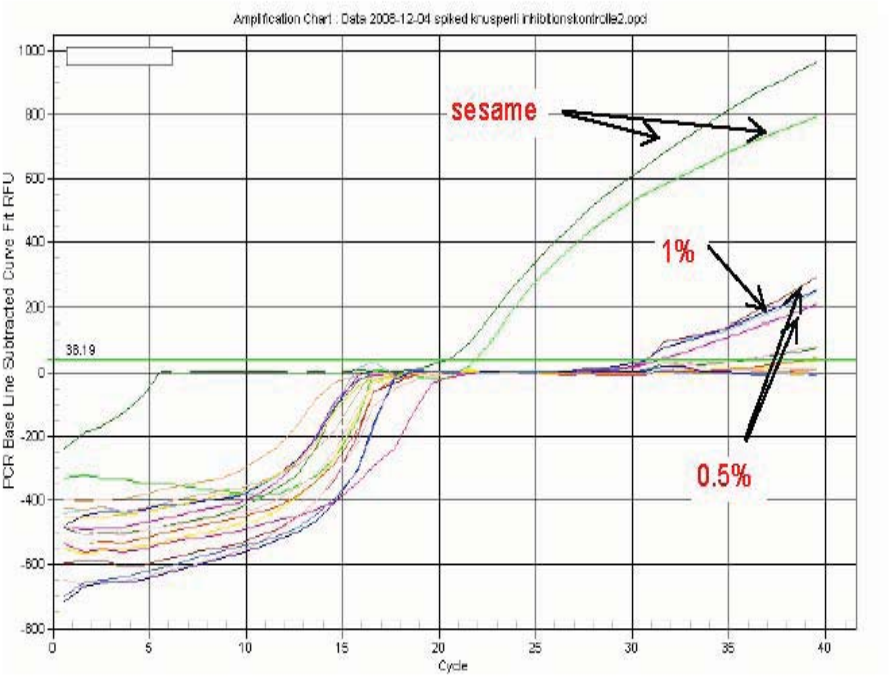

Figure 22: Amplification of DNA extracted with QIAmp Stool Kit from spiked muesli

Table 28: Ct values obtained for sesame spiked muesli

Spike level [%]	Ct value	Mean Ct value
Blank	- -	-
1	30.77 30.67	30.72
0.5	31.29 30.77	31.03

4.6 Comparison of the sandwich ELISA, the competitive ELISA and the PCR method

In contrast to the real-time PCR, both ELISAs could detect 0.001% sesame in each matrix. Diluting the extract 1:20 or 1:1 resulted in LODs of 44 ppm or 4 ppm, respectively, for the sandwich ELISA. The competitive ELISA showed a LOD of 90 ppm when diluting

the extract 1:20 and a LOD of 9 ppm when diluting the extract 1:1. For the real-time PCR method the LOD was 0.005% in whole wheat cookies and rusk.

Proteins could be extracted for ELISA experiments even from complex food matrices such as muesli or highly processed food such as whole wheat bread. DNA extraction turned out to be the most critical step for PCR analysis since from whole wheat bread DNA could not be extracted in sufficient amounts. Both ELISA methods are applicable for allergen determination in foods while the applicability for the real-time PCR depends on the matrix.

5 Experimental part

5.1 Protein extraction from sesame seeds

Reagents:

- PBS (Phosphate buffered saline): 3.9 g $NaH_2PO_4 \cdot 2H_2O$, 31.15 g $Na_2HPO_4 \cdot 2H_2O$ and 21.25 g NaCl were dissolved in 2.5 L H_2O_{dd} and stored at room temperature
- n-Hexan

Peeled, unroasted, white sesame was bought in a local supermarket. The sesame was grinded in a small kitchen blender. The obtained sesame paste was defatted by soxhlet-extraction with 200 mL n-hexane for 16 to 20 h and dried overnight. The dry, defatted sesame was extracted with PBS with constant stirring for 2 h. To 1 g sesame 15 ml PBS were added. After 2 h, the suspension was centrifuged at 4000 rpm at 4°C for 35 min (centrifuge from Sigma 4 K 10 with rotor 11140). The supernatant was filtered through a Whatman filter (Schleicher & Schuell 589/1 Schwarzband) and the filtrate was centrifuged at 10000 rpm for 5 min (Eppendorf centrifuge 5452). Then the supernatant was filtered again and the filtrate was aliquoted in Eppendorf tubes and stored at -20°C.

5.2 Protein extraction from food samples

Reagents:

- Extraction buffer: 6.06 g TRIS (Tris(hydroxymethyl)-aminomethan) and 11.69 g NaCl were dissolved in 900 mL H_2O_{dd}. The pH was adjusted to 8.15-8.25 with 1M HCl. The buffer was filled up to 1 L and stored at room temperature.

Food samples were bought in local supermarkets. After grinding the food samples in a small kitchen mixer, an aliquot of 10 g was weighed in and mixed with 50.0 mL of the extraction buffer. The suspension was homogenized with an Ultraturrax for 2 min. The extract was centrifuged at 4000 rpm at 20°C for 30 min. The supernatant was filtered through a Whatman filter and centrifuged at 10000 rpm for 5 min. The supernatant was

filtered again through a Whatman filter and aliquoted in 1.5 mL Eppendorf tubes. The protein extracts were stored at -20°C.

5.3 Spiking of food matrices

Three commercial food products, rusk, whole wheat cookies and muesli and one homemade product, whole wheat bread, were spiked with unroasted, peeled sesame seeds. Following spike levels were used: 1%, 0.5%, 0.1%, 0.05%, 0.01%, 0.005% and 0.001% sesame in the food matrix. First, sesame seeds were hackled in a small kitchen blender and mortared with mortar and pestle. In addition, about 700 g of blank food matrix were hackled in a big kitchen blender. For the 1% spike level, 99 g of the hackled food sample and 1 g of homogenized sesame were mixed for 10 min in a big kitchen blender. After blending the mixture 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 0.1% spike level, 10 g of the food matrix spiked with 1% sesame were mixed with 90 g of unspiked food matrix for 10 min. 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 0.01% spike level, 10 g of the food matrix spiked with 0.1% sesame were mixed with 90 g of unspiked food matrix. For the 0.05% spike level, 50 g of the food matrix spiked with 0.1% sesame were mixed with 50 g of unspiked food matrix. The other spike levels were prepared in the same manner. The spiked food samples were frozen at -20°C.

5.4 Determination of the protein concentration

5.4.1 Bradford assay

Reagents:

- **Brillant Blue G:**

  1 mg/mL bovine serum albumin (BSA) in PBS

  95% ethanol

88% phosphoric acid

Solutions:

- **Bradford stock solution:**

  100 mL 95% ethanol

  200 mL 88% phosphoric acid

  350 mg Brillant Blue G

  The solution is usable for an undefined time.

- **Bradford working solution:**

  425 mL bidistilled water

  15 mL 95% ethanol

  30 mL 88% phosphoric acid

  30 mL Bradford-stock-solution

The solution was filtered through a Whatman filter and stored in a dark bottle.

- PBS

Procedure

Starting from the 1 mg/mL BSA solution, the following standard solutions were prepared: 200 μg/mL, 150 μg/mL, 100 μg/mL, 50 μg/mL, 20 μg/mL and 10 μg/mL BSA in PBS. 100 μL of the BSA standard solution or the extract were pipetted into a half-micro cuvette and mixed with 1 mL of the Bradford working solution. After 2 min of incubation, the absorbance was measured at a wavelength of 595 nm.

5.4.2 Method by Warburg and Christian

After diluting the extract with PBS (usually 1:20-1:1000) the absorbance was measured at 280 nm (A_{280}) and 260 nm (A_{260}). The protein concentration (c_P in mg/mL) was

calculated according to the equation:

$$1.55 \cdot A_{280} - 0.76 \cdot A_{260} = c_P \tag{6}$$

5.5 Production and isolation of rabbit anti-sesame antibodies (IgG)

Rabbit antibodies against sesame proteins were produced by BioGenes (Berlin, Germany) by immunization of a rabbit with a protein extract (1 mg/mL) of sesame. The rabbit was immunized on the 1^{st}, 7^{th}, 14^{th}, and 28^{th} day. After further 21 days the last booster was carried out. The blood was collected seven days after the last booster.

Isolation of the antibodies

The isolation of the antibodies was carried out by caprylic acid/ammonium sulphate precipitation method.

Reagents

- PBS
- 60 mM acetate buffer, pH 4.0
- 0.1 N NaOH
- Caprylic acid
- 10 x PBS
- 1.0 N NaOH
- Ammonium sulphate

5.5.1 Isolation of the IgG fraction

1.5 mL of the antiserum were transferred into an Eppendorf tube and centrifuged for 3 min to remove the already precipitated proteins. After mixing 1.0 mL of the supernatant with 4 mL of acetate buffer the pH was adjusted with 0.1 N NaOH to 4.5.

Then 25 μL of caprylic acid were added drop by drop .After shaking the reaction tube for 30 min the solution was centrifuged at 5000 rpm for 30 min. The clear supernatant was filtered through a Whatman filter and mixed with 10 x PBS buffer (10 parts of the supernatant with one part of 10 x PBS). Then the pH was adjusted to 7.5 by adding 1.0 N NaOH.

After cooling the solution on ice, 0.227 g of ammonium sulphate per mL solution were slowly added in order to obtain 45% of the saturation concentration of ammonium sulphate. After stirring for 30 min the solution was stored at 4°C over night.

On the next day the tube was centrifuged at 4000 rpm for 15 min. The pellet (IgG) was resuspended in a small volume of PBS and dialyzed against PBS in order to remove ammonium sulphate residues.

5.5.2 Determination of the concentration of the isolated antibodies

The concentration of the isolated antibodies was determined with the Bradford assay as described in 5.4.1. However, for calibration standard solutions of human γ-globulin in the range from 25 to 10000 μg/mL PBS were used.

5.6 Production and isolation of chicken anti-sesame antibodies (IgY)

A chicken was immunized with a sesame protein extract (1 mg/mL) by M. Hermann (Institute for Molecular Pathology, University of Vienna, Austria). The chicken was immunized five times in intervals of one month. The egg laid one week after the last immunization was used for isolating the antibodies.

5.6.1 Isolation of the IgY fraction from the egg yolk

To isolate the antibodies from the egg yolk, the caprylic acid/ammonium sulphate precipitation method described in 5.5.1 was slightly modified. First egg white and yolk were carefully separated. The yolk was washed with H_2O_{dd}. After removing the vitelline membrane with a pair of tweezers the yolk was mixed with an equal volume of PBS. The emulsion was shaken at 900 rpm for 30 min and then centrifuged at 4000 rpm for 15 min. The supernatant was further centrifuged at 10000 rpm for 5 min. To the supernatant, the 4-fold volume of acetate buffer was added followed by adjusting the pH to 4.5 with 0.1 N NaOH. The rest of the steps were carried out as described in 5.5.

5.7 Enzyme linked immunosorbent assay (ELISA)

5.7.1 Reagents

- **Washing buffer:** A concentrate was prepared by dissolving 9.36 g $NaH_2PO_4 \cdot 2H_2O$, 74.76 g $Na_2HPO_4 \cdot 2H_20$, 51.0 g NaCl and 30 mL Tween 20 in 1 L H_2O_{dd}. The solution was stored at 4°C. In order to be used for washing the microtiter plates during the ELISA, 30 mL of the concentrate were diluted with 1.8 L H_2O_{dd}.

- **Coating buffer:** 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3 were dissolved in 1 L H_2O_{dd}. The solution was stored at room temperature.

- **Blocking solution:** The blocking solution, 2% (w/v) casein in PBS, was prepared by adding 2 g of casein to 100 mL PBS. The solution was stirred for 15 min and then filtered through a Whatman filter.

- **Citrate buffer:** 46.04 g potassiumdihydrogencitrat and 0.10 g sorbic acid were dissolved in 1 L H_2O_{dd}. The solution was stored at room temperature.

- **Substrate:** 0.375 g tetramethylbenzidine were dissloved in 5 mL dimethyl sulfoxide and 20 mL methanol. The solution was stored in a dark bottle at 4°C.

- **Substrate solution:** 25 ml of the citrate buffer, 500 μL of the substrate and 100 μL of 1% H_2O_2 were mixed. Since the solution is light-sensitive, it was prepared immediately before using it.

5.7.2 Instruments

All ELISA experiments were carried out in F96 nunc-Immuno plates from Nunc A/S (Roskilde, Denmark). The experiments were carried out either in triple or fourfold determination. During all incubation steps the plate was covered with aluminium foil and shaken on the micro plate shaker (IKA MTS 4). Between the incubation steps the plate was washed 3 times with 300 μL of washing buffer/well with the ImmunoWash (model 1575, BioRad Laboratories GmbH, Vienna Austria). The optical density was measured at 450 nm with the micro plate reader (model 680 XR, BioRad). Standard curves were established with the program Sigma-Plot 10.0. Non-linear regression was carried out using a sigmoid 4-parameter logistic function.

5.7.3 Sandwich ELISA

Antibodies:

- Polyclonal antibodies from rabbit (for preparation and purification see 5.5). The antibodies were stored at -20°C. After defreezing, the IgG fraction was stored at 4°C and could be used for weeks.

- Polyclonal antibodies from chicken (for preparation and purification see 5.6). The antibodies were stored at -20°C. After defreezing, the IgY fraction was stored at 4°C and could be used for weeks.

- Enzyme labeled goat anti-rabbit antibodies: ImmuoPure antibodies, host animal: goat, antigen: rabbit IgG (H+L), enzyme: horseradish peroxidase. Pierce (Thermo Fisher Scientific, Rockford, IL, USA). The antibodies were diluted with 50% glycerol and stored at -20°C.

Procedure

First, 200 μL of IgY solution, diluted 1:25000 in the coating buffer, were added to each well of the plate. The plate was covered with parafilm and incubated at 4°C for 16 hours.

After a washing step, 300 μL of the blocking buffer were added to each well. The incubation time was 60 min. After washing the plate, 200 μL/well of sesame protein standards (from 10 ng/mL to 10000 ng/mL) or the sample extract were added. The incubation time was 30 min.

After a washing step, 200 μL/well of the secondary antibody (IgG), diluted 1:5000 in PBS, were loaded and incubated for 30 min. After a washing step, 200 μL of the solution containing the enzyme labeled goat anti-rabbit antibodies, diluted 1:60 000 in PBS, were filled into each well. The incubation lasted 1 h. After washing the plate, 200 μL of the substrate solution were added and incubated for about 10 to 15 min (until an intense blue color was obtained). The reaction was stopped by adding 100 μL 1 M H_2SO_4.

Matrix effect

No matrix effect was found when analyzed by Gerda Redl at a 1:20 (1 mL plus 19 mL) matrix dilution in PBS. To check lower dilutions like 1:1 (1 mL plus 1 mL), 1:5 (1 mL plus 4 mL) and 1:10 (1 mL and 9 mL), an ELISA was carried out using a sesame calibration curve (10 ng/mL to 10000 ng/mL) in PBS. The unspiked commercial food products were extracted like described in 5.2 and stored at -20°C until they were used. The matrices were diluted in PBS and loaded in quadruplicates on the plate.

5.7.4 Competitive ELISA

Antibodies:

- Polyclonal antibodies from chicken (for isolation and purification see 5.6). The antibodies were stored at -20°C in aliquots of 200 μL. After defreezing the antibodies, they could be used 4 to 5 days stored at 4°C

- Enzyme labeled rabbit anti-chicken: ImmunoPure antibodies, host animal: rabbit, antigen: chicken IgY (H+L), enzyme: horseradish peroxidase (Pierce). The antibodies were dissolved with 50% glycerol and stored at -20°C.

Procedure

The plate were coated with 200 μL/well of sesame protein extract, diluted 1:6000 in coating buffer. The plate was covered with parafilm and incubated at 4°C overnight. After washing the plate, free binding sites were blocked with a 2% casein solution in PBS for 15 min. After a washing step, 50 μL/well of sesame standard (from 5 ng/mL to 10000 ng/mL in PBS) and 100 μL/well of IgY antibodies, diluted 1:10000 in PBS, were loaded. After an incubation period of 45 min the plate was washed. Next, 200 μL/well of enzyme labeled rabbit anti-chicken antibody, diluted 1:30000 in PBS, were added and the plate incubated for 1 h. After a washing step, 200 μL of the substrate solution was filled into each well. The reaction was stopped with 100 μL of 0.5 M H_2SO_4.

Cross reactivity test

Almond, sunflower, rye, Brazil nut, walnut, wheat, rice, honey, poppy seed, chocolate, oat, peanut and hazelnut were tested. The protein fraction was extracted as described in 5.2. The protein concentration was determined with the Bradford assay (see 5.4.1). Protein extracts and sesame standards were diluted in PBS and loaded onto the microtiter plate. The ELISA was carried out as described above. To compare results obtained with different plates, the OD values were normalized. For normalization, B_0 and NSB were measured on each plate. In order to determine B_0, instead of loading 50 μL of the sample or sesame protein standard, 50 μL of PBS and 100 μL of the diluted primary antibody solution were loaded in 6 to 9 wells. In order to determine the NSB value, instead of 50 μL of the sample or the sesame protein standard and 100 μL of the primary antibodies, 150 μL PBS were added in 6 to 9 wells.

5.7.5 Determination of the recovery of sesame

Three commercial blank food products (whole wheat cookies, muesli and rusk) and homemade blank whole wheat bread were spiked as described in 5.3. The proteins were extracted as described in 5.2. The nonfreezed extracts were diluted with PBS, the dilution factor depending on the spike level (see Table 29).

Table 29: Dilution of the sesame spiked extracts

Spike level [%]	Dilution factor
1	1:500
0.5	1:100
	1:50
0.1	1:50
	1:20
0.05	1:50
	1:20
0.01	1:10
	1:5
0.005	1:5
	1:1
0.001	1:1

The ELISAs were carried out as described in 5.7.3 and 5.7.4. For quantification, the OD value closest to the inflexion point of the calibration curve was used.

5.8 DNA extraction

5.8.1 DNA extraction with the CTAB method

Reagents:

- **CTAB extraction solution:** The extraction solution contained 20 g/L cetyltrimethyl-

ammonium bromide (CTAB), 1.4 M sodium chloride, 0.1 M Tris(hydroxymethyl)-aminomethane (Tris) and 0.02 M ethyldiaminetetraacetic acid. The pH was adjusted to pH 8 with HCl.

- **CTAB precipitation solution:** The solution was prepared by adding 0.04 M sodium chloride to 5 g/L CTAB.
- 1.2 M sodium chloride - solution
- chloroform
- 70% (v/v) ethanol
- isopropanol
- hydrochloric acid
- **proteinase K solution:** 100 mg of proteinase K were dissolved in 4 mL 50% glycerol, 10 mM Tris-HCl, adjusted to pH 8
- **RNase solution:** the stock solution (29 mg/mL) delivered from Sigma-Aldrich was diluted to a concentration of 20 μg/mL with 50% glycerol, 10 mM Tris-HCl, pH 8
- H_2O, bidistilled and autoclaved

Extraction of DNA

About 5 to 10 g of a food sample were grounded in a small kitchen blender. An aliquot of 90 to 110 mg was weighed in and mixed with 500 μL of the CTAB extraction solution. The mixture was incubated at 65°C for 30 min. Every 10 min the suspension was vortexed properly. After 15 μL of the proteinase K solution were added a further incubation step (65°C, 60 min) was carried out. Then, 20 μL of RNase solution were added. The mixture was vortexed properly and incubated at room temperature for 2 min. After centrifuging at 10000 rpm for 5 min the supernatant was transferred into a new microreaction tube and 200 μL chloroform were added. After vortexing for 1 min the mixture was centrifuged for about 5 min until the phases were separated. The aqueous

phase was transferred into a new microreaction tube. The solution was mixed with 2 parts by volume of CTAB precipitation solution. After a further incubation step of 60 min at room temperature, the solution was centrifuged for 5 min. The supernatant was removed and the precipitate dissolved in 350 μL of 1.2 M sodium chloride solution. After adding 350 μL chloroform the mixture was vortexed for 1 min and centrifuged until phase separation. The aqueous phase was transferred into a new microreaction tube and mixed with 0.6 parts by volume of isopropanol. After centrifuging for 5 min, the supernatant was removed and the sediment dissolved in 500 μL of ice cold 70% (v/v) ethanol. After vortexing and a further centrifugation step of 5 min the supernatant was removed with a pipette. The DNA pellet was dried at room temperature over night and then dissolved in either 50 or 100 μL H_2O_{dd}. After determining the concentration and the purity (see 5.8.3), the DNA extract was stored at -20°C. All DNA extractions were carried out in triplicates.

5.8.2 DNA extraction with the QIAmp Stool Kit

Reagents contained in the kit:

- QIAmp Mini Spin Columns
- Collection tubes (2 mL)
- InhibitEX Tablets
- Buffer ASL
- Buffer AL
- Buffer AW1 (concentrate)
- Buffer AW2 (concentrate)
- Buffer AE
- Proteinase K

- Ethanol 96%

Protocol

About 5 to 10 g of the food were ground in a kitchen blender. 180 to 220 mg of the sample were weighed in into a 1.5 mL microreaction tube. 1.6 mL Buffer ASL were added and the mixture vortexed for 1 min until the sample was well homogenized. The homogenate was centrifuged at full speed (14000 rpm) for 1 min and afterwards 1.4 mL of the supernatant were transferred into a new microreaction tube. The pellet was discarded. After adding one InhibitEX Tablet, the solution had to be vortexed until the tablet was completely suspended. After an incubation step for 1 min the sample was centrifuged at full speed for 3 min and the supernatant was transferred into a new microreaction tube and centrifuged again for 3 min at 14000 rpm. In a new microreaction tube, 600 μL of the supernatant were mixed with 25 μL proteinase K solution. 600 μL Buffer AL were added and the mixture vortexed for 15 s. A further incubation step for 15 min at 70°C was carried out. Then 600 μL of ethanol were added to the lysate and the mixture vortexed properly. 600 μL of the lysate were loaded onto a QIAmp spin column provided in a 2 mL collection tube. After a centrifugation step (1 min at 14000 rpm) the QIAmp spin column was transferred into a new 2 mL collection tube and a second aliquote (600 μL) of the lysate was added to the QIAmp spin column. After centrifuging the QIAmp spin column was transferred into a new collection tube; a third (600 μL) aliquot was added and centrifuged again. After the three centrifugation steps the filtrate in the collection tube was discarded. The QIAmp spin column was transferred into a new 2 mL collection tube. After 500 μL Buffer AW2 were added, a further centrifugation step was carried out at full speed for 3 min. The filtrate in the collection tube was discarded and the QIAmp spin column placed into a 1.5 mL microreaction tube. 200 μL Buffer AE were added and the mixture incubated for 1 min at room temperature. After centrifuging for 1 min the eluted DNA was stored at -20°C. All DNA extractions were carried out in duplicates.

5.8.3 Determination of the yield and quality of DNA extracts

The concentration and purity of the extracted DNA was determined by measuring the absorbance at 260 nm and 280 nm.

The obtained extracts were extracts diluted in micro cuvettes, either 1:5 with H_2O_{dd} for samples extracted by CTAB method or with Buffer AE for samples extracted by QIAmp stool kit to get a volume of 80 μL . As blank, H_2O_{dd} (CTAB method) or AE Buffer diluted 1:5 with H_2O_{dd} (QIAmp stool kit) was used.

The DNA concentration was calculated according to the equation

$$A_{260} \cdot F \cdot DF = c_{DNA} \tag{7}$$

The quality of the DNA extract was calculated as:

$$\frac{A_{260}}{A_{280}} = Q_{DNA} \tag{8}$$

The ratio should be between 1.8 and 2.0.

c_{DNA}...concentration of the DNA [μg/mL]

A_{260}...absorbance at 260 nm

A_{280}...absorbance at 280 nm

F...multiplication factor 50 for dsDNA

DF...dilution factor

Q_{DNA}...quality of DNA

5.9 Real-time PCR method

The real-time PCR method used in the present diploma thesis is part of a duplex assay developed by Schöringhumer et al. [41], allowing the simultaneous detection of trace amounts of sesame and hazelnut in food products. In the present diploma thesis the

real-time PCR method was used as singleplex PCR to detect sesame in food.

5.9.1 Reagents:

- **IQ Supermix:** The supermix consisted of 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 1.6 mM dTNPs, 50 units/mL iTaq DNA polymerase and 6 mM $MgCl_2$.

- **Taqman probe:** The TaqMan probe was synthesized by Metabion International AG (Martinsried, Germany) and was labeled with the reporter dye FAM (6-carboxyl-fluorescein) on the 5' end and the black hole quencher BHQ on the 3' end. The lyophilisate was dissolved in H_2O_{dd} and stored at -20°C.

- **Primers:** The forward and the reverse primer were designed with the software Beacon Designer 5.1 to be specific for a short sequence of the gene coding for the major allergen Ses i 1. With the Blast program no homology to other important food products was found. The primers were synthesized by Sigma Genosys (Steinheim, Germany). After dissolving the primers in H_2O_{dd} they were diluted 1:10 and filled into 200 μL microreaction tubes. The primers were stored at -20°C until used. The sequences of the primers and the probe and the optimal annealing temperatures are listed in Table 30.

Table 30: Sequence and optimal annealing temperature for primers and probe

Primer/probe	Sequence 5'-3'	Tm(°C)	Product size (bp)
TaqMan Ses i 1	FAM-CACCCTCCTGCTGCTGCTGCC-BHQ1	66.5	
Primer Ses i 1 forward	TGAGGAACGTGGACGAGAG	56.5	
Primer Ses i 1 reverse	CCCTAGCCCTCTGGTAAACC	57.1	117

5.9.2 Protocol

PCR runs were carried out in 96 well PCR plates (BioRad) in the iCycler thermocycler equipped with the IQ 5 multicolor real time PCR detection system (BioRad). The total reaction volume was 25 μL. All experiments were carried out in duplicates. In addition, two negative controls were run on each plate.

Real-Time PCR method used in the present diploma thesis

Each well contained 12.5 μL IQ Supermix, 7.5 μL mastermix and 5 μL of the template DNA (20 ng/μL). The mastermix was prepared by mixing 500 nM primer forward, 500 nM primer reverse, 200 nM of the TaqMan probe and H_2O_{dd}. The pipetting scheme is shown in Table 31.

A two-step real-time PCR program was used. The initial denaturation was carried out at 95°C for 3 min. Then 40 cycles were run, each cycle consisting of denaturation at 95°C for 10 s, followed by primer annealing and elongation at 54°C for 25 s.

Table 31: Pipetting scheme

	Concentration	Volume [μL]
IQ supermix		12.5
Ses i 1 primer forward	10 μM	1.25
Ses i 1 primer reverse	10 μM	1.25
Ses i 1 TaqMan probe	10 μM	0.5
DNA template	20 ng/μL	5.0
H_2O_{dd}		4.5
Total		25

5.9.3 Analysis of spiked samples

The four blank food matrices spiked with sesame (see 5.3) were extracted with the CTAB method or the QIAmp Stool Kit as described in 5.8. The DNA extracts were loaded in

concentrations of 20 ng/μL and were run in duplicates. In every PCR run two positive and two negative controls were run simultaneously to check the PCR performance.

The mastermix contained 27.5 μL primer forward, 27.5 μL primer reverse, 11 μL Taqman probe and 99 μL H_2O_{dd}. Into each well 7.5 μL mastermix, 12.5 μL Supermix and 5 μL template were loaded. The PCR program is described in 5.9.2.

5.9.4 Inhibition control

In the case of muesli, an inhibition control was carried out. One μL of DNA extract (100 ng/μL) from muesli which had been previously spiked with 1%, 0.1%, 0.01% or 0.001% sesame was loaded onto the plate and incubated at room temperature overnight. On the next day, 5 μL of sesame DNA (20 ng/μL) were added and the PCR run carried out as described in 5.9.2.

5.10 Chemicals

- Ammonium sulphate, Sigma
- Bovine serum albumin (BSA), Sigma
- Brilliant Blue G, Sigma-Aldrich
- Casein from cow milk, Sigma
- Dimethyl sulfoxide (DMSO), Sigma
- Acetic acid, Fluka
- Ethanol, Österreichische Alkoholhandels GmbH
- γ-Globulin (human), Sigma
- HCl, 37%, Merck
- H_2O_2, Sigma

- H_2SO_4, Merck
- Potassium dihydrogen citrate, Fluka
- $KH_2PO_4 \cdot 3H_2O$, Merck
- $K_2HPO_4 \cdot H_2O$, Merck
- Methanol, VWR
- NaCl, Merck, Sigma-Aldrich
- $NaH_2PO_4 \cdot 2H_2O$, Merck
- Na_2HPO_4, Merck
- Na_2CO_3, Sigma
- $NaHCO_3$, Merck
- NaOH pellets, Merck
- Sodium-acetate$\cdot 3H_2O$, Fluka
- NaN_3, Merck
- n-Hexane, Roth
- Octanoic acid, Merck
- Phosphoric acid, Merck
- Sorbic acid, Sigma
- Tetramethylbenzidine (TMB), Sigma
- Tween 20, Sigma
- Cetyltrimethylammoniumbromide (CTAB), Sigma-Aldrich
- Chloroform $\geq$ 99%, Roth

- Primer, Sigma-Genosys
- IQ^{TM} Supermix, BioRad
- Proteinase K, Sigma-Aldrich
- RNase, Sigma-Aldrich
- TaqMan probe, Metabion
- Tris (Tris(hydroxymethyl)aminomethane) TRIZMA base, Sigma-Aldrich
- QIAmp Stool Kit, Qiagen
- Ethylenediaminetetraacetic acid (EDTA), Merck

5.11 Equipment

Laboratory equipment

- Analytical balance: Mettler AT 400
- Pipettes: Eppendorf research 5000
  Eppendorf 100-1000 μL
  Gilson P200
  BioRad 0.5-10 μL
  BioRad 2-20 μL
  BioRad 20-200 μL
  BioRad 100-1000 μL
  Brand Transferpipette-8 20-200 μL
- Vortex: Janke & Kunkel, IKA-Labortechnik, VF2
- Centrifuge: Sigma 4 K 10 with Rotor 11140 Eppendorf Centrifuge 5452
- pH meter:Metrohm 691

- Drying oven, Memmert Modell 500
- Ultrasonic bath: Bandelin SONOREX SUPER RK103H
- Micro plate Shaker: MTS4, IKA-Labortechnik
- Photometer: Genesys 10 UV, Thermo Spectronic
- Ultraturrax T25, Janke & Kunkel, IKA-Labortechnik

ELISA-equipment

- ImmunoWash 1575, BioRad
- Microplate Reader 680 XR, BioRad

PCR-equipment

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

6 Conclusion

Sesame is one of the allergenic foods which has to be labeled in the food ingredient list according to EU regulations. Recently, in our research group three analytical methods have been developed allowing the detection of traces of sesame in food. These methods include a sandwich ELISA, a competitive ELISA and a real-time PCR method. The aim of the diploma thesis was to spike different food matrices with known amounts of sesame and to analyse the spiked samples with each of the three methods to compare their applicability and performance.

Four food matrices (rusk, whole wheat cookies, whole wheat bread and muesli) were spiked with sesame in concentrations from 0.001% to 1%. The protein fraction of the food samples were extracted with a protein extraction buffer containing Tris and NaCl. DNA was extracted either with the CTAB method or with a commercial DNA extraction kit.

When the protein extract was diluted 1:20 (1 mL + 19 mL), the limit of detection of the sandwich ELISA was found to be 44 ppm. Loading the protein extract diluted 1:1 (1 mL + 1 mL) resulted in a LOD of 4 ppm. The limit of detection of the competitive ELISA was higher. Diluting the food extracts 1:20 resulted in a LOD of 90 ppm, by using the extract diluted 1:1 a LOD of 9 ppm was achieved.

For whole wheat cookies the recoveries obtained with the sandwich ELISA were in the range from 80 to 100%. Only at a sesame concentration of 0.1% sesame, the recovery of sesame was 200%. With the competitive ELISA, recoveries >100% were obtained at lower spiking levels (0.05, 0.005 and 0.001%). With the real-time PCR method, sesame could be detected down to a spike level of 0.005%. The amplification efficiency in whole wheat cookies was 81.4%.

For whole wheat bread, in general too high recoveries (about 200%) were obtained with the sandwich ELISA. In contrast, with the competitive ELISA recoveries of 100% were achieved for the 0.005 and 0.001% spike level. However, for the other spike levels the recovery was less than 100%. Extracting a high amount of pure DNA from whole wheat bread with the CTAB method proved to be difficult. Low amounts of DNA were also obtained with the QIAmp Stool Kit. The LOD of the PCR method in whole wheat bread was determined to be 0.05% sesame.

In the case of rusk, for all spike levels recoveries > 100% were obtained with the sandwich ELISA. With the competitive ELISA, at lower spike levels even recoveries up to 300% were obtained. DNA extraction with the CTAB method yielded DNA of sufficient amount and purity. Sesame DNA could be amplified down to a sesame concentration of 0,005%, the amplification efficiency was 76%.

The analysis of sesame spiked muesli with the sandwich ELISA resulted in too high recoveries. In contrast, at higher spike levels recoveries of 100% were obtained with the competitive ELISA, only at a spike level of 0.001% the recovery was 300%. DNA extraction with the CTAB method yielded high DNA concentration and purity. Nevertheless, sesame DNA could not be amplified in any of the spiked samples. Inhibition control tests indicated that the results were not caused by enzyme inhibition. When DNA was extracted with the QIAmp Stool Kit, sesame DNA could be amplified down to a sesame concentration of 0.5% sesame.

The ELISA methods proved to be better applicable to the detection of traces of sesame in food. The LOD of both ELISA methods was found to be lower than the LOD of the PCR method. In addition, even from complex food matrices proteins could be extracted without any problems. Compared to the competitive ELISA, the advantages of the sandwich ELISA are the absence of matrix effects and its lower limit of detection.

Appendix

List of abbreviations

AG	antigen
B_0	blank
bp	base pair
c	concentration
Ct	treshold cycle
Da	dalton
DBPCFC	double blind placebo controlled food challenge
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotidtriphosphate
ELISA	enzyme linked immunosorbent assay
FRET	flourescence resonance energy transfer
H_2O_{dd}	bidistilled water
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
LOD	Limit of detection
LOQ	Limit of quantification
NSB	non specific bound
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
ppb	part per billion
ppm	parts per million
QCM	quartz crystal microbalance
rpm	rounds per minute
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
SPT	skin prick test
T_m	melting temperature

List of Tables

1 Symptoms of food allergy . . . 3

2 Protein concentration of food extracts tested for cross reactivity . . . 38

3 Recovery of sesame in spiked whole wheat cookies obtained with the sandwich ELISA . . . 43

4 Recovery of sesame in spiked whole wheat cookies obtained with the competitive ELISA . . . 44

5 Recovery of sesame in spiked whole wheat cookies obtained with sandwich and competitive ELISA . . . 45

6 Recovery of sesame in spiked whole wheat bread obtained with the sandwich ELISA . . . 45

7 Influence of the whole wheat bread matrix on the recovery of sesame . . . 47

8 Recovery of sesame in spiked whole wheat bread obtained with the competitive ELISA . . . 47

9 Recovery of sesame in spiked whole wheat bread obtained with sandwich and competitive ELISA . . . 48

10 Recovery of sesame in spiked rusk obtained with the sandwich ELISA . . . 49

11 Recovery of sesame in spiked rusk obtained with the competitive ELISA . 49

12 Recovery of sesame in spiked rusk obtained with sandwich and competitive ELISA . . . 50

13 Recovery of sesame in spiked muesli obtained with the sandwich ELISA . . 51

14 Recovery of sesame in spiked muesli obtained with the competitive ELISA 51

15 Recovery of sesame in spiked muesli obtained with sandwich and competitive ELISA . . . 52

16 Recovery of sesame in four spiked food matrices obtained with the sandwich ELISA . . . 53

17 Recovery of sesame in four spiked food matrices obtained with the competitive ELISA . . . 54

18 Limit of detection of the sandwich ELISA 58
19 Limit of detection of the competitive ELISA for sesame in whole wheat cookies 59
20 Limit of detection of the competitive ELISA for sesame in whole wheat bread 59
21 Limit of detection of the competitive ELISA for sesame in muesli 60
22 Limit of detection of the competitive ELISA for sesame in rusk 60
23 Comparison of the LOD for sesame of both ELISA methods 61
24 Concentration and purity of DNA extracted from spiked whole wheat bread by the CTAB method and by QIAmp Stool Kit 63
25 Concentration and purity of the DNA extracted from spiked rusk 64
26 Concentration and purity of the DNA extracted from spiked muesli by the CTAB method and by QIAmp Stool Kit 65
27 Ct values obtained for the spiked whole wheat bread 68
28 Ct values obtained for sesame spiked muesli 70
29 Dilution of the sesame spiked extracts 82
30 Sequence and optimal annealing temperature for primers and probe 87
31 Pipetting scheme 88

List of Figures

1 Stability and allergenicity of proteins 4
2 Principle of the competitive ELISA 24
3 Principle of the sandwich ELISA 25
4 Denaturation of double-stranded DNA in two single strands 30
5 Annealing of primers on the single strands 30
6 Elongation of the DNA templates by Taq DNA polymerase 30
7 Two phases of real-time PCR 33
8 Results of cross reactivity tested for almond, honey and sunflower 39
9 Results of cross reactivity tested for rye, Brazil nut and wheat 39
10 Results of cross reactivity tested for peanut and poppy seed 40
11 Results of cross reactivity tested for chocolate, oat and hazelnut 40
12 Optimization of the sesame protein solution volume for sandwich ELISA . 41
13 Influence of the whole wheat bread matrix on the calibration curve 46
14 Influence of the concentration of the enzyme labeled antibody (anti-IgG*) on the calibration curve 55
15 Influence of the concentration of the enzyme labeled antibody (anti-IgG*) on the calibration curve (2) 55
16 Calibration curve for sesame obtained after optimization of the sandwich ELISA 56
17 Influence of the concentration of the IgY on the calibration curve 57
18 Amplification of sesame DNA with different primer concentrations 66
19 Standard curve obtained by amplifying DNA extracted from spiked whole wheat cookies 67
20 Amplification of sesame DNA and DNA extracted from spiked whole wheat bread 68
21 Standard curve obtained by amplifying DNA extracted from spiked rusk . 69
22 Amplification of DNA extracted with QIAmp Stool Kit from spiked muesli 70

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German abstract

Vergleich von einem Sandwich und einem kompetitivem ELISA und einer real-time PCR Methode zur Detektion von potentiell allergenem Sesam in Lebensmitteln

Lebensmittelallergien treten in unserer Gesellschaft immer häufiger auf. Bereits 6-8% der Kinder leiden an einer Lebensmittelallergie. Symptome reichen von leichten Beschwerden wie Juckreiz oder Schnupfen bis hin zu Verdauungsbeschwerden und schlimmstenfalls zum anaphylaktischen Schock. Die einzige Möglichkeit eine allergische Reaktion zu vermeiden, ist der strikte Verzicht auf das jeweilige Lebensmittel. 14 Nahrungsmittelzutaten, die besonders häufig Allergien auslösen, müssen auf der Verpackung deklariert werden (EU-Richlinie (2007/68/EG). Auch Sesam gehört zu dieser Gruppe, denn bereits kleinste Spuren können eine allergische Reaktion auslösen. Es wurden bereits einige Methoden zur Detektion von allergenen Lebensmitteln entwickelt. ELISAs und Polymerase Kettenreaktion sind bislang die Methoden der Wahl. Beide weisen einige Vorsowie auch einige Nachteile auf. Es gibt jedoch kaum Studien, in denen die Methoden direkt miteinander verglichen wurden. Ziel dieser Arbeit war es, einen Sandwich und einen kompetitiven ELISA, und eine real-time PCR Methode zur Detektion von Spuren von Sesam in Lebensmitteln zu vergleichen.

Um die Methoden zu vergleichen, wurden 4 Lebensmittelmatrizes (Vollkornkeks, Vollkornbrot, Zwieback und Müsli) mit gemahlenen Sesamkörnern in verschiedenen Konzentrationen (0.001% - 1%) gespikt. Die Proteine wurden mit einem Extraktionspuffer, DNA mittels der CTAB-Methode oder dem QIAmp Stool Kit extrahiert. Während es bei der Proteinextraktion keine Schwierigkeiten gab, bereitete die Extraktion der DNA aus Vollkornbrot größere Probleme. Im Sandwich ELISA wurde bei einer 1:1 Verdünnung des Extrakts eine Nachweisgrenze (S/N=3) von 4 ppm und im kompetitiven ELISA von 9 ppm erreicht. Mit der PCR Methode wurde in Vollkornkeks und Zwieback eine Nachweisgrenze von 50 ppm erreicht. In Vollkornbrot betrug die Nachweisgrenze 500 ppm und in Müsli nur 5000 ppm.

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