Universität für Bodenkultur Wien

Interuniversitäres Department für
Agrarbiotechnologie Tulln, Analytikzentrum

M A S T E R A R B E I T

PERFORMANCE CHARACTERISTICS OF DIFFERENT ANTIBODIES FOR THE DETECTION OF FOOD ALLERGENS

zur Erlangung des akademischen Grades
Diplomingenieur (Dipl.Ing.)
der Universität für Bodenkultur Wien

Verfasser:	Denise Huber Bakk. Techn.
Matrikelnummer:	0271543
Studienrichtung:	Biotechnologie (066 418)
Betreuer:	Univ.Prof. Dipl.-Ing. Dr.techn. Rudolf KRSKA
Mitbetreuer:	Ass.Prof. Dipl.-Ing. Dr.nat.techn. Sabine BAUMGARTNER

Tulln, 16.03.2009

Acknowledgements

First of all I want to thank Sabine and Judith for their enduring support during my master thesis. Thanks to their good advice, my work concentrates on various aspects of analytical biochemistry and the characterisation of antibodies. I have acquired great knowledge with regard to scientific and administrative issues.
Rudolf Krska provided me with the opportunity to work at the IFA Tulln and its biochemistry team with whom I enjoyed numerous scientific discussions.
My studies were part of the PilotLaboratory “Rapid Test Systems for Allergenic Food Contaminants” of the Christian Doppler Research Association, which I am grateful to for the equipment I needed for my studies.
I also like to thank Marcella, who immunized the animals at the Veterinary University of Vienna.
Finally, I am deeply thankful to my family, my fiancé, Robert, and his family. Without my parents I would not have had any opportunity to study at the BOKU. Special thanks to Martha who checked the paper and the thesis for shortcomings in English. Each of them gave his/her best to help me complete this work.

Kurzfassung

In dieser Masterarbeit soll zum Einen gezeigt werden, ob sich polyklonale Antikörper von Hasen und Hühnern für den Einsatz in der Immunanalytik zum Nachweis von Lebensmittelallergenen eignen. Untersucht wurden Antikörper gegen Erdnuss, Haselnuss, α-Casein und Soja. Mittels ELISA, Elektrophorese und Western Blot wurden die Antikörper alleine und in Wechselwirkung miteinander untersucht. Es wurde bestätigt, dass Antikörper von Hasen um ein Zehnfaches sensitiver sind als Antikörper von Hühnern. Beide sind geeignet, als Bestandteil eines Immuntests in der Analytik eingesetzt zu werden.

Die aus den Hühnereiern gewonnenen Antikörper wurden mittels Affinitätschromatographie, die Antikörper aus den Hasenseren mittels Ammoniumsulfatfällung gewonnen, gereinigt und aufkonzentriert. Die Antikörper gegen Haselnuss, Erdnuss, α-Casein und Soja wurden auf Kreuzreaktivitäten getestet. Anhand unterschiedlicher Proben konnte gezeigt werden, dass die Antikörper gegen Erdnuss- und Sojaproteine keine Kreuzreaktivitäten aufweisen, während die Antikörper gegen Haselnuss eine sehr geringe Affinität gegenüber Erdnuss und Soja zeigten. Die Antikörper gegen α-Casein erkannten β- und κ-Casein zu einem geringen Prozentsatz, während α-Lactalbumin und β-Lactoglobulin keine Kreuzreaktivität verursachten.

Zum Anderen wurde die Effektivität von Blocklösungen untersucht. Das Blocken im Immuntest dient zur Vermeidung unspezifischer Reaktionen auf der Microtiterplatte und soll die Sensitivität des Antikörpers erhöhen. Die erhaltenen Testergebnisse waren in Abhängigkeit der Blocklösungen nicht wiederholbar. Daher wurden die bis dato verwendeten Blocklösungen in Frage gestellt und mit den üblichen Proteinblockern verglichen. Drei große Gruppen, Proteine, Kohlehydrate und synthetische Stoffe wurden in Bezug auf ihre Blockeffizienz bei unterschiedlichen Bedingungen getestet. Es wurden die Temperatur, die Zeit und die Pufferlösung variiert. Der Kohlehydrat-Blocker Ficoll, gelöst in Carbonatpuffer, erzielte die besten Ergebnisse. Er bietet ein dem meist verwendete Proteinblocker bovine serum albumin (BSA), in Carbonatpuffer gelöst, vergleichbares Bild. Polyvinylalkohol (PVA), in Phosphatpuffer gelöst, wies als einziger synthetischer Stoff eine ausreichende Blockkapazität auf. Während Ficoll und BSA bei Raumtemperatur und zwei Stunden

Inkubationszeit ausreichend geblockt hatten, wurde mit PVA bei 4 °C über Nacht geblockt, um denselben Effekt zu erzielen.
Interessant waren die Anwendungen der beiden Blocker, PVA und Ficoll im ELISA selbst. Mit den Antikörpern gegen α-Casein wurden Matrixeffekte untersucht. Dabei wurden zwei Proben ausgewählt, eine flüssige und einen feste, um herauszufinden, ob die Antikörper trotz ihrer Umgebung in der Matrix unverändert Ergebnisse liefern und alternative Blocklösungen (Ficoll und PVA) einen Einfluss auf die Antikörper im Test haben. Es konnte am Beispiel α-Casein sehr deutlich gezeigt werden, dass beide in der Analytik einsatzfähig sind, aber in den entsprechenden Matrizen validiert werden müssen.

Abstract

The aim of the master thesis is to understand the binding characteristics of the different antibodies and their comparison, regarding their origin and the food allergen detected. Antibodies against peanut, hazelnut, α-casein and soy were dealt with in the studies. Affinity chromatography was used to purify and concentrate the antibodies from eggs and ammonium precipitation was applied for the antibodies from rabbit sera. Enzyme-linked immunosorbent assay (ELISA), electrophoresis and Western Blot were performed to evaluate the antibodies used and to determine cross-reactivities as well as matrix effects. The methods used had to be previously customized and especially the ELISA assays were adjusted for the detection of food allergens in general. For instance, blocking reduces non-specific binding to the microtiter plate to a minimum. For detecting food allergens, the problem with protein blocking solutions is obvious: The blocker might interfere with the antibodies of the assay and lead to false positive results. Therefore, other blocking solutions are greatly needed. There are some alternatives like synthetic blockers or carbohydrates. Comparisons of these different blocking agents, namely proteins, carbohydrates and synthetic blockers, were made at different reaction conditions. The incubation periods and temperatures were varied, as well as the pH. The best combinations were evaluated and compared, in respect of their blocking efficiency. The two best non-proteinaceous blockers, i.e. polyvinylalcohol and Ficoll, were subsequently applied to ELISA tests. The outcome of these studies was highly satisfying: The studies showed that Ficoll and PVA did as well as BSA in buffer solution. Hence, they can be considered as alternative blocking reagents for ELISA, especially for the detection of food allergens. Still, matrix effects occur which need to be checked for each matrix individually in validation studies.

Contents

List of Figures

List of Tables

Abbreviations

BCA	bicinchoninic acid
BSA	bovine serum albumin
DONS	6,6'-deoxy-2-naphthalenesulfonic acid
EDTA	ethylenediaminetetraacidic acid
ELISA	enzyme-linked immunosorbent assay
FPLC	fast protein liquid chromatography
MES	N-morpholino-2-ethanesulfonic acid
MOPS	N-morpholino-2-propanesulfonic acid
NaCl	sodium chloride
PBS	phosphate buffered saline
PEG	polyethylenglycol
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TMB	3,3',5,5' tetramethylbenzidine
UV/VIS	ultraviolet/visible light

1 Introduction

A wide range of people is allergic to various food additives or basic food elements. It often happens that industrially processed food contains traces of allergens which are not marked on the final product. The easiest and most effective way of preventing food allergies, is to forgo the offending food [1]. However, it is becoming more and more difficult to avoid food allergens totally in everyday life. Hence, biochemists have a significant responsibility in the field of analysis. Antibodies are required to find the allergens hidden. The fastest way is to obtain polyclonal antibodies from rabbit or chicken. More time consuming is the production of monoclonal antibodies which can be 10 times more sensitive than the polyclonal antibodies. In the human body, the response of the immune system to food allergens is immune globulin E (IgE). The body regards the food allergen as a foreign invader and stimulates the B-cells to produce plasma cells which secrete the antibody IgE. These antibodies attach to mast cells and if the body is confronted with the same allergen again, the mast cells release granules with cytokines or histamines or other transmitters. This process, known as IgE-mediated allergy, may lead to a series of reactions causing damage to the intestinal morphology, immune function disorders, growth depression and diarrhoea, rashes or even death.

The identification of allergies is as important as the detection of these food allergens in food. There are some common methods in biochemistry in order to analyze the food allergens and their corresponding antibodies. Both are characterized by and used in rapid analytical methods such as an enzyme-linked immunosorbent assay (ELISA). ELISAs are a major tool for biochemists and analysts. The ELISA offers a wide range of possibilities concerning the specific and sensitive reaction mechanism of antibody and antigen interactions. The food allergens chosen were all tested in two different ELISA formats and under different conditions. The antibodies were tested for their sensitivity and compared with each other. In order to ensure the specificity of the assay, the material of the microtiter plate shall not react with the other components involved in the ELISA reaction. Blocking the bottom of the microtiter plate after coating is important for the sensitivity and specificity of the following reactants which shall bind to the coated protein only and not onto the plate. Non-specific binding is detrimental for the assay, in addition saturation of the unoccupied sites must be achieved [2]. The most effective and common blocking solutions consist of bovine serum albumin or casein, in other words proteins in buffer solution. Hence, non-proteinaceous blocking reagents were resorted to the study.

Tween 20, often used as detergent in washing buffers, was tested for its ability in immunoblotting (nitrocellulose membrane) and caused some artefacts, thus leading to a misinterpretation of results [3]. Therefore it cannot be used alone as a blocking agent. Tween 20 and some other detergents were checked for their ability to influence protein coating onto polystyrene microtiter plates [4], since Tween 20 in PBS buffer was reported to prevent non-specific protein binding to polystyrene [5].

Other studies proved that the alternative synthetic blocker PVA reduced non-specific binding in enzyme-linked immunosorbent assays [6], as part of a post-coating solution and for stabilization of the test kits itself [7, 8] as well as for serum pre-incubation of e.g. Luminex assays [9]. The blocking capacity of PVP was demonstrated in [2] and in Western blotting by reducing the background signal without decreasing specific immunoreactivity [10]. Both, PVA and PVP were used in ELISAs for the detection of antibodies bound to virus-like particles. PVA reduced the amount of non-specific binding of antibodies, whereas PVP increased the sensitivity of antibody detection [11, 12]. Additionally, PEG was described as alternative blocker [2]. It was used as a block copolymer in a sandwich immunosorbent assay system with magnetic beads and turned out to be more effective than the conventional protein blocker BSA [13].

When working with food allergens, a more or less universal blocking agent would be of integral importance for the assay development. Despite the efficiency of protein blockers, they are not the first choice in this case as they can possibly interfere with the analyt and the antibody. For example, defatted milk powder would not be used for the determination of any milk allergen in an ELISA. The application of BSA might be critical as well. Although e.g. defatted milk protein and casein proved to be most effective for the blocking of the polystyrene microtiter plates [14], other possibilities must be taken into account, if the allergen α-casein should be measured. Fish gelatine was described as an excellent blocker for nitrocellulose membranes for Western Blot. Hence, it was also tested on polystyrene plates as protein blocker.

In respect of the specific ELISA systems, there are no general categorisations of blocking activity to refer to. The proteins have mainly been chosen after empirical testing and by convenience [14]. The alternative blockers PVA, PVP and PEG [2] were tested in our ELISA system for food allergens, in comparison with conventional protein blockers, BSA and fish gelatine. Besides, carbohydrates i.e. dextrans, Ficoll and trehalose were taken into the series of tests to check their blocking efficiency and to evaluate a possible usage. In the first experiments only the blocking agents were coated onto the plate and their blocking

efficiency was tracked by running simple standard curves on these plates. No dose-dependent signal changes were expected, which proved an efficient blocking strategy. Afterwards the best-suited blockers were checked in two ELISA formats with different polyclonal antibodies for the determination of α-casein and peanut for their suitability.

Cross-reactivity studies for all food allergens mentioned were performed based on two different experiments: The cross-reacting substance can either be treated like a sample therefore the amount of foreign protein determined gives an idea of the percentage of cross-reactivity between the sample and the antibody tested. The cross-reacting substance can also be measured in a standard serial row and compared with the standard curve of the food allergen and its parameters. For α-casein, matrix effects were determined to see the performance characteristics of the antibodies and the influence of the blocking reagent in matrices.

2 Material and Methods

2.1 Reagents and Instrumentation

For the preparation of the IgY-antibodies, polyethylenglycol (M_W~8 kDa) was bought from BioChemika Fluka AG (Buchs, Switzerland). The clean-up of IgY-antibodies was performed with FPLC and UV-detector from Pharmacia GE Health Care (Uppsala, Sweden), using a column, containing 2-mercaptopyridine coupled to sepharose (5 mL) from Amersham (London, Great Britain). The column had a binding capacity of 100 mg IgY-antibody per 5 mL gel. For the preparation of rabbit-antibodies, $(NH_4)_2SO_4$ from J.T. Baker (Deventer, Netherlands) and dialysis tubes (Spectra/Pol molecularporous membrane tubing, diameter of 32 mm, molecular weight cut-off 6-8 kDa) from Spectra Labs (Rancho Dominguez, United States) were used. The centrifuge Allegra™ X-22R was obtained from Beckman Coulter (Fullerton, United States) and allowed to cool the samples during centrifugation. The UV/VIS spectrometer Lambda2S was bought from Perkin Elmer (Waltham, United States). Water was purified by reverse osmosis before use. The folded filters, type 595 ½, diameter of 150 mm and pore size of 4-7 µm, were bought from Whatman Schleicher und Schuell (Dassel, Germany), the glassfiber filters, type MN85/70 BF, diameter of 45 mm and pore size of 0.6 µm, from Millipore (Massachusetts, United States), the cellulose acetate filters with glassfiber prefilters and a pore size of 0.45 µm from Sartorius Stedim Biotech (Aubagne Cedex, France). The BCA Protein Assay Kit was bought from Pierce (Rockford, United States). The whole set-up for electrophoresis and Western Blot, including the gels (NuPAGE, 12% Bis/Tris; used with MES or MOPS running buffer, separation range of 1 – 200 kDa), nitrocellulose membranes, loading buffers (NuPAGE LDS) and chambers (XCell II Blot Modul Novex), was purchased from Invitrogen (Carlsbad, United States). SimplyBlue SafeStain was used for the coomassie blue staining.

Non-binding, medium and high binding 96-well microtiter plates from Greiner Bio One (Kremsmuenster, Austria) were used. The microtiter plate washer, Tecan 96PW™, and the 12-channel ELISA reader, Sunrise™, with Magellan5 software were obtained from Tecan Austria GmbH (Groedig, Austria).

The food allergens were self-prepared because there was no standard material available. Soybean flour (not roasted), α-casein, α-lactalbumin, β-casein, β-lactoglobulin and κ-casein were purchased from Sigma Aldrich (Vienna, Austria). Milk powder was purchased from

Roth (Karlsruhe, Germany). Other foods were bought in a local food store. Zuckertüten were obtained from Auer (Vienna, Austria), Eierbiskotten from Manner (Vienna, Austria), soy milk vanilla from Alpro Soya (Düsseldorf, Germany) and Latella from Tirol Milch (Woergl, Austria). All antibodies were prepared in house, the sera and eggs were collected at the University of Veterinary Medicine in Vienna. The labelled antibodies anti-rabbit-IgG-HRP, anti-chicken-IgG-HRP and anti-mouse-IgG-HRP were obtained from Sigma Aldrich (Vienna, Austria), as well as Ficoll (M_W~400 kDa), polyvinylpyrrolidone (M_W 40-50 kDa), bovine serum albumin (Fraction V, ≥ 96%), fish gelatine (gelatine from cold water fish skin) and polyvinylalcohol (M_W 13-23 kDa). D(+)-trehalose (>99% purity) was purchased from Roth (Karlsruhe, Germany), polyethylenglycol (M_W~20 kDa) from BioChemika Fluka AG (Buchs, Switzerland) and two different dextrans (M_W 40 and 2 000 kDa) from Pharmacia Biotech (Vienna, Austria). The salts for phosphate and carbonate buffers, citric acid and sulphuric acid were bought from Merck (Vienna, Austria). Potassium sorbic acid, polyoxyethylene-(20)-sorbitan monolaurate (Tween20), sodium azide, 3,3',5,5' tetramethylbendizidine (TMB), hydrogen peroxide and dimethyl sulphoxide were bought from Sigma Aldrich (Vienna, Austria). For silver staining and Western blotting further chemicals were needed: From Sigma Aldrich (Vienna, Austria) EDTA, Trisbase, Bis/Tris and Bicine were obtained. From Roth (Karlsruhe, Germany) SDS, MOPS, glycerol, acetone, MES and methanol were purchased. Formaldehyde, acetic acid, glycine, glutardialdehyde, sodium thiosulfate*5 H_2O, sodium acetate and carbonate and silver nitrate were bought from Merck (Vienna, Austria). Ethanol was received from J.T. Baker (Deventer, Netherlands). DONS (6,6'-deoxy-2-naphthalenesulfonic acid) was bought from Fluka Chemika (Buchs, Switzerland).

2.2 Stock solutions and buffers

For the IgY preparation 0.2 M PBS buffer, containing 2 M NaCl, pH 7.5, and IgY buffer, a dilution of 0.2 M PBS buffer to 0.1 M PBS buffer, were used. For the clean-up, three buffers were needed: Buffer A (binding buffer) consisted of buffer B and 0.5 M K_2SO_4, buffer B (elution buffer) of 20 mM Na_2HCO_3, pH 7.5 and buffer C (cleaning buffer) of buffer B and 30% isopropanol. For the rabbit-antibody ammonium sulphate precipitation 50 mM PBS buffer was needed. It was diluted from 0.2 M PBS buffer.

MOPS buffer contained 50 mM MOPS, 50 mM Trisbase, 1 mM EDTA and 0.1% SDS, pH 7.7. A 20 fold MOPS buffer was prepared and stored at 4 °C, it was diluted before use. MES buffer was followed the same protocol, consisting of 50 mM MES, 50 mM Trisbase, 0.1% SDS and 1 mM EDTA, pH 7.3.

Coating buffer (pH 9.6) contained 12 mM sodium carbonate, 38 mM sodium hydrogencarbonate and 0.01% sodium azide. 0.2 M PBS buffer containing 0.36 M NaCl, pH 7.5, and the extraction buffer of 50 mM PBS containing 1 M NaCl, pH 7.6, were made separately. However, 0.2 M PBS buffer was diluted for the application as 10 mM PBS washing buffer, adding 0.1% Tween 20. Blocking solutions were used depending on the studies: 1% blocker in PBS or coating buffer (except for 3% PVP and 5% trehalose). The assay buffer consisted of 50 mM phosphate buffer and 0.1% Tween 20. For the substrate solution 12.5 mL substrate buffer pH 4.0, containing 0.2 M citric acid and 0.01% potassium salt of sorbic acid, were mixed with 2.5 µL hydrogen peroxide (30% w/v) and 100 µL tetramethylbenzidine (TMB) stock solution. TMB stock solution included 1.25% (w/v) tetramethylbenzidine dissolved in 5 mL dimethyl sulphoxide, then adding 25 mL methanol. 1 M H_2SO_4 was used as stop solution.

For silver staining there were several solutions needed: The fixation solution contained 30 mL ethanol and 10 mL acetic acid, filled up with deionised water to 100 mL. The incubation solution consisted of 30% ethanol, 0.05 M sodium acetate, 0.5% glutardialdehyde and 0.8 mM sodium thiosulfate*5 H_2O, filled up to 250 mL with deionised water. The silver staining solution contained 0.8 mM silver nitrate and 0.02% formaldehyde, filled up to 250 mL with water. The developing solution consisted of 0.02 M sodium carbonate and 0.01% formaldehyde, filled up to 300 mL. The stop solution consisted of 1% glycine in deionised water. 10% glycerol solution in deionised water was used for preserving the silver stained gel.

The transfer buffer for the Western Blot consisted of 25 mM bicine, 25 mM Bis/Tris and 1 mM EDTA. Before use, methanol was added, e.g. 50 mL buffer plus 100 mL methanol and 850 mL deionised water. 2% BSA were dissolved in 50 mM PBS buffer for the blocking solution. 0.2 M PBS buffer was used as washing buffer, adding 0.1% Tween 20. 0.01 M Tris/HCl buffer, pH 6.0 was used before starting the colour reaction. Substrate solution consisted of 3.9% (w/v) TMB, 7.2% (w/v) DONS, 10 mL ethanol, 30 mL citrate buffer (0.15 M, ph 5.0) and 5 µl H_2O_2 (added short before use).

2.3 Affinity chromatography

Affinity chromatography is a chromatographic tool to separate biochemical mixtures. The term covers many similar methods based on a unique principle: the reversible interaction between a protein and the immobilized ligand. For our purposes, it purifies and concentrates the IgY-antibodies [15] based on protocols [16, 17, 18]. The columns consisted of 2-mercaptopyridine coupled to sepharose [19]. The IgY-antibodies were bound to mercaptopyridine via thiol-bindings. Sepharose was the carrier material. Three different buffer solutions were applied: to bind the antibodies to the column (binding buffer containing carbonate buffer and potassium sulphate), then to elute them with a different buffer solution (carbonate buffer, pH 7.5) and to clean the column with the cleaning buffer. The antibodies then bind to 2-mercaptopyridine and the remaining substances are washed out. Finally, the antibodies can be eluted and collected in a purified and concentrated form.

2.4 Ammonium sulphate precipitation

A useful method of concentration and an initial step in purification is ammonium sulphate precipitation. Salting out of proteins by using ammonium sulphate is one of the most widely known methods of purifying and concentrating enzymes or antibodies, particularly at the laboratory scale. Increases in the ionic strength of the solution cause a reduction in the repulsive effect of charges between identical molecules of a protein. It also reduces the forces holding the solvation shell around the protein molecules. As soon as these forces are sufficiently reduced, the protein will precipitate. Ammonium sulphate is both convenient and effective because it is highly soluble, cheap, lacks toxicity to most proteins and has a stabilizing effect. The protein concentration, temperature and pH are kept constant. The concentration of the salt needed to precipitate a protein will vary depending on its concentration. However, fractionation of protein mixtures by the stepwise increase in the ionic strength can be a very effective way of a partial purification.
In case of our rabbit-antibodies, the clean-up was started with a precipitation of 30% ammonium sulphate and repeated with 40%. Then the antibodies precipitated and were collected.

2.5 Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method to separate proteins according to their molecular weight as well as their electrophoretic motility. It allows the characterization of proteins, regarding their composition and their purity [20]. The buffer solution is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in the mass to charge ratio, for each protein has an isoelectric point and a molecular weight particular to its primary structure (native electrophoresis). SDS binds in a ratio of approximately 1.4 g SDS per 1 g protein [21] yielding an approximately uniform mass to charge ratio for most proteins. The distance of migration through the gel can be assumed to be directly related to the size of the protein. A loading dye is added to the protein solution to track the progress of the protein solution through the gel during the electrophoretic run.
Staining of the gel can either be performed with coomassie blue or silver nitrate. Classical coomassie blue staining can usually detect a 50 ng protein band, whereas silver staining increases the sensitivity 50 times [22].

2.6 Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs) are a useful tool for biochemists and immunologists to detect antigens or antibodies in samples. For protein detection, ELISA is one of the most suitable analytical rapid methods [23, 24]. It is as reliable as PCR-based techniques for the detection of potentially allergenic residues [25, 26]. Both allow the detection of protein in very small concentrations (pg mL^{-1}). Especially for the food allergens soy and lupine various studies are available [25, 26, 27, 28]. The ELISA assays have different formats for the polystyrene, 96-well-microtiter plates, which can be modified, depending on the conditions (see figure 1 and 2).

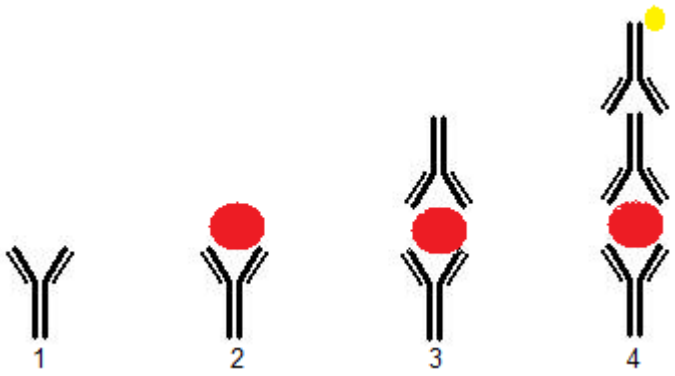

Figure 1 Indirect Sandwich ELISA format.1 Analyt-specific antibody was coated on the microtiter plate, 2 standard was added, 3 secondary analyt-specific antibody was added, 4 species-specific antibody labelled was added.

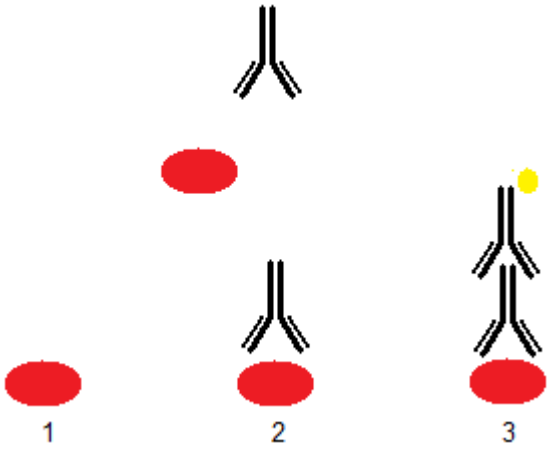

Figure 2 Indirect competitive ELISA format. 1 standard was coated on the microtiter plate, 2 analyt-specific antibody and standard were added; antibodies, which were bound to free standard, were washed out and did not bind to the standard bound 3 species-specific antibody labelled was added.

2.7 Western Blot

Western Blotting can be performed after the electrophoresis. The separated proteins from the gel of the SDS-PAGE are transferred to a nitrocellulose membrane. The proteins are immobilised in the membrane and can be used to check the antibodies for cross-reactivities [29]. The immunoassay on the membrane is similar to an ELISA assay. The format is competitive, for the protein itself is immobilised on the membrane in this case.

3 Experiments

3.1 Preparation and clean-up of the antibodies from chicken and rabbit

3.1.1 Preparation of IgY-antibodies from eggs

The egg shell was broken and the yolk separated from the egg white, without destroying the yolk's membrane. The yolk was rinsed with water, opened with tweezers and the content of five yolks (of five eggs consecutively laid) collected. The same amount of IgY buffer was added to the yolk and stirred for 15 minutes. Then 3.5% (w/v) PEG 8000 were added, the mixture was stirred for another 15 minutes and shaken for 15 minutes as well. The following centrifugation was performed at 9500 rpm, at 10 °C for 15 minutes. The liquid phase contained the antibodies and was filtered through a gauze tissue before storing the extract at -20 °C. Small volumes for immediate use were kept at 4 °C overnight and centrifuged again for three minutes on the next day before clean-up.
The clean-up of the chicken antibodies was performed using affinity chromatography. At first, the peristaltic pump must be rinsen with buffer A, the column then attached to the pump and equilibrated with five volumes of buffer A. At least 10 mL of the sample were applied to the column and rinsed with 15 mL of buffer A. In the meantime the FPLC and the UV detector were switched on and the cleaned frits put into buffer A and B. The affinity chromatography was started with 0% buffer B and the flow rate was raised step wise (1, 5, 10 mL/min) before switching to 100% of buffer B. Then the column was attached to the FPLC. The clean-up was performed with a flow rate of 5 mL min^{-1}, starting with buffer A to allow binding to the column and switch to buffer B to elute the sample and collect it in a fraction size of 2.5 mL, which takes half a minute for each fraction. The column was cleaned with buffer C and for longer storage with 20% ethanol, as well as the FPLC system.

3.1.2 Preparation of rabbit-antibodies from sera

The sera from rabbit were cooled at 4 °C before use. The clean-up procedure was a stepwise ammonium sulphate precipitation. The first precipitation at 30% ammonium sulphate was performed while stirring for an hour at 4 °C. 25 minutes centrifugation at 4 °C

with 9500 rpm followed and the supernatant was precipitated again at the same conditions, at 40% ammonium sulphate. After centrifugation the antibody was found in the pellet, which was washed with an ammonium sulphate solution of 40% and centrifuged again. The pellet was dissolved in as much of 50 mM PBS buffer as needed. The dialysis tubes were rinsed with warm water and deionised water before tested to be dense. The dissolved pellet was put into the tube and dialyzed at 4 °C in deionised water overnight. The water was changed once and dialyzed again for another four hours. The final volume of antibody solution was used for electrophoresis and the determination of the amount of antibody via photometer.

3.2 Preparation of the food allergen

Milk proteins were kept at -20 °C and dissolved in 0.2 M PBS buffer, while stirring at 50 °C. The concentrations were determined by BCA test.

The extraction of food allergens was performed the same way for all food samples and powders, only dependent on the amount of fat in the sample. The food samples were stored at -20 °C, which facilitated grinding. 30 g of ground sample were mixed with 300 mL of acetone at 4 °C with the ultraturax, twice for five minutes. The mixture was filtered over glassfiber filters, the acetone solution, including the dissolved fat, reused after distillation. The sample was dried at room temperature overnight. Then the extraction of the desired protein followed. 1 g dried, defatted sample was extracted with 10 mL preheated extraction buffer, being 50 mM PBS buffer, for 15 minutes at 60 °C in the water bath. After ten minutes centrifugation at 9500 rpm the supernatant was filtered with folded filters and cellulose acetate filters with glassfiber prefilters and kept at 4 °C until the protein concentration was determined with a BCA test.

3.3 Determination of the amount of protein with BCA test

The BCA test kit was bought and used following to the protocol. The sample was measured undiluted and in dilutions (1:10, 1:50 and 1:100). The dilutions were made with 0.2 M PBS buffer. 20 µL of each dilution and ready-to-use albumin standards were put into the wells of a non-binding microtiter plate and 200 µL of colouring reagent added. The colouring reagent included 25 mL of reagent A and 500 µL of reagent B. The test was performed at

37 °C for 30 minutes. Then the absorbances were measured with the ELISA reader. The results were calculated by ValiData software using Windows Excel and the obtained standard curve.

3.4 Checking the food allergens and their antibodies via SDS-PAGE

12% Bis/Tris gel was used for electrophoresis. Only MOPS buffer was prepared freshly. The loading buffer was stored at 4 °C. The sample contained 5-7 µg protein and 2.5 µL loading buffer. The rest was filled up to 10 µL with deionised water. The 10 µL of sample were heated at 70 °C for 10 minutes and put onto the gel. The electrophoresis was run for an hour at 4 °C at 200 V and 300 mA. The gel was washed three times in deionised water and then stained with coomassie blue for an hour while shaking. It was destained in deionised water overnight.

3.5 Blocking studies for the ELISA assays

At first, tests with glycerol solution and PVA blocking were checked to find out about the blocking abilities of the solutions. Then various blockers were tested for their blocking capacity. Common blockers [23, p.62] were compared with synthetic and carbohydrate blockers.

3.5.1 Indirect Immunoassay for α-casein for the blocking studies

Coating and blocking of the microtiter plate

After coating 1 µg mL^{-1} of IgY-anti-α-casein, the first assays performed were blocked with 50% glycerol solution at room temperature (two microtiter plates) or 1% PVA in coating buffer at 37 °C (two microtiter plates) for two hours, every other two frozen in 50% glycerol solution at -20 °C afterwards.

For the great deal of blocking studies, plates were coated with blocking solutions of 1% BSA, 1% fish gelatine, 1% PVA, 1% PEG, 1% Ficoll, 1% dextran 40, 1% dextran 2000, 3% PVP and 5% trehalose in coating buffer at pH 9.6 or in PBS buffer at pH 7.6. 300 µL of blocking solution were filled in each well and the sealed plate was incubated at 4 °C

overnight, at room temperature for two hours or at 37 °C for two hours. Afterwards it was washed three times with washing buffer containing Tween 20.

Assay procedure

100 µL of α-casein standards (dilutions with assay buffer from 0 to 130 ng mL^{-1}) were filled into each cavity and shaken at room temperature for one hour. The plate was washed again three times with washing buffer containing Tween 20. 100 µL of rabbit-anti-α-casein (diluted 1:10 000 with assay buffer) were added and shaken at room temperature for one hour. After washing the plate three times with washing buffer containing Tween 20, 100 µL of anti-rabbit-HRP antibody (diluted 1:50 000 with assay buffer) were put into each well and shaken at room temperature for an hour. After the final procedure of triple washing with washing buffer containing Tween 20, 100 µL of substrate solution were filled into each well and shaken at room temperature for 30 minutes protected from light. Eventually, 100 µL stop solution were added and the absorbance was measured at 450 nm with an ELISA reader. The four-parameter curves were calculated by Magellan5 software, based on the measured absorbances.

3.6 Checkerboard titration of soy antibody in the competitive ELISA format

3.6.1 Indirect Competitive Immunoassay for soy

Coating of the microtiter plate

Soy bean standard was coated in a concentration of 500 ng mL^{-1}, diluted in coating buffer. 100 µL thereof were put into each cavity and incubated at 4 °C overnight. Washing was performed three times with phosphate buffer without Tween 20.

Blocking of the microtiter plate

300 µL of 1% Ficoll in coating buffer (pH 9.6) were put into each well and incubated at room temperature for two hours. Then triple washing with washing buffer containing Tween 20 followed.

Assay procedure

75 µL of soy bean standard (dilutions with assay buffer from 0 to 5000 ng mL^{-1}) were filled into each cavity. 25 µL rabbit-anti-soy (diluted 1:5 000 with assay buffer) were added immediately and shaken at room temperature for an hour. After washing the plates three times, 100 µL of anti-rabbit-HRP (diluted 1:50 000 with assay buffer) were put into each well and shaken at room temperature for an hour. 100 µL of substrate solution were added after triple washing and shaken for 30 minutes, protected from light. Eventually, 100 µL of stop solution were added. The absorbances were obtained with the ELISA reader and the four-parameter curve derived by Magellan5 software using Eq.1 and 2.

$$y = d + \frac{a-d}{1+\left(\frac{x}{c}\right)^b} \qquad (1)$$

$$x = c\left(\frac{a-d}{y-d} - 1\right)^{\frac{1}{b}} \qquad (2)$$

x	concentration of the sample/standard [ng/mL]
y	absorption
a,b,c,d	parameters

3.7 Checkerboard titration of hazelnut antibodies in the competitive and the Sandwich ELISA format

3.7.1 Indirect Sandwich Immunoassay for hazelnut

Coating of the microtiter plate

IgY-anti-hazelnut was diluted with coating buffer up to a final concentration of 1 µg mL^{-1}, rabbit-anti-hazelnut up to 0.5 µg mL^{-1}. For one Sandwich format 100 µL of IgY-anti-hazelnut were coated on the high binding microtiter plate and incubated at 4 °C overnight. For the other Sandwich format, rabbit-anti-hazelnut was coated. The washing step after coating was performed three times with phosphate buffer without Tween 20.

Blocking of the microtiter plate

300 µL of 1% PVA in 10 mM PBS buffer were added into each well of the microtiter plate and blocked at 4 °C. Afterwards the plates were washed three times with phosphate buffer containing Tween 20.

Assay procedure

100 µL of standard (dilutions with assay buffer from 0 to 400 ng mL^{-1}) were filled into each cavity and shaken at room temperature for an hour. The plates were washed again three times with washing buffer containing Tween 20. 100 µL of secondary antibody, for the first format rabbit anti-hazelnut (diluted 1:10 000 with assay buffer), for the other format IgY-anti-hazelnut (diluted 1:1000 with assay buffer), were added and shaken at room temperature for an hour. After triple washing of the plate with washing buffer containing Tween 20, 100 µL of anti-rabbit-HRP antibody (diluted 1:50 000 with assay buffer) or anti-IgY-HRP antibody (diluted 1:30 000 with assay buffer) were put into each well and shaken at room temperature for an hour. After the final triple washing procedure, 100 µL of substrate solution were filled into each cavity and shaken at room temperature for 30 minutes (for coating rabbit-anti-hazelnut 15 minutes were enough), protected from light. Finally 100 µL stop solution were added and the absorbance was measured at 450 nm with an ELISA reader. A four-parameter equation (Eq. 1) was used to describe the standard curves.

3.7.2 Indirect Competitive Immunoassay for hazelnut

Coating of the microtiter plate

Hazelnut standard was coated in a concentration of 500 ng mL^{-1}, diluted in coating buffer. 100 µL thereof were put into each cavity and incubated at 4 °C overnight. Washing was performed three times with phosphate buffer without Tween20.

Blocking of the microtiter plate

300 µL of 1% PVA in 10 mM PBS buffer (pH 7.6) were put into each well and incubated at 4 °C overnight. Then triple washing with washing buffer containing Tween 20 followed.

Assay procedure

75 µL of hazelnut standard (dilutions with assay buffer from 0 to 5000 ng mL^{-1}) were filled into each cavity. 25 µL of IgY-anti-hazelnut (diluted 1:1 000 with assay buffer) or rabbit-anti-hazelnut (diluted 1:10 000 with assay buffer) were added immediately and shaken at room temperature for an hour. After washing the plates three times, 100 µL of anti-IgY-HRP (diluted 1:30 000 with assay buffer) or anti-rabbit-HRP (diluted 1:50 000 with assay buffer) were put into each well and shaken at room temperature for an hour. The rest of the assay was performed as described for the sandwich ELISA.

3.8 Checkerboard titration of α-casein antibodies in the competitive and the Sandwich ELISA format

3.8.1 Indirect Sandwich Immunoassay for α-Casein

Coating of the microtiter plate

IgY-anti-α-Casein and rabbit-anti-α-casein were diluted with coating buffer up to a final concentration of 1 µg mL^{-1}. For one Sandwich format 100 µL of IgY-anti-α-casein were coated on the high binding microtiter plate and incubated at 4 °C overnight. For the other Sandwich format, rabbit-anti-α-casein was coated. The washing step after coating was performed three times with phosphate buffer without Tween 20.

Blocking of the microtiter plate

300 µL blocking solution were added into each well of the microtiter plate. For comparison of different blockers and different conditions the sealed plate was blocked with: 1% PVA in 10 mM PBS buffer (pH 7.6) at 4 °C overnight or 1% Ficoll in coating buffer (pH 9.6) for two hours at room temperature. Afterwards the plates were washed three times with phosphate buffer containing Tween 20.

Assay procedure

100 µL of standard (dilutions with assay buffer from 0 to 200 ng mL^{-1}) were filled into each cavity and shaken at room temperature for one hour. The plate was washed three times with washing buffer containing Tween 20. 100 µL of secondary antibody, for the first format rabbit anti-α-Casein (diluted 1:10 000 with assay buffer), for the other format IgY-anti-α-

casein (diluted 1:1000 with assay buffer), were added and shaken at room temperature for an hour. Triple washing of the plate with washing buffer containing Tween 20 was performed and 100 µL of anti-rabbit-HRP antibody (diluted 1:50 000 with assay buffer) or anti-IgY-HRP antibody (diluted 1:30 000 with assay buffer) were put into each well and shaken at room temperature for an hour. After the final triple washing procedure, 100 µL of substrate solution were filled into each cavity and shaken at room temperature for 30 minutes, protected from light. Finally 100 µL stop solution were added and the absorbance was measured at 450 nm with an ELISA reader. A four-parameter equation (Eq. 1) was formed to describe the standard curves.

3.8.2 Indirect Competitive Immunoassay for α-Casein

Coating of the microtiter plate

For a competitive ELISA, α-casein standard was coated with 500 ng mL^{-1}, diluted in coating buffer. 100 µL thereof were put into each cavity and incubated at 4 °C overnight. Washing was performed three times with phosphate buffer without Tween 20.

Blocking of microtiter plate

For comparison of different blockers and different conditions the sealed plate was blocked with: 1% PVA in 10 mM PBS buffer (pH 7.6) at 4 °C overnight, 1% Ficoll or 1% BSA in coating buffer (pH 9.6) for two hours at room temperature. 300 µL of blocking solution were put into each well. Then triple washing with washing buffer containing Tween 20 followed.

Assay procedure

75 µL of α-casein standard (dilutions with assay buffer from 0 to 5000 ng mL^{-1}) were filled into each cavity. 25 µL of IgY-anti-α-casein (diluted 1:1 000 with assay buffer) or rabbit-anti-α-casein (diluted 1:10 000 with assay buffer) were added immediately and shaken at room temperature for one hour. After washing the plate three times, 100 µL of anti-IgY-HRP (diluted 1:30 000 with assay buffer) or anti-rabbit-HRP (diluted 1:50 000 with assay buffer) were put into each well and shaken at room temperature for one hour. The rest of the assay was performed as for the sandwich ELISA.

3.9 Checkerboard titration of peanut antibodies in the competitive and the Sandwich ELISA format

3.9.1 Indirect Sandwich Immunoassay for peanut

Coating of the microtiter plate

Rabbit-anti-peanut was diluted with coating buffer to a final concentration of 0.1 µg mL^{-1}, IgY-anti-peanut up to 2 µg mL^{-1}. For one Sandwich format, 100 µL of IgY-anti-peanut were coated on the high binding microtiter plate and incubated at 4 °C overnight. For the other Sandwich format, rabbit-anti-peanut was coated. The washing step after coating was performed three times with phosphate buffer without Tween 20.

Blocking of the microtiter plate

300 µL blocking solution were added into each well of the microtiter plate. For comparison of different blockers and different conditions the sealed plate was blocked with: 1% PVA in 10 mM PBS buffer (pH 7.6) at 4 °C overnight or 1% Ficoll in coating buffer (pH 9.6) for two hours at room temperature. Afterwards the plates were washed three times with phosphate buffer containing Tween 20.

Assay procedure

100 µL of peanut standard (dilutions with assay buffer from 0 to 400 ng mL^{-1}) were filled into each cavity and shaken at room temperature for an hour. The plate was washed again three times with washing buffer containing Tween 20. 100 µL of the secondary antibody, for the first format rabbit-anti-peanut (diluted 1:10 000 with assay buffer), for the other format IgY-anti-peanut (diluted 1:1000 with assay buffer) were added and shaken at room temperature for an hour. After washing the plate three times with washing buffer containing Tween 20, 100 µL of anti-rabbit-HRP antibody (diluted 1:50 000 with assay buffer) or anti-IgY-HRP antibody (diluted 1:30 000 with assay buffer) were put into each well and shaken at room temperature for an hour. After the final triple washing procedure, 100 µL of substrate solution were filled into each cavity and shaken at room temperature for 30 minutes, protected from light. Finally 100 µL stop solution were added and the absorbance was measured at 450 nm with an ELISA reader. A four-parameter equation (Eq. 1) was formed to describe the standard curves.

3.9.2 Indirect Competitive Immunoassay for peanut

Coating of the microtiter plate

For a competitive ELISA, peanut standard was coated with 500 ng mL^{-1}, diluted in coating buffer. 100 µL thereof were put into each cavity and incubated at 4 °C overnight. Washing was performed three times with phosphate buffer without Tween 20.

Blocking of the microtiter plate

For comparison of different blockers and different conditions the sealed plate was blocked with: 1% PVA in 10 mM PBS buffer (pH 7.6) at 4 °C overnight or 1% Ficoll in coating buffer (pH 9.6) for two hours at room temperature. 300 µL of blocking solution were put into each well. Then triple washing with washing buffer containing Tween 20 followed.

Assay procedure

75 µL of peanut standard (dilutions with assay buffer from 0 to 5000 ng mL^{-1}) were filled into each cavity. 25 µL of IgY-anti-peanut (diluted 1:1 000 with assay buffer) or rabbit-anti-peanut (diluted 1:10 000 with assay buffer) were added immediately and shaken at room temperature for an hour. After washing the plate three times, 100 µL of anti-IgY-HRP (diluted 1:30 000 with assay buffer) or anti-rabbit-HRP (diluted 1:50 000 with assay buffer) were put into each well and shaken at room temperature for an hour. The rest of the assay was performed as for the sandwich ELISA.

3.10 Cross-reactivity studies for anti-peanut and anti-hazelnut antibodies

Two different aspects were observed for determining cross-reactive agents for anti-hazelnut and anti-peanut antibodies: At first, food samples in question [30] were extracted like food allergen standards and the protein content was defined with BCA tests. The extracts were used like hazelnut and peanut standard: They were diluted in a serial row and all measured and described as standard curves. The following concentrations of the extracts were measured: 1000, 500, 100, 10, 1, 0.1, 0.01 µg mL^{-1}. The scheme of the ELISA is shown in table 1.

Table 1 Scheme of the ELISA microtiter plate for determining cross-reactivities.

	standard	sample	sample	sample
	column 1-3 [ng/mL]	column 4-6 [ng/mL]	column 7-9 [ng/mL]	column 10-12 [ng/mL]
A	5000	1000 000	1000 000	1000 000
B	2000	500 000	500 000	500 000
C	1000	100 000	100 000	100 000
D	500	10 000	10 000	10 000
E	100	1000	1000	1000
F	50	100	100	100
G	10	10	10	10
H	0.01	0.01	0.01	0.01

The ELISA format was the indirect competitive format. 1% Ficoll in coating buffer was used for blocking for two hours at room temperature. The antibodies from rabbit were diluted 1:5000, the antibodies from chicken 1:500. The ELISA reader and Magellan5 software derived standard curves based on a fitted four-parameter model (see Eq. 1). The parameter “c” of Eq. 1 is the IC_{50}-value of the curve. This value can directly be compared within the standards. IC_{50}-values describe the concentration of the test substance [TS] (in ng mL^{-1}) and the cross-reacting substance [CS] (in ng mL^{-1}) required for 50% reduction of the absorbance [31].

$$\text{rel. cross-reactivity}\,[\%] \;=\; \frac{[\text{TS}]}{[\text{CS}]} \cdot 100 \qquad (3)$$

In case of most of the food samples tested, the IC_{50} were not reliable because the software was not able to describe the curve properly or the values were much too high.

Hence, the extracts were measured as samples as well. The standard curve of hazelnut and peanut were formed for calculating the measured samples, which were measured undiluted and in a dilution of 1:10. Magellan5 software calculated results of concentrations for the samples, based on the standard curve for hazelnut or peanut. This calculated concentration [S] (in ng mL^{-1}) was multiplied with the dilution factor [DIL] and divided by the concentration of the extract [STD] (in mg mL^{-1}) (divided by 10^6 to have the same units) and multiplied by 100 to receive a value in percent.

$$\text{rel. cross-reactivity}\,[\%] \;=\; \frac{[\text{S}] \cdot [\text{DIL}]}{[\text{STD}]} \cdot 100 \qquad (4)$$

3.11 Cross-reactivity studies for anti-α-casein and anti-soy antibodies

Only one analysis was performed to determine cross-reactivities for anti-α-casein and anti-soy antibodies. The food samples in question [30] were extracted like food allergen standards and the protein content was defined by BCA tests. The extracts were used like α-casein and soy standard: They were diluted in a serial row and all measured and described as standard curves. The following concentrations of the extracts were measured: 1000, 500, 100, 10, 1, 0.1, 0.01 µg mL^{-1}. The scheme of the ELISA is shown in table 2.

Table 2 Scheme of the ELISA microtiter plates for determining cross-reactivities.

	standard	sample	sample	sample
	column 1-3 [ng/mL]	column 4-6 [ng/mL]	column 7-9 [ng/mL]	column 10-12 [ng/mL]
A	5000	1000 000	1000 000	1000 000
B	2000	500 000	500 000	500 000
C	1000	100 000	100 000	100 000
D	500	10 000	10 000	10 000
E	100	1000	1000	1000
F	50	100	100	100
G	10	10	10	10
H	0.01	0.01	0.01	0.01

The ELISA format was the indirect competitive format. 1% Ficoll in coating buffer was used to block the microtiter plate for two hours at room temperature. The antibodies from rabbit were diluted 1:5000, the antibodies from chicken 1:500. The ELISA reader and Magellan5 software derived standard curves based on a four-parameter model (see Eq. 1). The parameter “c” of Eq. 1 is the IC_{50}-value of the curve and again this value can directly be compared with the other standards, using Eq. 3.

To prove that anti-α-casein antibodies are cross-reactive with β-casein and κ-casein, but not with α-lactalbumin or β-lactoglobulin, additional methods were used: electrophoresis, silver nitrate staining and Western blotting.

3.12 Checking α-casein and its antibodies via SDS-PAGE, Silver Staining and Western Blot analysis

3.12.1 SDS-PAGE

The anti-α-casein antibodies were checked for cross reactivity with all milk proteins in ELISA assays. Additionally, a Western Blot was performed to see which bands of the proteins were bound to the antibody. Hence, two 12% Bis/Tris gels were run with MES running buffer and the following samples twice (to be able to cut the gel in halfs afterwards): milk powder, α-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin (see table 3).

Table 3 Order for half a gel; repeated after one lane free.

Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
standard	milk powder	α-casein	β-casein	κ-casein	α-lactalbumin	β-lactoglobulin

Each sample contained 5-7 µg protein and 2.5 µL loading buffer. The rest was filled up to 10 µL with deionised water. They were heated at 70 °C for 10 minutes and then put onto the gel. Both gels were run in parallel at 4 °C and for an hour at 200 V and 300 mA. One gel was used for Western blotting and one gel was stained with coomassie blue. The bands were hard to find and not very intense, therefore the incubation was performed longer than an hour and half of the gel was used for silver staining, which was more sensitive than the coomassie blue staining.

3.12.2 Silver Staining

The gel was washed with deionised water before fixing the bands for half an hour with 100 mL of an ethanol/acetic acid mixture. The incubation was performed overnight in the incubation solution. Washing was performed three times for five minutes with deionised water before silver nitrate was added and incubated for 20 minutes. The development took four minutes before stopping in stop solution for ten minutes. Then the gel was washed three times for five minutes with deionised water and preserved in glycerol solution for 30 minutes.

3.12.3 Western Blot

One membrane was used for the Western Blot. The sponges were put into transfer buffer solution, bubbles banished and the nitrocellulose membrane laid in buffer solution as well. The blot was built as the following: 2 sponges, filter papers, gel from electrophoresis, nitrocellulose membrane, filter papers, 2 sponges (see figure 3).

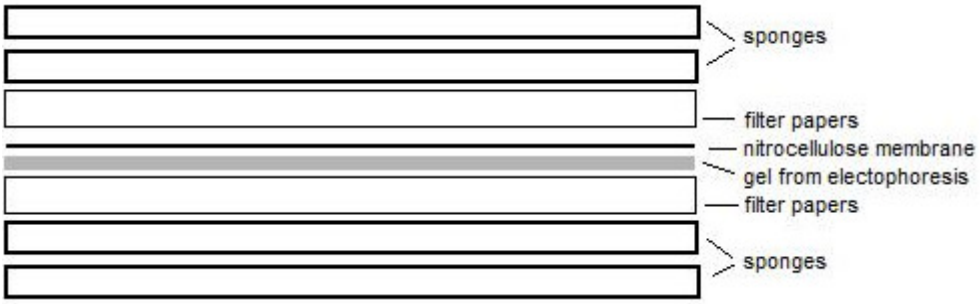

Figure 3 Blot apparatus for Western Blot.

Everything was pressed together in the blot apparatus and the chamber filled with transfer buffer. The transfer was performed for an hour at 30 V and 170 mA. Eventually, the gel was empty and the membrane was cut in halfs to go ahead with the immunoblotting. The membranes were blocked in 2% BSA solution overnight. Then the membranes were washed twice for ten minutes with 0.2 M PBS buffer containing 0.1% Tween 20. The incubation of the primary antibody was performed for an hour. IgY-anti-α-casein was diluted 1:25, rabbit-anti-α-casein was diluted 1:50 with PBS buffer. One membrane was incubated with one primary antibody each. They were treated separately from then on. Triple washing was performed for ten minutes, each with PBS buffer containing Tween 20. The incubation of the secondary, labelled antibody was performed for an hour. Anti-IgY-HRP was diluted 1:1000, anti-rabbit-HRP was diluted 1:1500 with PBS buffer. They were put onto the corresponding membranes. After triple washing the membranes were put into Tris/HCl solution for one minute before the substrate solution was added. After five to ten minutes the reaction was stopped by rinsing with deionised water.

3.13 Comparison of different extraction buffers for the extraction of α-casein

Different extraction buffers were tested for their suitability of extracting food allergens. The extraction temperature and buffer were varied.

The following buffers were used: coating buffer (pH 9.6), 50 mM PBS buffer, containing 1 M NaCl, 0.2 M PBS buffer and one extraction buffer from an available test kit from R-Biopharm. Instead of the assay buffer the dilutions of the standards were performed in different extracts. The extraction procedure was followed to the extraction of food allergen standards (see chapter 3.2) with different extraction buffers, once performed at 60 °C, another time at room temperature. This led to eight different results for each sample tested. The ELISA format used was the indirect competitive format for IgY- and rabbit-anti-α-casein (see chapter 3.8.2). Blocking was performed with 1% Ficoll in coating buffer for two hours at room temperature.

Table 4 shows the scheme of the ELISA assays for the comparison of different extraction buffers for the blank materials: candy cone (Zuckertüte), cookie (Biskotten), soy milk and whey drink (Latella).

Table 4 Scheme of the ELISA format for testing various buffers and blank materials. Sample 1 = candy cone, sample 2 = cookie, sample 3 = soy milk, sample 4 = whey drink; RT = room temperature.

	1-3: α-casein standards	4-6: sample 1	7-9: sample 2
A carbonate buffer RT			
B 50 mM PBS RT			
C 0.2 M PBS RT			
D extraction buffer RT			
E carbonate buffer 60°C			
F 50 mM PBS 60°C			
G 0.2 M PBS 60°C			
H extraction buffer 60°C			
	1-3: α-casein standards	4-6: sample 3	7-9: sample 4
A carbonate buffer RT			
B 50 mM PBS RT			
C 0.2 M PBS RT			
D extraction buffer RT			
E carbonate buffer 60°C			
F 50 mM PBS 60°C			
G 0.2 M PBS 60°C			
H extraction buffer 60°C			

3.14 Determination of matrix effects for α-casein

Two different, blank matrices were extracted with Ridascreen extraction buffer: soy milk (liquid) and cookie (solid). The extraction was performed at 60 °C for 15 minutes. The extracts of the blank matrices were used like assay buffer to dilute the α-casein standard in the matrix solutions. The ELISA assay was performed in an indirect competitive format, as described in chapter 3.8.2. The blocking was performed with 1% Ficoll in coating buffer for two hours at room temperature. The scheme of the microtiter plate is shown in table 5.

Table 5 Determination of matrix effects in cookie and soy milk matrix.

	α-casein standard	cookie matrix	soy milk matrix
	column 1-3 [ng/mL]	column 4-6 [ng/mL]	column 7-9 [ng/mL]
A	5000	5000	5000
B	2000	2000	2000
C	1000	1000	1000
D	500	500	500
E	100	100	100
F	50	50	50
G	10	10	10
H	0.01	0.01	0.01

4 Results and Discussion

4.1 Affinity chromatography for the clean-up of IgY-antibodies

During the FPLC clean-up the amount of protein was measured in the fractions at 280 nm. The fractions with the highest absorbances were checked additionally via photometer at 280 nm. Those with an absorbance higher then 0.3 were collected and pooled. The value of the absorbance (considering the dilution) was divided by the extinction coefficient ε of IgY, which is 1.33 [18], to receive the protein concentration of the sample, according to the Lambert-Beer law.

$$A = \epsilon \cdot l \cdot c \qquad (5)$$

A	Absorbance
ε	extinction coefficient; for IgY ε=1.33
l	length of the light path; l=1 cm
c	concentration of the sample [mg/mL]

Table 6 Absorbance values for IgY-anti-α-casein.

fraction	absorbance value
3	0.487
4	1.332
5	0.405

Table 6 shows the absorbances of the fractions for IgY-anti-α-casein. The absorbance for the pooled IgY-anti-α-casein sample was 2.041 and therefore the protein amount was 1.53 mg mL^{-1}.

Table 7 Absorbance values for IgY-anti-hazelnut.

fraction	Absorbance value
3	1.776
4	3.200
5	1.249
6	0.486

Table 7 lists the absorbances measured for IgY-anti-hazelnut. The absorbance value for the pooled IgY-anti-hazelnut sample was over 3.0 and therefore diluted 1:5. The measured value for the dilution was 0.76 and the protein amount was 2.86 mg mL^{-1}.

Table 8 Absorbance values for IgY-anti-peanut.

fraction	Absorbance value
3	1.043
4	2.415
5	0.674

Table 8 shows the absorbances of the fraction of IgY-anti-peanut. The absorbance value for the pooled IgY-anti-peanut sample was 2.6 and therefore diluted 1:5 as well. The measured value for the dilution was 0.59 and the protein amount was 2.22 mg mL^{-1}.
There was enough material of the IgY-antibodies against peanut and hazelnut for the whole master thesis. Only for the studies of the α-casein antibodies, new IgY-anti-α-casein was required.

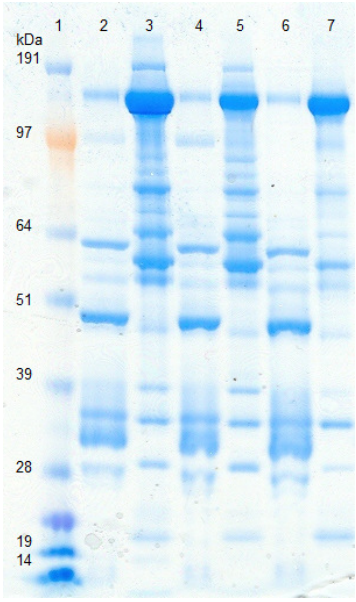

Figure 4 12% Bis/Tris gel in MOPS buffer. In lane 1 there is the standard ladder. Lane 2 shows IgY-anti-hazelnut in egg york extract, lane 3 the cleaned IgY-anti-hazelnut. Lane 4 shows IgY-anti-peanut in egg york extract, lane 5 the cleaned IgY-anti-peanut. Lane 6 shows IgY-anti-α-casein in egg york extract, lane 7 the cleaned IgY-anti-α-casein.

The IgY-antibodies have a size of 180 kDa. The cleaned antibodies have much broader bands at 180 kDa (see lane 3, 5, 7 in figure 4) than the extracts before the affinity chromatography (see lane 2, 4, 6). The efficiency of the clean-up was shown, but there were still a lot of other bands to be seen. Hence, the cleaning was not optimized as far as the extract only contains IgY, but the amount of the antibody was raised significantly.

4.2 Ammonium sulphate precipitation of the rabbit-antibodies

The amount of antibody in solution after ammonium sulphate precipitation and dialysis was determined via photometer at 280 nm. After dialysis there was little precipitate, which was dissolved in 50 mM PBS buffer and measured as well. The absorbances for the supernatant and the precipitate are listed in table 9.

Table 9 Absorbance values for the supernatant and the precipitate of different rabbit-antibodies.

antibodies	absorbance values of supernatant	absorbance values of precipitate
rabbit-anti-α-casein	3.2 → 1:5 dilution: 1.006	3.2 → 1:5 dilution: 0.956
rabbit-anti-hazelnut	3.2 → 1:5 dilution: 1.667	3.2 → 1:5 dilution: 1.154
rabbit-anti-peanut	3.2 → 1:10 dilution: 1.171	3.3 → 1:5 dilution: 1.546

In the precipitate there was antibody as well as in the supernatant, which is proved by the electrophoresis (see figure 5).

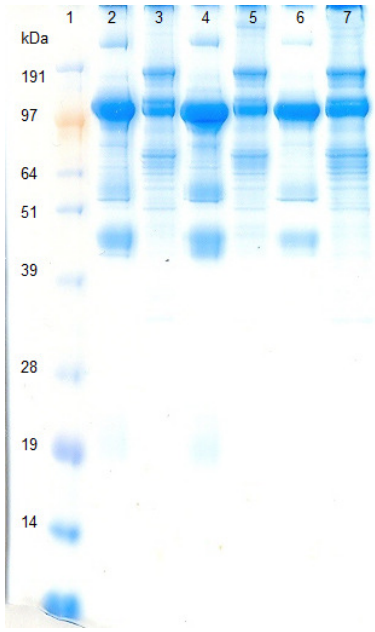

Figure 5 12% Bis/Tris gel with MOPS running buffer. It shows rabbit-antibodies after 40% $(NH_4)_2SO_4$ precipitation and dialysis. Lane 1 shows the standard ladder. Lane 2 shows the supernatant of rabbit-α-casein, lane 3 the precipitate. Lane 4 shows the supernatant of rabbit-hazelnut, lane 5 the precipitate. Lane 6 shows the supernatant of rabbit-peanut, lane 7 the precipitate.

Approximately 150 kDa is the size of the rabbit-IgG, which can be seen in all lanes in figure 5. All solutions contain rabbit-antibody. Therefore the supernatant and the precipitate solutions were pooled and measured again.

Table 10 Absorbance values for the pooled rabbit-antibody solutions (in dilution 1:5).

antibodies	absorbance values
rabbit-anti-α-casein	0.957
rabbit-anti-hazelnut	1.520
rabbit-anti-peanut	1.430

Table 10 shows the absorbances for three rabbit-antibodies. The concentration of antibody could be calculated according to Eq. 5 (considering the dilution) and an extinction coefficient of 1.45 [31]. Rabbit-anti-α-casein had a concentration of 3.3 mg mL^{-1}, rabbit-anti-hazelnut of 5.2 mg mL^{-1} and rabbit-anti-peanut of 5.2 mg mL^{-1}. The dilutions of 1:5 could be used for ELISA assays as well because the concentrations were high enough.
The rabbit-antibodies were extracted more often during the master thesis because of different reasons: The antibody lost activity or it was precipitated once again to establish a better cleaning of the antibody from other proteins or it was simply all used.

4.3 Determination of the protein amount in food allergens with the BCA test

All samples and the dilutions were measured in triplicate. The average value and the resulted standard deviations are listed in table 11. The software ValiData calculated concentrations out of these data and the calibration curve (obtained by eight measured standard concentrations). The dilutions were considered and a final standard concentration declared. The calculated concentrations are listed in table 11.

Table 11 Calculated concentrations of three food allergens with BCA test.

food allergen	mean abs.	std. dev.	dilution	calculated conc. regarding dilution [mg/mL]	final conc. [mg/mL]
α-casein	0.924	0.005	-	1.18	**1.40**
	0.156	0.002	1:10	1.68	
	0.039	0.006	1:50	1.27	
hazelnut	1.599	0.011	1:10	21.7	**23.10**
	0.443	0.003	1:50	23.4	
	0.239	0.001	1:100	24.4	
peanut	1.129	0.005	1:10	15.2	**15.30**
	0.286	0.007	1:50	14.3	
	0.161	0.006	1:100	16.2	

The samples for cross-reactivity studies were all defatted and extracted the same way as the preparation of the food allergen standards (see chapter 3.2). The BCA results are listed in table 12.

Table 12 Listed results of the concentrations of food allergens for cross reactivity studies.

food allergen	protein concentration [mg/mL]
milk powder	19
β-casein	10
κ-casein	10
α-lactalbumin	10
β-lactoglobulin	34.2
soy bean	25
BSA	90
egg white	85.8
egg york	21.5
lupine	11.5
linseed	11.1
cashew nut	20.4
cocos	6.89
almond	15.4
puppy seed	32.3
pistachio	25.8
sesame	16.0
sun flower	49.9
walnut	11.0

For milk powder, the extraction efficiency was determined: The BCA test detected 19 mg mL^{-1} of protein in the extract. Milk powder itself [32] should have a protein content of 36% (given on the label), therefore 36 g of protein per 100 g milk powder and 0.36 g in 1 g. 1 g milk powder was extracted in 10 mL, resulting in 0.36 g protein in 10 mL. In 1 mL there should be 0.036 g (=36 mg) of protein. The concentration calculated is 36 mg mL^{-1}. Therefore, the extraction efficiency is only (19/36 =) 52.8%.

4.4 Electrophoresis of the food allergens and their antibodies from rabbit and chicken

For the cross-reactivity studies a range of various food samples and other proteins were extracted and characterized by means of electrophoresis to check the amount of protein which was calculated with the BCA tests and to see the different proteins of different size within the extracts (see figure 6 to 8).

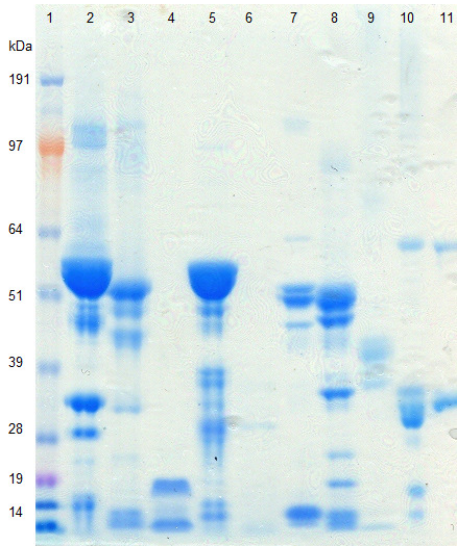

Figure 6 12% Bis/Tris gel with MOPS running buffer. Lane 1 shows the standard ladder. Lane 2 shows peanut, lane 3 hazelnut, lane 4 walnut, lane 5 almond, lane 6 soy bean, lane 7 sunflower, lane 8 pistachio, lane 9 egg white, lane 10 milk powder, lane 11 shows α-casein.

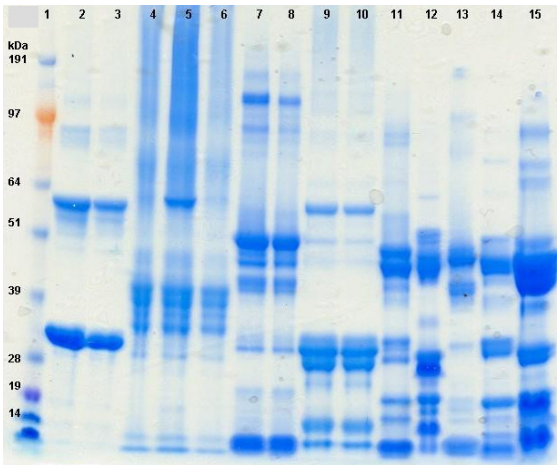

Figure 7 12%Bis/Tris gel, MOPS running buffer. The standard ladder is in lane 1. Lane 2 shows α-casein from 08.10.08, lane 3 α-casein from 29.05.08, lane 4 shows egg white, extracted at 60 ℃, lane 5 shows egg white, extracted at room temperature, lane 6 shows egg white from 26.02.08, lane 7 shows hazelnut from 07.10.08, lane 8 hazelnut from 18.07.08, lane 9 shows milk powder from 07.10.08, lane 10 milk powder, extracted on 26.02.08, lane 11 shows cashew nut, lane 12 coco, lane 13 linseed, lane 14 puppy seed and lane 15 shows sesame.

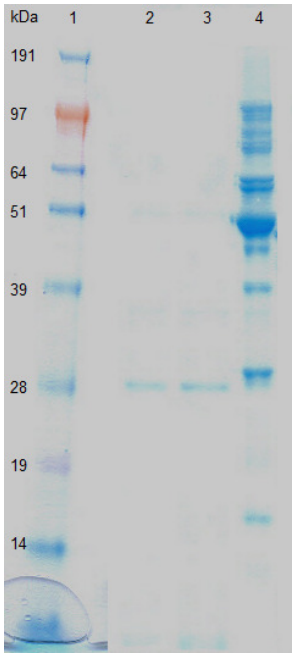

Figure 8 12% Bis/Tris gel, MOPS running buffer. The standard ladder is in lane 1. Lane 2 shows defatted soy bean (4 mg mL^{-1}), lane 3 shows non-defatted soy bean (2.34 mg mL^{-1}) and lane 4 shows non-roasted soy (bought from Sigma; 25 mg mL^{-1}).

Three different soy bean extracts were made. One sample was defatted and compared before and after defatting. There were no bands visible on the gel (see lane 2 and 3 in figure 8). The third soy bean extract was made out of soy bean flour from Sigma Aldrich and yielded in a much higher protein amount, namely 25 mg mL^{-1}, and clearly visible bands on the gel (lane 4). Therefore, this extract was used for cross reactivity studies and as food allergen for the characterization of the rabbit-anti-soy antibody.

4.5 Results of the blocking studies

The need for these blocking studies became evident as soon as the results of blocking with glycerol solution were received. The assays were performed in parallel. The first microtiter plate was coated with IgY-anti-α-casein and blocked with 1% PVA in coating buffer, the second microtiter plate was coated with IgY-anti-α-casein and blocked with glycerol solution, the third plate was coated with IgY-anti-α-casein, blocked with 1% PVA in coating buffer and frozen with 50% glycerol solution and the fourth plate was coated with IgY-anti-α-casein and frozen without blocking (see table 13-14 and figure 9).

Table 13 Obtained absorbances for indirect Sandwich immunoassays for α-casein. Blocking was performed with PVA at 37 °C or glycerol solution at room temperature for two hours.

	blocked with PVA; R= 0.999						blocked with glycerol solution; R=0.998				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		Measured absorbances			average value	std.dev.
130	1.063	1.041	0.969	1.024	0.049		1.549	1.568	1.506	1.541	0.032
65	0.803	0.642	0.760	0.735	0.083		1.214	1.199	1.050	1.154	0.091
32	0.598	0.601	0.463	0.554	0.079		0.883	0.849	0.842	0.858	0.022
16	0.358	0.313	0.363	0.345	0.028		0.519	0.486	0.512	0.506	0.017
12	0.324	0.295	0.314	0.311	0.015		0.458	0.398	0.409	0.422	0.032
8	0.230	0.215	0.226	0.224	0.008		0.374	0.282	0.290	0.315	0.051
4	0.138	0.139	0.144	0.140	0.003		0.162	0.183	0.186	0.177	0.013
0.01	0.056	0.071	0.066	0.064	0.008		0.056	0.061	0.068	0.062	0.006

Table 14 Obtained absorbances for indirect Sandwich immunoassays for α-casein. The plate was blocked with PVA at 37 °C for two hours and frozen in glycerol solution or frozen without blocking.

	blocked with PVA and frozen; R=0.999						no blocking and frozen; R=0.999				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		Measured absorbances			Average value	std.dev.
130	0.757	0.816	0.837	0.803	0.041		1.894	1.949	1.899	1.914	0.030
65	0.566	0.562	0.540	0.556	0.014		1.460	1.463	1.416	1.446	0.026
32	0.375	0.405	0.387	0.389	0.015		1.123	1.022	0.971	1.039	0.077
16	0.233	0.245	0.220	0.233	0.013		0.717	0.710	0.558	0.662	0.090
12	0.191	0.182	0.169	0.181	0.011		0.595	0.554	0.554	0.568	0.024
8	0.114	0.178	0.152	0.148	0.032		0.283	0.431	0.412	0.375	0.081
4	0.085	0.107	0.093	0.095	0.011		0.240	0.218	0.224	0.227	0.011
0.01	0.060	0.070	0.061	0.064	0.006		0.068	0.069	0.074	0.070	0.003

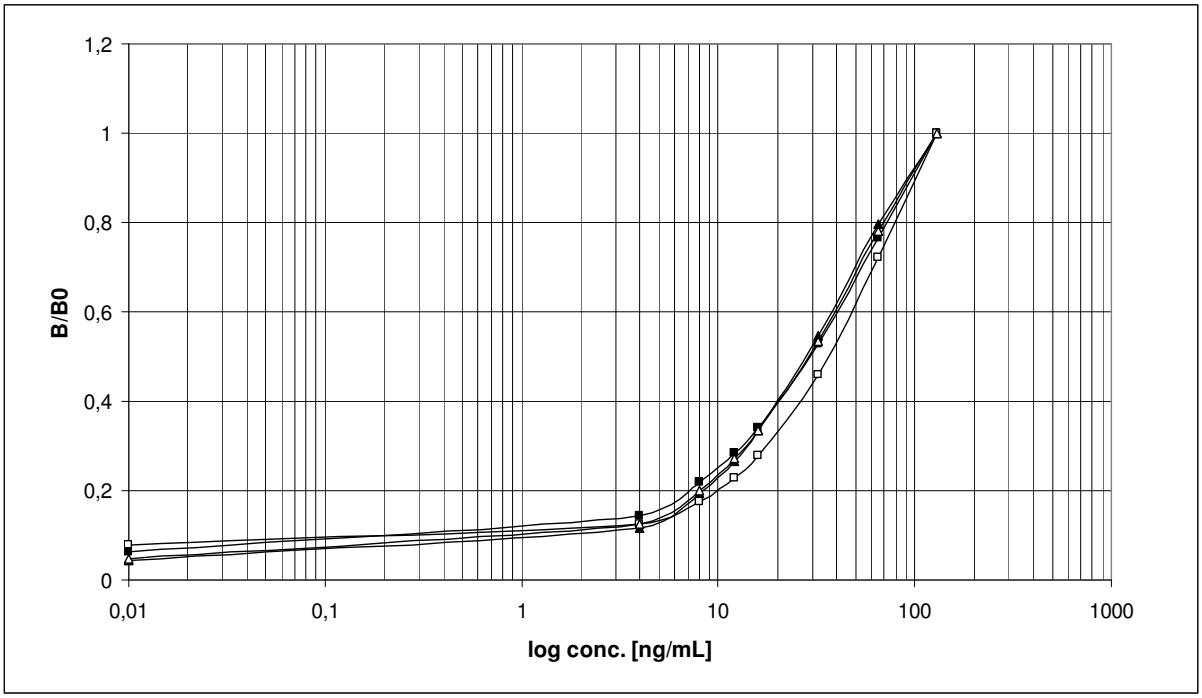

Figure 9 Indirect Sandwich immunoassay for α-casein. Four different assays, all coated with 1 µg mL^{-1} of IgY-anti-α-casein: Plate 1 was blocked with PVA (■), plate 2 with glycerol solution (▲), plate 3 was blocked with PVA and frozen in glycerol solution at -20 °C (□) and plate 4 was frozen without blocking at -20 °C in glycerol solution (Δ).

The results were all the same: All assays yielded in great standard curves. The arising question was if glycerol solution was able to block unspecific binding as well as PVA. The answer is no, glycerol solution has no blocking capacity (see table 15).

Table 15 Obtained absorbances for the glycerol blocking study. The coating and blocking was performed in one step only with 50% glycerol solution at room temperature for two hours.

standard conc. [ng/mL]	measured absorbances			average value	std.dev.
130	1.225	1.199	1.218	1.214	0.013
65	0.788	0.810	0.665	0.754	0.078
32	0.472	0.504	0.715	0.564	0.132
16	0.251	0.274	0.293	0.273	0.021
12	0.223	0.226	0.146	0.198	0.045
8	0.148	0.171	0.144	0.154	0.015
4	0.109	0.108	0.087	0.101	0.012
0.01	0.050	0.064	0.056	0.057	0.007

It shows the rise of the absorbances with the rising standard concentration. This leads to the assumption that the proteins in the standard solution are stuck to the surface. If there were no such trend visible, glycerol solution would be an effective blocking agent. The question is, if glycerol solution has no blocking capacity and the results of the PVA blocking were the same (see figure 9), wether PVA blocks effectively. The answer is no (see table 16) because again, the absorbances rise with the standard concentration.

Table 16 Obtained absorbances for glycerol blocking study. The coating and blocking was performed with 1% PVA in coating buffer at 37 °C for two hours.

standard conc. [ng/mL]	measured absorbances			average value	std.dev.
130	0.628	0.615	0.603	0.615	0.013
65	0.389	0.367	0.366	0.374	0.013
32	0.248	0.218	0.210	0.225	0.020
16	0.143	0.141	0.125	0.136	0.010
12	0.101	0.099	0.094	0.098	0.004
8	0.091	0.079	0.087	0.086	0.006
4	0.070	0.076	0.076	0.074	0.003
0.01	0.051	0.049	0.063	0.054	0.008

Hence, the need for a general blocking solution was great. For dealing with different food allergens, there should be one blocking solution available for all of them. Protein blockers are the most common, but there might be one big problem arising with the use of protein blockers for food allergen assays: The food allergen itself is a protein and in case of α-casein, the protein blockers α-casein or milk powder cannot be used for obvious reasons.

Hence, the use of non-proteinaceous blockers was in question. Three different groups of chemicals were tested: synthetic blockers and carbohydrates in comparison to the common protein blockers. The protein blockers were tested to receive data for proving the effectiveness of other blockers. The results of the studies are shown in table 17 and graphically in figure 10.

Table 17 Minimal and maximal absorbances are listed for various blocking reagents, tested at different conditions (measured at 450 nm). Differences (Δabs) were calculated by subtraction of abs_{min} from abs_{max}. Each four-parameter curve was formed by Magellan software, based on the measurement of samples in triplicate. Abs_{min} and abs_{max} are parameters from this four-parameter curve. Buffer A = 10 mM PBS buffer, pH 7.6; Buffer B = carbonate buffer, pH 9.6.

incubation temperature and period		**37 °C 2 h**			**room temperature 2 h**			**4 °C overnight**		
	buffer solution	abs_{min}	abs_{max}	Δabs	abs_{min}	abs_{max}	Δabs	abs_{min}	abs_{max}	Δabs
proteins										
1 % BSA	A	0.183	0.230	**0.047**	0.160	0.248	**0.087**	0.193	0.284	**0.091**
	B	0.182	0.207	**0.025**	0.158	0.182	**0.024**	0.239	0.201	**0.038**
1 % fish gelatine	A	0.379	0.470	**0.092**	0.306	0.560	**0.254**	0.417	0.734	**0.317**
	B	0.304	0.367	**0.063**	0.306	0.342	**0.036**	0.350	0.386	**0.036**
synthetic blockers										
1 % PVA	A	0.104	0.787	**0.682**	0.133	0.491	**0.358**	0.067	0.240	**0.173**
	B	0.107	0.509	**0.402**	0.116	0.736	**0.620**	0.060	0.728	**0.668**
3 % PVP	A	0.128	1.709	**1.580**	0.128	2.099	**1.971**	0.130	1.637	**1.507**
	B	0.155	1.698	**1.543**	0.154	1.653	**1.499**	0.129	1.587	**1.458**
1 % PEG	A	0.116	1.311	**1.196**	0.081	1.465	**1.384**	0,091	1.240	**1.148**
	B	0.139	1.457	**1.318**	0.127	1.544	**1.416**	0,101	1.165	**1.064**
carbohydrates										
1 % dextran40	A	0.134	2.266	**2.132**	0.157	2.604	**2.447**	0.133	1.665	**1.532**
	B	0.146	2.132	**1.986**	0.143	1.761	**1.619**	0.161	1.009	**0.848**
1 % dextran2000	A	0.140	1.515	**1.375**	0.125	1.547	**1.422**	0.156	0.828	**0.672**
	B	0.115	0.516	**0.401**	0.110	0.537	**0.427**	0.127	0.406	**0.279**
1 % ficoll	A	0.141	0.473	**0.332**	0.135	0.314	**0.178**	0.102	0.331	**0.229**
	B	0.204	0.182	**0.023**	0.130	0.184	**0.054**	0.123	0.209	**0.086**
5 % trehalose	A	0.221	2.318	**2.097**	0.183	1,820	**1.638**	0.331	1.634	**1.303**
	B	0.163	2.164	**2.000**	0.135	1.629	**1.493**	0.268	1.667	**1.399**

Abs_{min} corresponds to the lowest absorbance gained with zero protein concentration in the standard solution and abs_{max} shows the highest absorbance gained with the highest standard solution, 130 ng mL^{-1}, respectively. The parameter “a” in equation 1 is abs_{min}, the parameter “d” is abs_{max}.

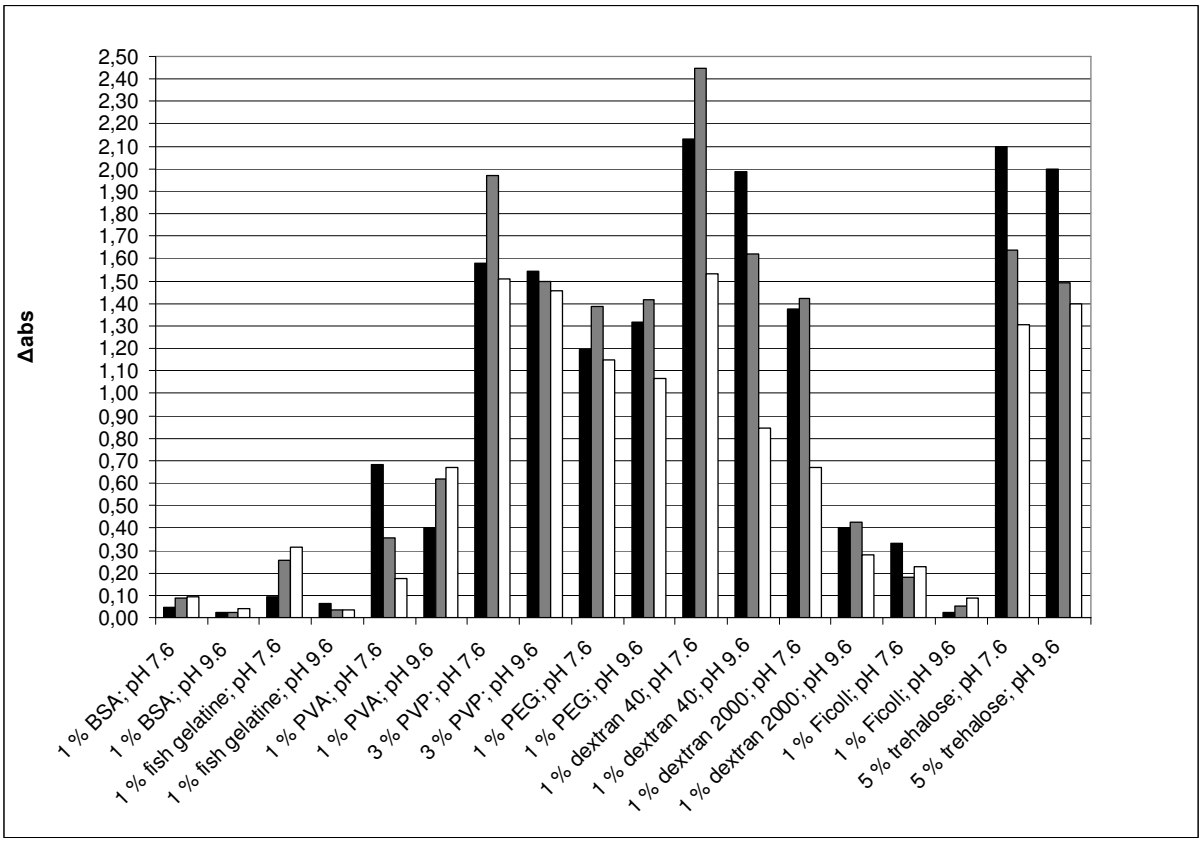

Figure 10 Summarized data of all Δabs values (450 nm) from table 17. Tested conditions: 37 °C 2 hours (■), room temperature 2 hours (■), 4 °C overnight (□) for each blocking reagent at pH 7.6 (10 mM PBS buffer) and at pH 9.6 (coating buffer).

Three main groups of blocking reagents were tested, proteins, synthetic reagents and carbohydrates. At first, the blocking reagents were used for blocking the plate without any other coating substance at three different temperatures and times (37 °C / 2 h, room temperature / 2 h and 4 °C / overnight). Two different buffers at pH 7.6 and pH 9.6 were applied. The standard curves formed, resulted from the application of α-casein and the corresponding polyclonal antibody, which was detected with a second HRP-labelled antibody. Two protein blockers, BSA and fish gelatine, and three synthetic blockers, PVA, PVP and PEG were used respectively. Furthermore, carbohydrates with high molecular weight were used to determine their blocking capacity and efficiency.

Blank values, preferably at absorbances around 0.1 or below, indicate an optimal blocking reagent. Higher values are an indication of non-specific binding of the standard on the microtiter plate, thus signalling insufficient blocking efficiency of the blocking reagents used. Table 17 lists the minimal and maximal absorbances, obtained by Magellan software5, and calculated differences in absorbances (Δabs) for all the blockers used at the different reaction conditions. Δabs values show the behaviour of the curve with regard to rising standard concentrations.

BSA and fish gelatine were expected to be very reliable protein blockers. However, in reality even 1% BSA in coating buffer (pH 9.6), as common blocker, yielded absorbances up to 0.2, but there was no trend visible between blank and highest standard concentration used (130 ng mL^{-1}), which showed that BSA was very effective (Δabs < 0.1, figure 10) in all conditions. Fish gelatine behaved a bit differently. At pH 7.6, incubated at room temperature and 4 °C, higher Δabs of 0.254 and 0.317 were detected, which corresponded to a visible rise in the standard curve. A comparison of the blocking efficiency of BSA with fish gelatine revealed that the latter could also be used as alternative protein blocking reagent at pH 9.6 at all incubation conditions, but for pH 7.6 only the incubation at 37 °C for 2 hours showed sufficient efficiency (see figure 10). There was no enhancing trend evident for increasing standard concentrations. In general 1% fish gelatine reached higher abs_{min} values than BSA, around 0.4 (see table 17), which has to be taken into account during the assay development.

Polyvinylalcohol, polyvinylpyrrolidone and polyethylenglycol are the three synthetic blocking reagents employed. As it can be seen from the Δabs values only 1% PVA at pH 7.6 incubated at 4 °C overnight showed sufficient blocking capacity. In prior studies PVP was considered an alternative synthetic blocker, but neither 3% PVP in coating buffer nor in PBS buffer showed any blocking effects. Curves were obtained, ranging in the absorbance from 0.1 to 2.1. The same effect was shown for 1% polyethylenglycol in coating and PBS buffer. The results were standard curves ranging in the absorbance from around 0.1 to 1.6. Yet, these results are not satisfactory. PVP and PEG can therefore not be recommended as alternative blocking agents. Only 1% PVA in PBS buffer incubated at 4 °C overnight achieved an absorbance maximum of 0.2. The Δabs reached only 0.173 (see table 17) in this case, indicating a comparable blocking capacity to BSA blocking. Hence, 1% PVA could be taken into account for the blocking in ELISA assays at pH 7.6. Higher amounts of PVA were not subject of these experiments because of the increasing insolubility at higher concentrations.

Finally, carbohydrates were tested as alternative blocking agents because of the great amount of hydroxyl groups available for the interaction with the activated surface of the microtiter plate. The same property is valid for synthetic reagents: They have nothing in common with the proteins to be detected, consequently no interference with the food allergens is expected in the ELISA. The following carbohydrates were tested: Ficoll, dextran 40, dextran 2000 and trehalose. All carbohydrates were used as 1% solutions except for trehalose, which was a 5% solution dissolved in coating buffer or PBS buffer.

Ficoll is a polysaccharide made of saccharose cross-linked with epichlorohydrin to form a highly branched, high molecular weight and hydrophilic polysaccharide, which behaves neutrally and shows good solubility in aqueous solutions. The size of the Ficoll used was at about 400 kDa.

Dextran is a complex branched glucan synthesised by lactic acid bacteria. Different molecular weights are available. For this study, dextrans of an average size of 40 kDa and 2000 kDa were used. Trehalose was applied as stabilising agent for plate storage in the dry state due to its high water retention capability. As disaccharide it contains two α-1,1-glycosidic linked glucose molecules. Trehalose was not able to cover the empty sites on the microtiter plate, as expected. 5% trehalose was dissolved in coating and PBS buffer, but no blocking effects were observed, as shown in the resulting Δabs from 1.3 to 2.1 (see table 17). Standard curves were obtained within a range in absorbance from 0.2 – 2.0. 1% of dextran 40 was dissolved in coating and PBS buffer, but the blocking results were poor. 1% dextran 2000 did better in both buffer solutions, but there was still a visible trend with increasing standard concentrations. Blocking with 1% Ficoll was efficient for all incubation parameters used at pH 9.6 (Δabs < 0.1), but not at pH 7.6, where Δabs up to 0.33 were measured.

Based on these findings, Ficoll and PVA were further investigated for their suitability as alternative blockers. Hence, indirect Sandwich and competitive ELISA formats were used to prove the blocking abilities in the assays.

4.6 Indirect competitive Immunoassay for soy bean

For the detection of soy bean there was only one antibody available: rabbit-anti-soy antibody. It is not possible to receive antibodies from chicken for this food allergen. Therefore, no Sandwich immunoassay was performed. Only one competitive format was characterized, basically for the cross-reactivity studies. Rabbit-anti-soy antibody was compared with the α-casein antibodies. The data from table 18 were measured with the ELISA reader, the curves formed by Magellan5 software and the calculation of B/B_0 by Microsoft Excel.

Table 18 Obtained absorbances for the competitive ELISA for soy bean.

	rabbit-anti-soy 1:1000; R=0.992						rabbit-anti-soy 1:5000; R=0.995				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.396	0.404	0.390	0.397	0.007		0.289	0.309	0.296	0.298	0.010
2000	0.647	0.704	0.537	0.629	0.085		0.457	0.485	0.411	0.451	0.037
1000	0.829	0.882	0.483	0.731	0.217		0.644	0.619	0.562	0.608	0.042
500	1.104	1.137	0.637	0.959	0.280		0.771	0.818	0.628	0.739	0.099
100	1.731	1.721	1.388	1.613	0.195		1.267	1.394	1.052	1.238	0.173
50	1.868	1.864	0.903	1.545	0.556		1.385	1.415	0.853	1.218	0.316
10	2.001	2.024	1.447	1.824	0.327		1.615	1.633	1.245	1.498	0.219
0.01	1.949	2.051	1.988	1.996	0.051		1.661	1.685	1.201	1.516	0.273
	rabbit-anti-soy 1:10 000; R=0.998						rabbit-anti-soy 1:50 000; R=0.988				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	Std.dev.
5000	0.179	0.205	0.198	0.194	0.013		0.112	0.131	0.125	0.123	0.010
2000	0.257	0.277	0.268	0.267	0.010		0.128	0.138	0.134	0.133	0.005
1000	0.332	0.434	0.350	0.372	0.054		0.138	0.163	0.145	0.149	0.013
500	0.455	0.491	0.503	0.483	0.025		0.184	0.174	0.113	0.157	0.038
100	0.755	0.787	0.813	0.785	0.029		0.233	0.266	0.159	0.219	0.055
50	0.840	0.851	0.862	0.851	0.011		0.257	0.257	0.130	0.215	0.073
10	1.063	1.067	0.964	1.031	0.058		0.268	0.295	0.790	0.247	0.061
0.01	0.980	1.080	1.013	1.024	0.051		0.269	0.318	0.300	0.296	0.025

Table 18 shows the differences in absorbance for the different dilutions, from 1:1000 to 1:50 000, of rabbit-anti-soy antibody. The average values for the measured absorbances show the decrease from lowest to highest standard. The biggest differences from blank to highest standard are found in the dilutions 1:1000 and 1:5000, whereas the given absorbances for the dilution of 1:10 000 of the antibody already vary in a range from 0.2 to 1.0, corresponding to an optimized standard curve in buffer solution.

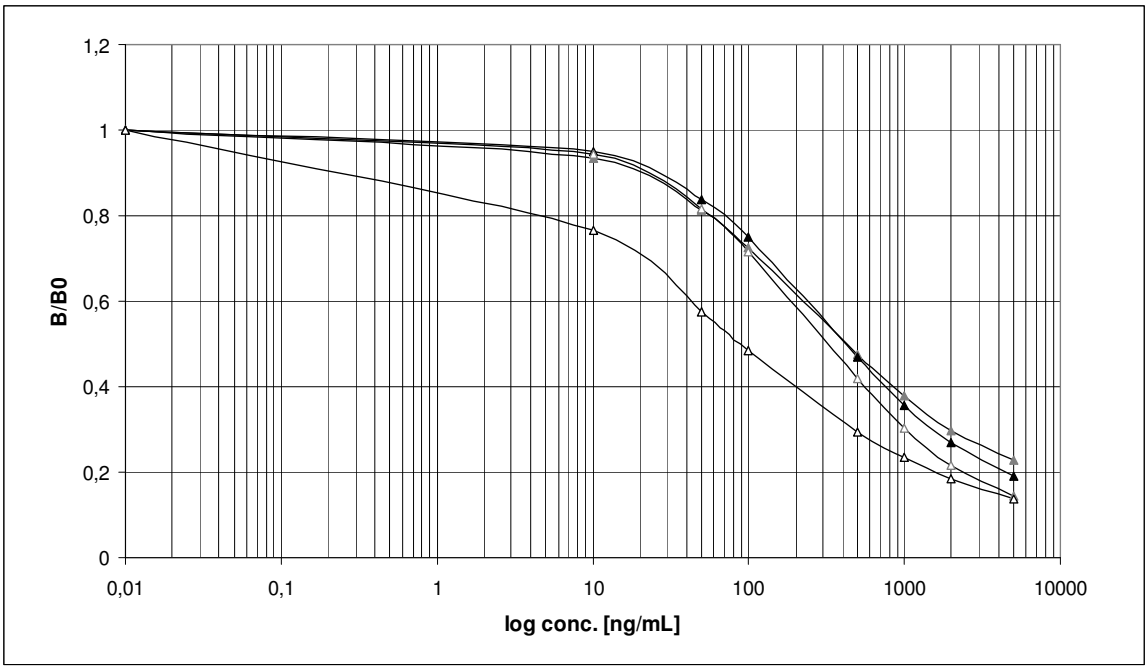

Figure 11 Soy bean competitive ELISA. The blocking was performed with 1% Ficoll in coating buffer (pH 9.6). Rabbit-anti-soy antibody was diluted 1:1000 (▲), 1:5000 (▲), 1:10 000 (Δ) and 1:50 000 (Δ).

Looking at figure 11, the standard curves obtained for rabbit-anti-soy were compared and the dilution 1:5000 (▲) was chosen for further studies. The behaviour of the curves is similar for the dilutions 1:1000, 1:5000 and 1:10 000, but table 18 shows that the range in absorbance is bigger for 1:5000 than for 1:10 000, which is important for the cross-reactivity studies.

These dilutions and criteria were regarded for all food allergens. Not all curves of the first experiments are shown in the master thesis, just the ones which were chosen to obtain the best results.

4.7 Indirect Sandwich and competitive Immunoassays for hazelnut

There were two antibodies available for hazelnut: from rabbit and from chicken. The antibodies were characterized separately in the competitive ELISA format and together in the Sandwich ELISA formats. The data from table 19 were measured with the ELISA reader and the standard curves formed by Magellan5 software. The B/B_0 and B/B_{max} values were calculated by Microsoft Excel.

Table 19 Obtained absorbances for the Sandwich ELISA for hazelnut.

	coating IgY-anti-hazelnut						coating rabbit-anti-hazelnut				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
400	1.38	1.401	1.400	1.394	0.012		2.654	2.631	2.233	2.506	0.237
200	1.068	1.087	1.000	1.052	0.046		2.511	2.562	2.037	2.370	0.290
100	0.830	0.818	0.820	0.823	0.006		2.267	2.265	1.735	2.089	0.307
60	0.653	0.625	0.634	0.637	0.014		2.031	2.126	1.445	1.867	0.369
30	0.475	0.470	0.470	0.472	0.003		1.496	1.475	1.047	1.339	0.253
10	0.352	0.385	0.377	0.371	0.017		0.950	0.947	0.607	0.835	0.197
5	0.268	0.216	0.223	0.236	0.028		0.738	0.602	0.520	0.620	0.110
0.01	0.185	0.161	0.178	0.175	0.012		0.200	0.239	0.389	0.276	0.100

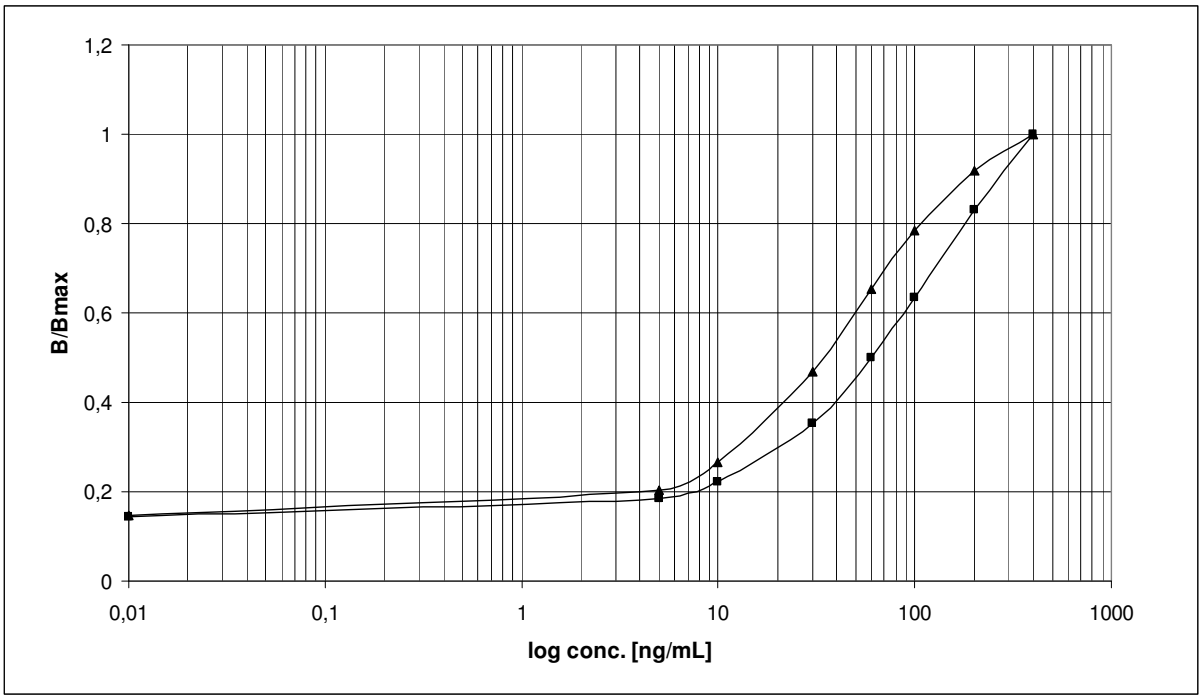

Figure 12 Hazelnut Sandwich ELISA. Blocking was performed with 1% PVA in 10 m PBS buffer. Two formats were performed: coating 1 µg mL^{-1} IgY-anti-hazelnut (■) and coating rabbit-anti-hazelnut (▲).

The data from table 19 show that both Sandwich formats work well. The range in absorbance from lowest to highest standards is greater for coating rabbit-anti-hazelnut and use IgY-anti-hazelnut as secondary antibody than the other way round. Figure 12 shows that this antibody combination shows a more sensitive curve comparing the B/B_{max} values because the IC_{50}-value is further to the left (IC_{50} of the first Sandwich format (▲) is 51.4 ng mL^{-1}, IC_{50} of the latter (■) is 128.0 ng mL^{-1}).

Table 20 lists the experimental data for the competitive ELISA for hazelnut.

Table 20 Obtained absorbances for the competitive ELISA assays for hazelnut.

	IgY-anti-hazelnut						rabbit-anti-hazelnut				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.120	0.178	0.175	0.158	0.033		0.318	0.417	0.379	0.371	0.050
2000	0.228	0.244	0.223	0.232	0.011		0.744	0.740	0.671	0.718	0.041
1000	0.271	0.301	0.313	0.295	0.022		1.003	0.929	0.690	0.874	0.164
500	0.418	0.426	0.424	0.423	0.004		1.096	1.147	0.961	1.068	0.096
100	0.804	0.743	0.652	0.733	0.076		1.588	1.545	1.807	1.647	0.141
50	1.019	0.890	0.769	0.893	0.125		1.977	1.802	1.772	1.850	0.111
10	0.865	0.857	0.908	0.877	0.027		2.064	2.172	1.819	2.018	0.181
0.01	0.985	0.977	0.947	0.970	0.020		2.091	2.001	1.881	1.991	0.105

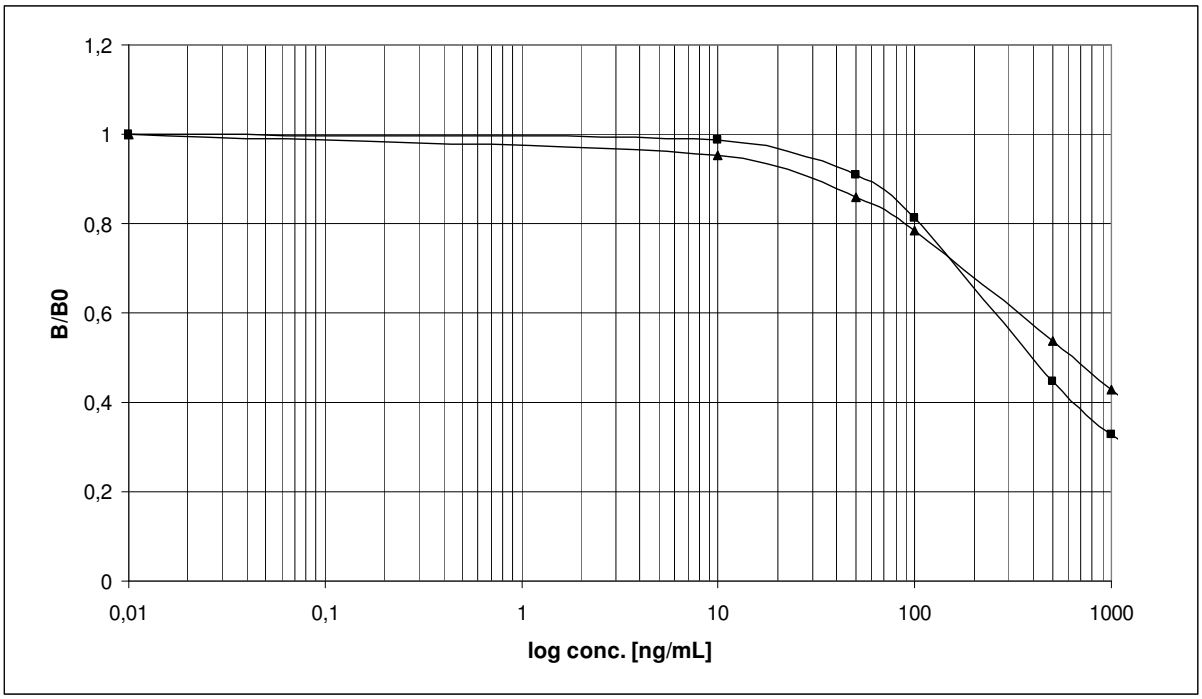

Figure 13 Hazelnut competitive ELISA. Blocking was performed with 1% PVA in 10 mM PBS buffer. Two antibodies were characterized: IgY-anti-hazelnut (■), diluted 1:1000 and rabbit-anti-hazelnut (▲), diluted 1:10000.

In figure 13 both antibodies show a good performance in the competitive ELISA format. The dilutions of the antibodies indicate that IgY-anti-hazelnut is less sensitive than rabbit-anti-hazelnut. The dilution varies by a factor of 10, which is typical for the difference in origin. The antibodies behave as expected. All of the rabbit and chicken antibodies for the detection of food allergens which were tested during the master thesis showed the same behaviour and differences in sensitivity.

4.8 Indirect Sandwich and competitive ELISA assays for α-casein

Chicken anti-α-casein and rabbit anti-α-casein antibodies were coated and blocked with 1% Ficoll in coating buffer (pH 9.6) for two hours at room temperature or with 1% PVA in PBS buffer (pH 7.6) at 4 °C overnight. Four-parameter equations were formed with the measured absorbances by Magellan5 software. The measurements were all performed in triplicate. B/B_0 and B/B_{max} were calculated out of the absorbances derived from the four-parameter curves.

Table 21 Measured absorbances for two Sandwich formats. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received by coating IgY-anti-α-casein, the values on the right by coating rabbit-anti-α-casein.

	Ficoll blocking; IgY-anti-α-casein; R=0.996						Ficoll blocking; rabbit-anti-α-casein; R=0.922				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		Measured absorbances			Average value	std.dev.
200	0.796	0.733	0.770	0.766	0.032		1.221	1.033	1.059	1.104	0.102
150	0.782	0.781	0.735	0.766	0.027		1.694	1.007	1.753	1.485	0.415
100	0.757	0.742	0.716	0.738	0.021		0.851	0.847	0.874	0.857	0.015
60	0.656	0.633	0.611	0.633	0.023		0.743	0.757	0.764	0.755	0.011
30	0.423	0.445	0.431	0.433	0.011		0.596	0.616	0.596	0.603	0.012
10	0.224	0.206	0.176	0.202	0.024		0.438	0.448	0.435	0.440	0.007
5	0.139	0.103	0.105	0.116	0.020		0.573	0.370	0.355	0.433	0.122
0.01	0.074	0.071	0.052	0.066	0.012		0.393	0.377	0.285	0.352	0.058

Table 22 Measured absorbances for two Sandwich formats. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received by coating IgY-anti-α-casein, the values on the right by coating rabbit-anti-α-casein.

	PVA blocking; IgY-anti-casein; R=0.999						PVA blocking; rabbit-anti-casein; R=0.911				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		Measured absorbances			average value	Std.dev.
200	1.388	1.288	1.202	1.293	0.093		1.067	0.999	0.907	0.991	0.080
150	1.295	1.238	1.234	1.256	0.034		0.881	0.883	0.820	0.861	0.036
100	1.177	1.044	1.095	1.105	0.067		0.823	0.827	0.768	0.806	0.033
60	0.922	0.876	0.815	0.871	0.054		0.806	0.681	0.664	0.717	0.078
30	0.510	0.510	0.534	0.518	0.014		0.569	0.568	0.520	0.552	0.028
10	0.263	0.253	0.274	0.263	0.011		0.421	0.509	0.454	0.461	0.044
5	0.193	0.196	0.191	0.193	0.003		0.355	0.384	0.485	0.408	0.068
0.01	0.153	0.152	0.154	0.153	0.001		0.342	0.773	0.290	0.468	0.265

Tables 21 and 22 show the experimental data for the assays in Sandwich format, blocked with Ficoll (table 21) and PVA (table 22). Each assay could be compared directly at the two different blocking conditions and they perfectly matched. There was a difference between the two formats, depending on the antibody used for coating and for detecting α-casein. The curves for rabbit-anti-α-casein coating started with higher blank values and showed a much lower increase in absorbance than the curves obtained with IgY-anti-α-casein coating.

The same assays without coated antibody showed good blocking effects in both assay formats for the corresponding blocking solutions. Hence, only one blocking curve is shown in figure 14. No curve was achieved, indicating the good inhibition by the blocking solution.

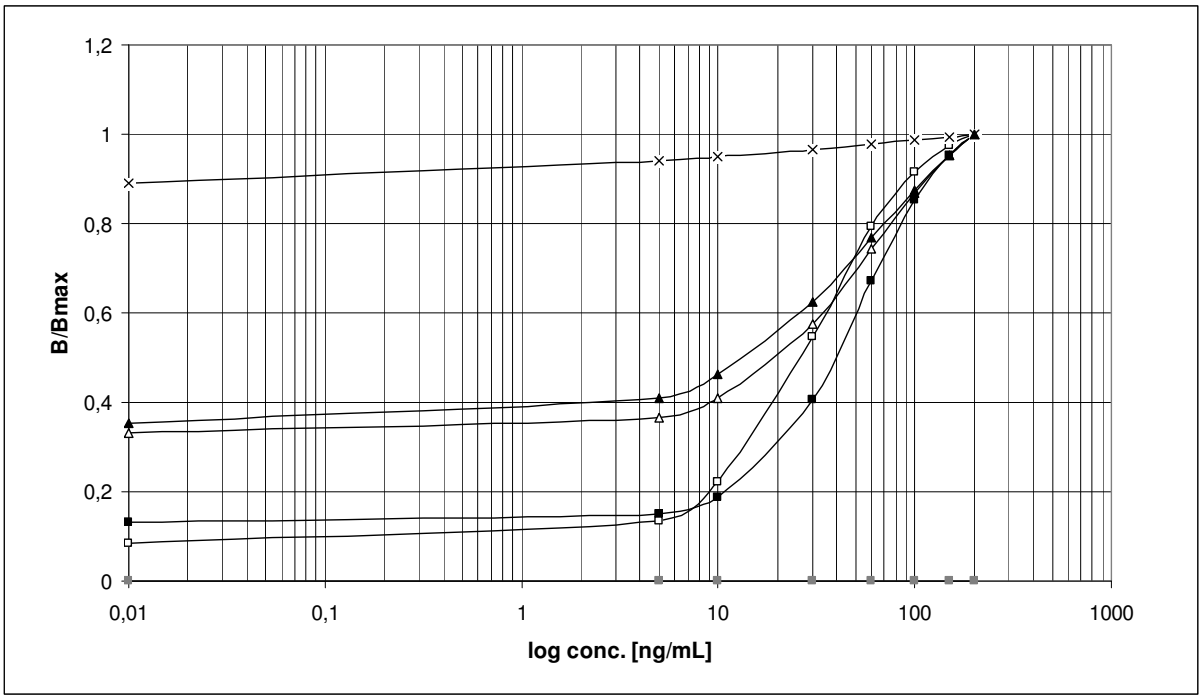

Figure 14 α-casein Sandwich ELISA: Blocking was performed with PVA at pH 7.6 or with Ficoll at pH 9.6. Two Sandwich formats were tested: coating 1 µg mL^{-1} rabbit-anti-α-casein with PVA blocking (▲), coating 1 µg mL^{-1} rabbit-anti-α-casein with Ficoll blocking (Δ), coating 1 µg mL^{-1} IgY-anti-α-casein with PVA blocking (■), coating 1 µg mL^{-1} IgY-anti-α-casein with Ficoll blocking (□). The assays were performed without coating rabbit-antibody before to check the blocking efficiency too: PVA blocking in the sandwich format with IgY-anti-α-casein is shown (-x-).

For the competitive ELISAs blocking was performed with 1% Ficoll or 1% BSA in coating buffer for two hours at room temperature or 1% PVA in PBS buffer at 4 °C overnight. Tables 23 (blocked with BSA), 24 (blocked with Ficoll) and 25 (blocked with PVA) show the experimental data for figure 15.

Table 23 Measured absorbances for two competitive format. The blocking was performed with 1% BSA in coating buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	BSA blocking; IgY-anti-α-casein; R=0.973						BSA blocking; rabbit-anti-α-casein, R=0.999				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	Std.dev.
5000	0.189	0.217	0.175	0.194	0.021		0.329	0.276	0.271	0.292	0.032
2000	0.321	0.327	0.295	0.314	0.017		0.484	0.411	0.410	0.435	0.042
1000	0.484	0.544	0.494	0.507	0.032		0.572	0.538	0.524	0.545	0.025
500	0.684	0.731	0.709	0.708	0.024		0.667	0.649	0.681	0.666	0.016
100	1.194	1.162	1.320	1.225	0.084		1.049	1.035	1.062	1.049	0.014
50	1.246	1.355	1.488	1.363	0.121		1.182	1.146	1.191	1.173	0.024
10	1.262	1.232	1.172	1.222	0.046		1.366	1.378	1.366	1.370	0.007
0.01	1.317	1.174	1.109	1.200	0.106		1.524	1.438	1.492	1.485	0.043

Table 24 Measured absorbances for two competitive formats. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	Ficoll blocking; IgY-anti-α-casein; R=0.996						Ficoll blocking; rabbit-anti-α-casein; R=0.997				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.141	0.158	0.175	0.158	0.017		0.203	0.203	0.191	0.199	0.007
2000	0.246	0.287	0.318	0.284	0.036		0.411	0.446	0.410	0.422	0.021
1000	0.411	0.421	0.412	0.415	0.006		0.595	0.654	0.634	0.628	0.030
500	0.453	0.503	0.485	0.480	0.025		0.827	0.835	0.729	0.797	0.059
100	0.775	0.754	0.732	0.754	0.022		1.243	1.211	1.226	1.227	0.016
50	0.754	0.847	0.742	0.781	0.057		1.375	1.317	1.302	1.331	0.039
10	0.984	0.939	0.893	0.939	0.046		1.495	1.500	1.496	1.497	0.003
0.01	1.026	0.995	0.945	0.989	0.041		1.538	1.589	1.561	1.563	0.026

Table 25 Measured absorbances for two competitive formats. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	PVA blocking; IgY-anti-α-casein; R=0.999						PVA blocking; rabbit-anti-α-casein; R=0.999				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	Std.dev.
5000	0.240	0.245	0.251	0.245	0.006		0.534	0.426	0.404	0.455	0.070
2000	0.497	0.489	0.504	0.497	0.008		0.502	0.481	0.545	0.509	0.033
1000	0.772	0.744	0.690	0.735	0.042		0.842	0.836	0.845	0.841	0.005
500	1.067	1.113	1.011	1.064	0.051		1.048	1.117	1.121	1.095	0.041
100	1.545	1.535	1.501	1.527	0.023		1.384	1.375	1.341	1.367	0.023
50	1.546	1.621	1.596	1.588	0.038		1.361	1.515	1.410	1.429	0.079
10	1.639	1.741	1.690	1.690	0.051		1.490	1.438	1.490	1.473	0.030
0.01	1.638	1.725	1.699	1.687	0.045		1.442	1.512	1.439	1.464	0.041

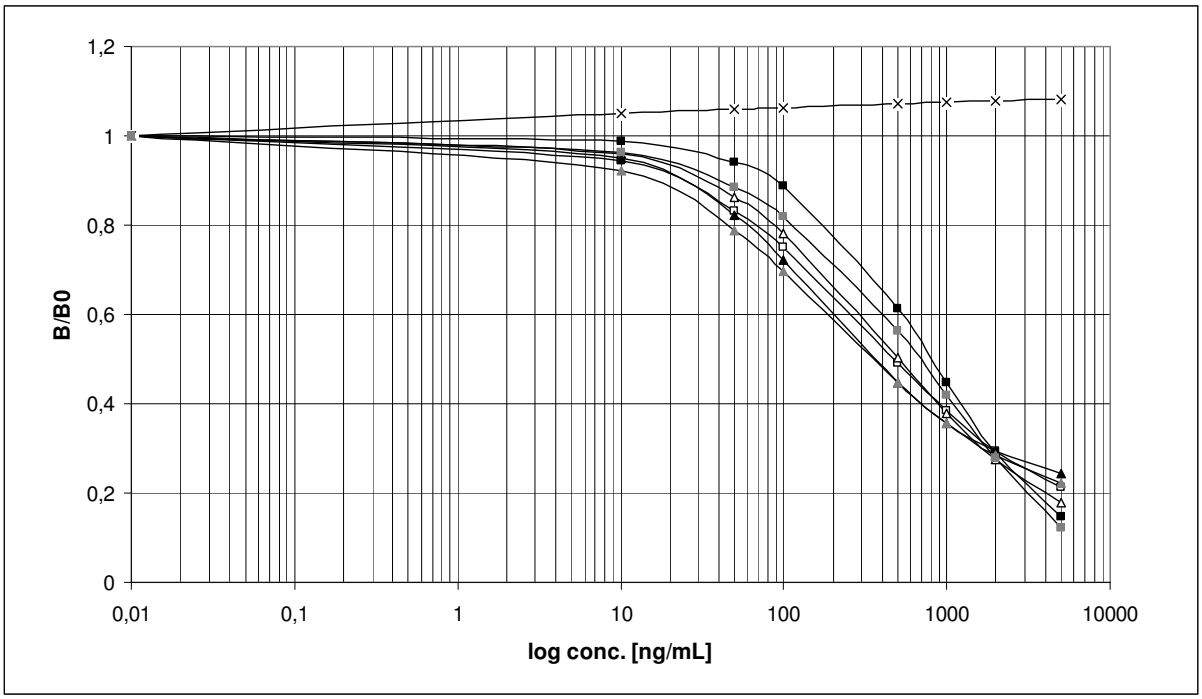

Figure 15 α-casein competitive ELISA: The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. 500 ng mL^{-1} of α-casein were coated on the microtiter plate and blocked with PVA at pH 7.6, BSA at pH 9.6 or Ficoll at pH 9.6. Rabbit-anti-α-casein with PVA blocking (▲), rabbit-anti-α-casein with BSA blocking (▲) and rabbit-anti-α-casein with Ficoll blocking (Δ) were diluted 1:10 000. IgY-anti-α-casein with PVA blocking (■), IgY-anti-α-casein with BSA blocking (■) and IgY-anti-α-casein with Ficoll blocking (□) were diluted 1:1000. The assays were performed without coating α-casein before to check the blocking efficiency as well: PVA blocking with rabbit-anti-α-casein is shown (-x-).

The results were calculated in the same way as for the sandwich ELISA. The B/B_0 values were calculated from the absorbances, derived from four-parameter curves from Magellan5 software. Again, only one curve is shown for the blocking capacity of the two blocking reagents. Two aspects in figure 15 are worth considering: The standard curves for rabbit-anti-α-casein showed the same behaviour no matter if blocked with BSA, Ficoll or PVA. The blocking solutions were equally suitable. The same was shown for IgY-anti-α-casein, but the IC_{50}-value of the curve which was blocked with PVA (IC_{50} = 785.1 ng mL^{-1}) was higher than the one blocked with Ficoll (IC_{50} = 341.0 ng mL^{-1}). The standard curve obtained with BSA blocking can be found in between these two curves. Hence, the efficiencies of PVA and Ficoll blocking were proven.

By comparing the two types of polyclonal antibodies according to their different origin, different sensitivities can be shown with the competitive ELISA format (figure 15). The rabbit antibody was diluted 10 times higher than the chicken antibody. However, the antibodies are equally sensitive within the appropriate dilutions in the competitive ELISA

format. The curves were all very close and had a similar shape. In particular, the standard curves of both antibodies which were blocked with Ficoll were nearly identical.

4.9 Indirect Sandwich and competitive ELISA assays for peanut

Chicken and rabbit antibodies were coated onto the microtiter plate and blocked with 1% Ficoll in coating buffer for two hours at room temperature or 1% PVA in 10 mM PBS buffer at 4 °C overnight. The measurements were performed in triplicate and Magellan5 software formed a four-parameter curve out of these values, which was then applied for calculating B/B_0 and B/B_{max} values for each concentration measured (experimental data in table 26 and 27). These data can be seen in figure 16 for the sandwich ELISA for peanut.

Table 26 Measured absorbances for two Sandwich formats. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received by coating IgY-anti-peanut, the values on the right by coating rabbit-anti-peanut.

	Ficoll blocking; IgY-anti-peanut; R=0.998						Ficoll blocking; rabbit-anti-peanut; R=0.984				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
400	1.910	2.038	1.804	1.917	0.117		1.900	1.672	1.661	1.744	0.135
200	1.733	1.715	1.729	1.726	0.009		1.680	1.434	0.934	1.349	0.380
100	1.445	1.467	1.513	1.475	0.035		1.243	1.192	1.061	1.165	0.094
60	1.291	1.261	1.327	1.293	0.033		0.874	0.885	0.819	0.859	0.035
30	1.054	1.078	1.119	1.084	0.033		0.764	0.723	0.737	0.741	0.021
10	0.695	0.822	0.750	0.756	0.064		0.559	0.590	0.596	0.582	0.020
5	0.531	0.552	0.556	0.546	0.013		0.481	0.562	0.536	0.526	0.041
0.01	0.146	0.147	0.135	0.143	0.007		0.234	0.239	0.231	0.235	0.004

Table 27 Measured absorbances for two Sandwich formats. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received by coating IgY-anti-peanut, the values on the right by coating rabbit-anti-peanut.

	PVA blocking; IgY-anti.peanut; R=0.994						PVA blocking; rabbit-anti-peanut; R=0.999				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
400	2.078	2.146	2.104	2.109	0.034		1.628	1.560	1.618	1.602	0.037
200	2.251	2.059	2.011	2.107	0.127		1.445	1.472	1.333	1.417	0.074
100	1.670	1.699	1.735	1.701	0.033		1.403	1.271	1.287	1.320	0.072
60	1.492	1.520	1.541	1.518	0.025		1.274	0.520	0.992	0.929	0.381
30	1.229	1.280	1.302	1.270	0.037		0.864	0.834	0.788	0.829	0.038
10	1.128	0.920	0.920	0.989	0.120		0.475	0.519	0.481	0.492	0.024
5	0.660	0.669	0.604	0.644	0.035		0.364	0.354	0.381	0.366	0.014
0.01	0.188	0.255	0.214	0.219	0.034		0.145	0.142	0.200	0.162	0.033

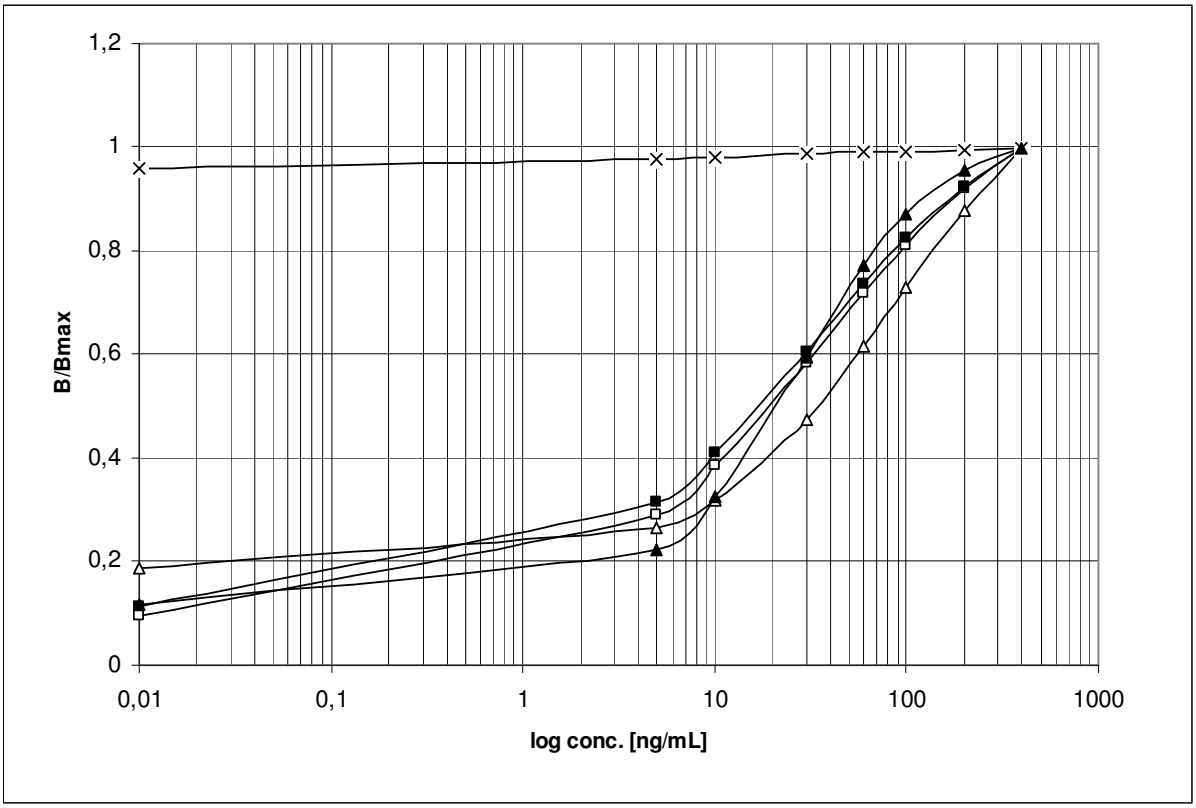

Figure 16 Peanut Sandwich ELISA: Blocking was performed with PVA at pH 7.6 or with Ficoll at pH 9.6. Two Sandwich formats were tested: coating 0.1 µg mL^{-1} rabbit-anti-peanut with PVA blocking (▲), coating 0.1 µg mL^{-1} rabbit-anti-peanut with Ficoll blocking (Δ), coating 2 µg mL^{-1} IgY-anti-peanut with PVA blocking (■), coating 2 µg mL^{-1} IgY-anti-peanut with Ficoll blocking (□). The assays were also performed without coating antibody before to check the blocking efficiency: PVA blocking with IgY-anti-peanut is shown here (-x-).

The curves can be compared in respect of the blocking solution and of the antibodies. All curves were very close and showed the same behaviour. Both Ficoll and PVA proved to be effective blockers and yielded results that perfectly matched.

The assays were performed without coating antibodies before as well. One outcome is shown in figure 16 because they did not differ in behaviour. It was a straight line close to 1 for B/B_{max}, lacking non-specific binding.

The competitive ELISA assays were equally performed with chicken and rabbit antibodies and blocked with 1% Ficoll in coating buffer for two hours at room temperature or with 1% PVA in 10 mM PBS buffer at 4 °C overnight. The calculation of the curves was done as described for the sandwich ELISA format. Table 28 and 29 show the experimental data for the competitive ELISA which can be seen in figure 17.

Table 28 Measured absorbances for two competitive formats. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received using IgY-anti-peanut, the values on the right by using rabbit-anti-peanut.

	Ficoll blocking; IgY-anti-peanut; R=0.999						Ficoll blocking; rabbit-anti-peanut; R=0.988				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.325	0.337	0.361	0.341	0.018		0.407	0.410	0.397	0.405	0.007
2000	0.410	0.457	0.421	0.429	0.025		0.440	0.444	0.447	0.444	0.004
1000	0.569	0.533	0.524	0.542	0.024		0.478	0.495	0.499	0.491	0.011
500	0.720	0.719	0.716	0.718	0.002		0.518	0.588	0.523	0.543	0.039
100	1.303	1.288	1.254	1.282	0.025		0.673	0.776	0.728	0.726	0.052
50	1.540	1.392	1.479	1.470	0.074		0.775	0.904	0.825	0.835	0.065
10	1.776	1.807	1.769	1.784	0.020		1.083	1.134	1.149	1.122	0.035
0.01	1.858	1.818	1.967	1.881	0.077		0.909	1.210	1.151	1.090	0.160

Table 29 Measured absorbances for two competitive formats. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received using IgY-anti-peanut, the values on the right by using rabbit-anti-peanut.

	PVA blocking; IgY-anti-peanut; R=0.998						PVA blocking; rabbit-anti-peanut; R=0.998				
standard conc. [ng/mL]	measured absorbances			Average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.208	0.193	0.214	0.205	0.011		0.297	0.295	0.296	0.296	0.001
2000	0.253	0.251	0.257	0.254	0.003		0.354	0.361	0.358	0.358	0.004
1000	0.287	0.318	0.287	0.297	0.018		0.362	0.391	0.387	0.380	0.016
500	0.328	0.369	0.357	0.351	0.021		0.448	0.479	0.495	0.474	0.024
100	0.707	0.749	0.739	0.732	0.022		0.722	0.768	0.729	0.740	0.025
50	0.915	1.066	1.000	0.994	0.076		0.868	0.909	0.903	0.893	0.022
10	1.426	1.540	1.492	1.486	0.057		1.086	1.180	1.151	1.139	0.048
0.01	1.393	1.775	1.737	1.635	0.210		1.227	1.223	1.235	1.228	0.006

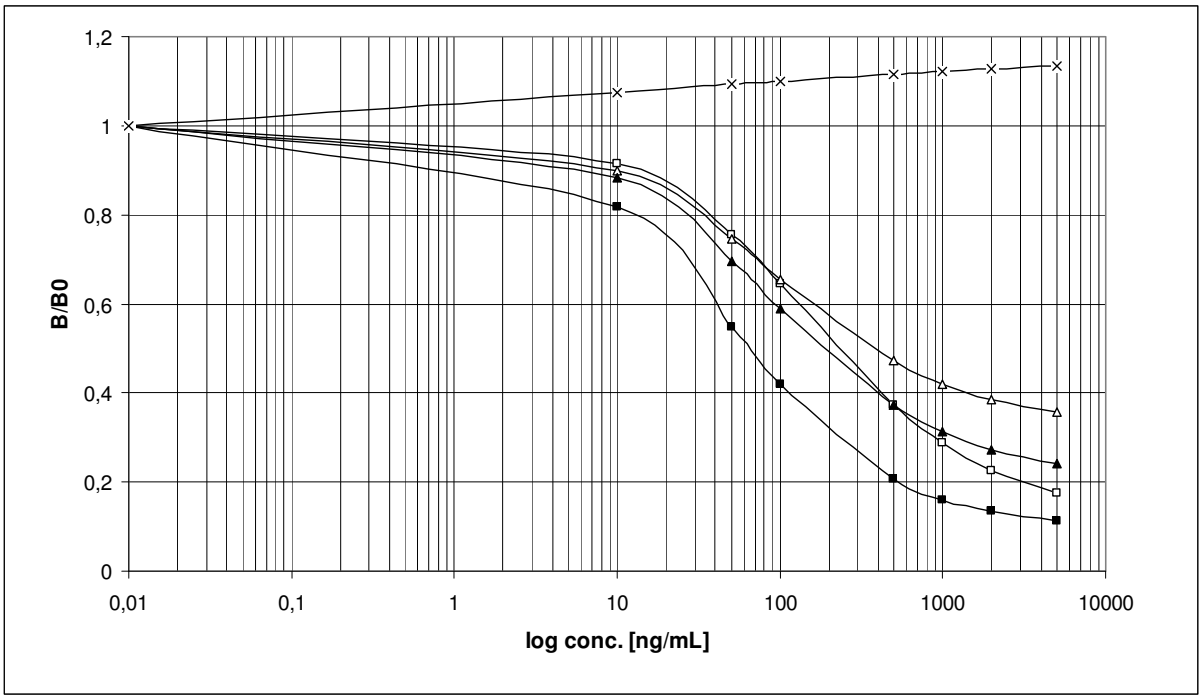

Figure 17 Peanut competitive ELISA: The assay was performed with rabbit-anti-peanut and IgY-anti-peanut. 500 ng mL^{-1} of peanut were coated on the microtiter plate and blocked with PVA at pH 7.6 or Ficoll at pH 9.6. Rabbit-anti-peanut with PVA blocking (▲) and rabbit-anti-peanut with Ficoll blocking (Δ) were diluted 1:10 000. IgY-anti-peanut with PVA blocking (■) and IgY-anti-peanut with Ficoll blocking (□) were diluted 1:1000. Again the assays were run without coating peanut before: PVA blocking and the performance of rabbit-anti-α-casein is shown (-x-).

Figure 17 shows the results of the competitive ELISA formats for peanut detection which are comparable for both blocking solutions. Parallel standard curves were obtained for each antibody in regard to the blocking solution used. The IC_{50}-values for rabbit-anti-peanut antibody differ slightly between Ficoll and PVA blocking. For IgY-anti-peanut antibody the IC_{50}-values differ by a factor of 3, but the behaviour of the curve is similar for both blocking reagents. The blocking solutions were both effective.

The assays prepared without coating peanut did not show OD values above 0.2. Therefore the blocking efficiency of Ficoll and PVA was established once again.

4.10 Results of the cross-reactivity studies for hazelnut and peanut

Two different sets of tests were performed to determine cross-reactive agents for hazelnut and peanut: At first, the extracts of various food samples were treated like standards, therefore they were diluted in a serial row and measured like the food allergen standard

itself in a competitive ELISA format (see table 30 and figure 18 for the examples of hazelnut, peanut and almond).

Table 30 Obtained absorbances for the cross-reactivity study of anti-hazelnut antibodies for hazelnut, peanut and almond.

hazelnut	IgY-anti-hazelnut						rabbit-anti-hazelnut				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.809	0.714	0.690	0.738	0.063		0.678	0.733	0.870	0.760	0.099
2000	1.127	1.070	1.073	1.090	0.032		1.339	1.046	0.984	1.123	0.190
1000	1.437	1.478	1.406	1.440	0.036		1.545	1.405	0.996	1.315	0.285
500	1.723	1.702	1.623	1.683	0.053		1.626	1.499	1.686	1.604	0.095
100	2.009	2.082	1.953	2.015	0.065		1.852	1.857	1.714	1.808	0.081
50	2.125	2.088	2.019	2.077	0.054		1.991	2.002	1.776	1.923	0.127
10	2.128	2.154	2.064	2.115	0.046		2.156	1.877	2.105	2.046	0.149
0.01	2.162	2.14	2.135	2.146	0.014		2.177	2.105	2.081	2.121	0.050
peanut	IgY-anti-hazelnut						rabbit-anti-hazelnut				
standard conc. [µg/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
1000	1.499	0.813	1.446	1.253	0.382		0.984	1.037	1.155	1.059	0.088
500	1.804	1.739	1.738	1.760	0.038		1.428	1.353	1.394	1.392	0.038
100	2.099	0.500	2.091	1.563	0.921		2.105	2.037	1.810	1.984	0.154
10	2.263	1.995	2.209	2.156	0.142		2.016	1.834	2.032	1.961	0.110
1	2.207	2.082	2.195	2.161	0.069		1.847	1.966	2.084	1.966	0.119
0.1	2.165	2.081	2.202	2.149	0.062		2.082	2.008	1.789	1.960	0.152
0.01	2.174	2.089	2.216	2.160	0.065		2.072	2.026	1.897	1.998	0.091
0.00001	2.143	2.049	2.133	2.108	0.052		1.988	2.002	2.006	1.999	0.009
almond	IgY-anti-hazelnut						rabbit-anti-hazelnut				
standard conc. [µg/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
1000	2.020	2.050	1.824	1.965	0.123		2.120	2.102	2.056	2.093	0.033
500	2.089	2.105	1.952	2.049	0.084		1.943	2.003	1.896	1.947	0.054
100	2.127	2.178	1.981	2.095	0.102		2.023	2.141	2.080	2.081	0.059
10	2.194	2.227	2.034	2.152	0.103		2.033	1.963	1.879	1.958	0.077
1	2.177	2.252	2.067	2.165	0.093		1.948	2.038	1.882	1.956	0.078
0.1	2.152	2.207	2.046	2.135	0.082		1.847	1.839	1.946	1.877	0.060
0.01	2.146	2.205	2.038	2.130	0.085		1.940	1.918	1.834	1.897	0.056
0.00001	2.129	2.185	2.041	2.118	0,073		2.276	2.132	2.160	2.189	0.076

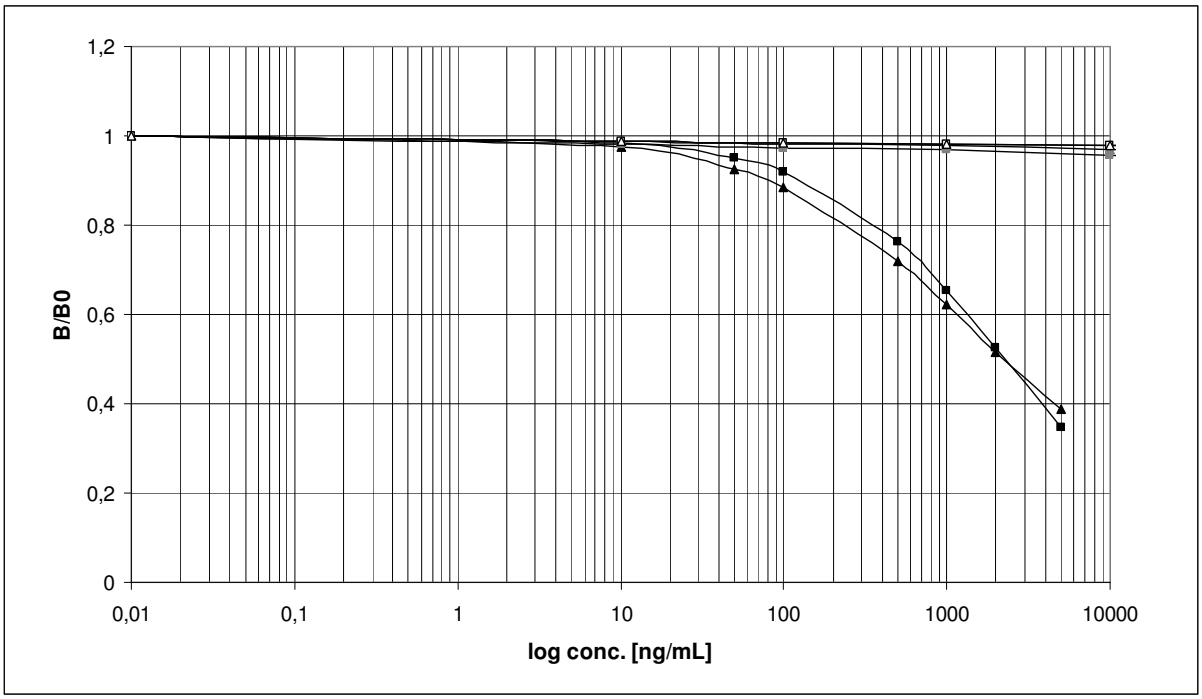

Figure 18 Cross-reactivity study for IgY-anti-hazelnut and rabbit-anti-hazelnut. Hazelnut standard was detected with IgY-anti-hazelnut (■) and rabbit-anti-hazelnut (▲), peanut detected with IgY-anti-hazelnut (■) and rabbit-anti-hazelnut (▲) and almond detected with IgY-anti-hazelnut (□) and rabbit-anti-hazelnut (Δ).

The obtained standard curves for anti-hazelnut antibodies, peanut and almond show already, that there is no cross reactivity for almond and for peanut within the tested concentration range. For the determination of cross-reactivity in percent, the IC_{50}-value was required. Magellan5 software gives four parameters to form the fitted curves, which were calculated out of the measurement data. The third parameter "c" is the IC_{50}-value. Table 31 lists these values for the obtained curves above.

Table 31 Listed IC_{50}-values for hazelnut, peanut and almond.

	IgY-anti-hazelnut	rabbit-anti-hazelnut
hazelnut	2474.6	1422.3
peanut	$1.7*10^9$	$5.4*10^9$
almond	$4.1*10^{23}$	14.3

Eq. 3 gives the percentage of cross-reactivity for anti-hazelnut antibodies, but the IC_{50}-values here were too high for peanut and not reliable for almond. Hence, secondly, the extracts were treated like samples and therefore measured undiluted and in a dilution of 1:10. The absorbances and the calculated concentrations are listed in table 32.

Table 32 Obtained absorbances and calculated concentrations by Magellan5 software. -- = these concentrations could not be calculated because the corresponding absorbances were out of range of the hazelnut standard curve.

absorbances				calc.conc. [ng/mL]		
	IgY-anti-hazelnut	rabbit-anti-hazelnut			IgY-anti-hazelnut	rabbit-anti-hazelnut
peanut	0.28	0.39		peanut	--	--
1:10	1.01	1.52		1:10	1328.90	810.43
almond	1.28	1.83		almond	736.50	390.38
1:10	1.80	2,52		1:10	218.45	--

The concentration of the peanut extract was 15 mg mL^{-1}, of the almond extract 15.5 mg mL^{1}. Eq. 4 gives the result of the cross-reactivity in percent. Table 33 shows the outcome of all those studies.

Table 33 Summarized results for the cross reactivity studies for anti-hazelnut and anti-peanut antibodies; n.d. = non- detectable

	IgY-anti-hazelnut	rabbit-anti-hazelnut		IgY-anti-peanut	rabbit-anti-peanut
hazelnut	--	--		n.d.	n.d.
peanut	0.09%	0.05%		--	--
cashew nut	n.d.	n.d.		n.d.	n.d.
egg york	n.d.	n.d.		n.d.	n.d.
egg white	n.d.	n.d.		n.d.	n.d.
coco	n.d.	n.d.		n.d.	n.d.
linseed	n.d.	n.d.		n.d.	n.d.
lupine	0,01%	n.d.		n.d.	n.d.
almond	n.d.	n.d.		n.d.	n.d.
milk powder	n.d.	n.d.		n.d.	n.d.
puppy seed	n.d.	n.d.		n.d.	n.d.
pistachio	n.d.	n.d.		n.d.	n.d.
sesame	n.d.	n.d.		n.d.	n.d.
soy bean flour	0.01%	0.01%		n.d.	n.d.
sun flower kernel	n.d.	n.d.		n.d.	n.d.
walnut	n.d.	n.d.		n.d.	n.d.

Both antibodies against hazelnut cross-reacted a little with peanut (IgY-anti-hazelnut: 0.09% and rabbit-anti-hazelnut: 0.05%) and soy bean flour (both: 0.01%). IgY-anti-hazelnut was additionally cross-reactive with lupine (0.01%). They were not cross-reactive with cashew nut, egg york, egg white, coco, linseed, almond, milk powder, puppy seed, sun flower kernels and walnut.

The antibodies for the detection of peanut were not cross-reactive with any of all the samples tested. It was expected for soy bean flour or other nuts to be similar to the peanut

food allergen, but the studies showed that these assumptions were not true. There is no further cleaning of the anti-peanut antibodies essential. For the anti-hazelnut antibodies it was not necessary to clean with an affinity column against peanut because the cross-reactivities were too low.

4.11 Results of the cross-reactivity studies for α-casein and soy bean

To determine cross-reactive agents for α-casein and soy bean, the extracts of various food samples were treated like standards, therefore they were diluted in a serial row and measured like the food allergen standard itself (see table 34 and figure 19 for the examples of α-casein, β.casein and κ-casein) in a competitive ELISA format.

Table 34 Obtained absorbances for the cross-reactivity study of anti-α-casein antibodies for α-casein, β – casein and κ-casein.

α-casein	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.245	0.226	0.205	0.225	0.020		0.297	0.237	0.234	0.256	0.036
2000	0.532	0.497	0.444	0.491	0.044		0.483	0.409	0.391	0.428	0.049
1000	0.835	0.854	0.702	0.797	0.083		0.713	0.632	0.616	0.654	0.052
500	1.304	1.147	1.227	1.226	0.079		0.933	1.012	0.957	0.967	0.041
100	1.753	1.748	1.701	1.734	0.029		1.523	1.517	1.496	1.512	0.014
50	1.828	1.790	1.747	1.788	0.041		1.806	1.710	1.716	1.744	0.054
10	1.803	1.892	1.764	1.820	0.066		1.909	1.953	1.903	1.922	0.027
0.01	1.912	1.853	1.820	1.862	0.047		2.022	2.055	2.016	2.031	0.021
β -casein	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [µg/mL]	measured absorbances			average value	Std.dev.		measured absorbances			average value	std.dev.
1000	0.222	0.256	0.238	0.239	0.017		0.288	0.290	0.288	0.289	0.001
500	0.288	0.293	0.321	0.301	0.018		0.285	0.341	0.329	0.318	0.029
100	0.653	0.667	0.690	0.670	0.019		0.604	0.680	0.620	0.635	0.040
10	1.622	1.645	1.635	1.634	0.012		1.423	1.477	1.416	1.439	0.033
1	1.829	1.814	1.842	1.828	0.014		1.811	1.836	1.742	1.796	0.049
0.1	1.823	1.805	1.815	1.814	0.009		1.918	1.935	1.917	1.923	0.010
0.01	1.759	1.843	1.776	1.793	0.044		1.908	2.002	1.842	1.917	0.080
0.00001	1.809	1.808	1.806	1.808	0.002		1.974	1.999	1.940	1.971	0.030
κ-casein	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [µg/mL]	measured absorbances			average value	Std.dev.		measured absorbances			average value	std.dev.
1000	0.172	0.159	0.177	0.169	0.009		0.153	0.174	0.147	0.158	0.014
500	0.206	0.239	0.235	0.227	0.018		0.162	0.210	0.180	0.184	0.024
100	1.824	0.286	0.284	0.798	0.889		0.279	0.300	0.288	0.289	0.011
10	1.201	1.171	1.232	1.201	0.031		1.025	1.027	1.037	1.030	0.006
1	1.751	1.787	1.804	1.781	0.027		1.737	1.692	1.623	1.684	0.057
0.1	1.804	1.816	1.805	1.808	0.007		1.861	1.934	1.863	1.886	0.042
0.01	1.811	1.788	1.821	1.807	0.017		2.012	1.942	1.808	1.921	0.104
0.00001	1.793	1.830	1.812	1.812	0.019		1.960	1.977	1.876	1.938	0.054

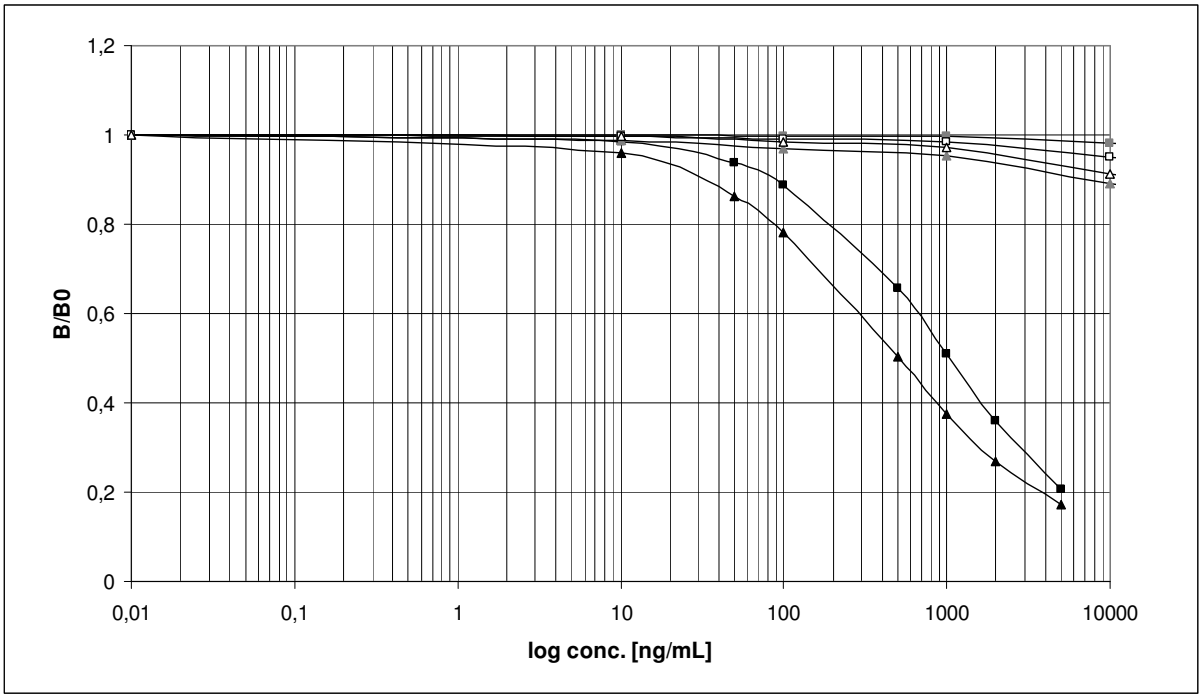

Figure 19 Cross-reactivity study for IgY-anti-α-casein and rabbit-anti-α-casein. α-casein standard was detected with IgY-anti-α-casein (■) and rabbit-anti-α-casein (▲), β-casein detected with IgY-anti-α-casein (■) and rabbit-anti-α-casein (▲) and κ-casein was detected with IgY-anti-α-casein (□) and rabbit-anti-α-casein (Δ).

The obtained standard curves show already, that both anti-α-casein antibodies were cross-reactive with other milk allergens. For the determination of cross-reactivity in percent, the IC_{50}-value was required again. Table 35 lists these values for the obtained curves above.

Table 35 Listed IC_{50}-values for α-casein, β-casein and κ-casein.

	IgY-anti-α-casein	rabbit-anti-α-casein
α-casein	993.4	431.8
β-casein	67334	14007
κ-casein	29633	9665.1

The relative cross-reactivities were calculated using Eq. 3. Table 36 shows the summarized data of all studies done for anti-α-casein and anti-soy antibodies.

Table 36 Summarized results of the cross-reactivity studies for anti-α-casein and anti-soy antibodies; n.d. = non detectable.

	rabbit-anti-soy		IgY-anti-α-casein	rabbit-anti-α-casein
soy bean flour	--		n.d.	n.d.
α-casein	n.d.		--	--
β-casein	n.d.		1.5%	3.1%
κ-casein	n.d.		3.4%	4.5%
α-lactalbumin	n.d.		n.d.	n.d.
β-lactoglobulin	n.d.		n.d.	n.d.
milk powder	n.d.		27.9%	34.5%
BSA	n.d.		n.d.	n.d.
egg york	n.d.		n.d.	n.d.
egg white	n.d.		n.d.	n.d.
peanut	n.d.		n.d.	n.d.
linseed	n.d.		n.d.	n.d.
lupine	<0.01%		n.d.	n.d.

Both antibodies for the detection of α-casein were cross-reactive with β- and κ-casein, two milk proteins. They were not cross-reactive with α-lactalbumin and β-lactoglobulin, two whey proteins. Hence, electrophoresis, silver nitrate staining and Western Blot were used to determine cross-reactivity of anti-α-casein antibodies for milk proteins (results shown in the following chapter). Both anti-α-casein antibodies were not cross-reactive with soy bean flour, BSA, egg york, egg white, peanut, linseed and lupine.

The rabbit-anti-soy antibody was not cross-reactive with any of the substances checked. Less than 0.01% reacted with lupine. Again, cross-reactivity with peanut was expected, but the studies showed that rabbit-anti-soy is not cross-reactive and therefore needed no further cleaning procedures.

4.12 Results of the electrophoresis of milk allergens, rabbit-anti-α-casein and IgY-anti-α-casein

Gels from SDS-PAGE were stained with coomassie blue and silver nitrate. They can be compared directly in figure 20.

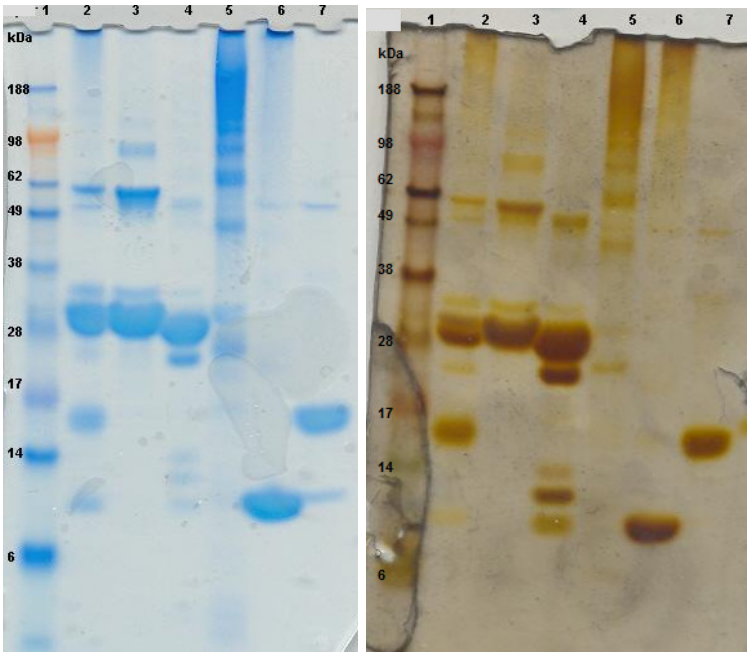

Figure 20 12% Bis/Tris gel, MES running buffer. Lane 1 shows the standard ladder. Lane 2 shows milk powder standard, lane 3 shows α-casein, lane 4 β-casein, lane 5 κ-casein, lane 6 α-lactalbumin and lane 7 shows β-lactoglobulin. On the left side, the gel was stained with coomassie blue, on the right side with silver nitrate.

Both gels showed the same bands of the milk proteins. The silver nitrate staining showed additional bands compared with the coomassie blue staining (see lane 4, between 6 and 14 kDa in figure 20). Further characterization was done by means of Western blotting with the two antibodies detecting α-casein.

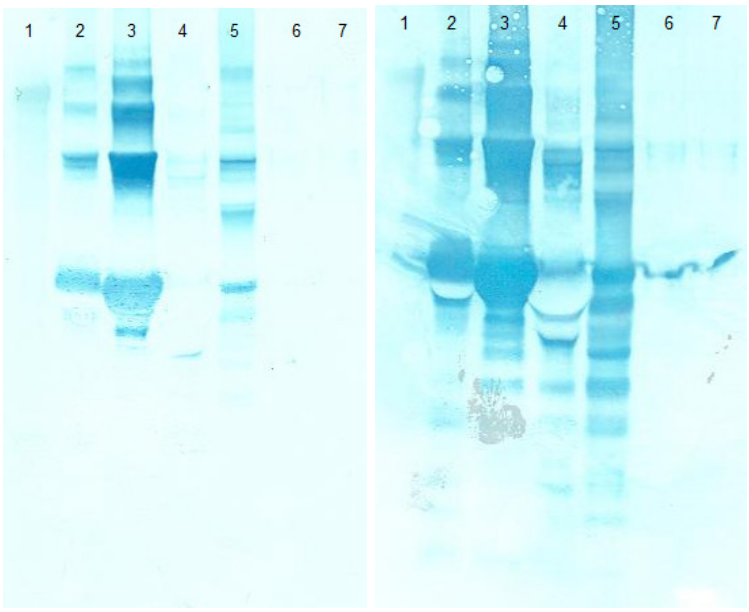

Figure 21 Nitrocellulose membranes from the Western Blot. Lane 1 shows the standard ladder. Lane 2 shows milk powder, lane 3 shows α-casein, lane 4 β-casein, lane 5 κ-casein, lane 6 α-lactalbumin and lane 7 shows β-lactoglobulin. The primary antibody, IgY-anti-α.casein, was used on the left membrane, on the right side rabbit-anti-α-casein was applied.

Seeing figure 21, both antibodies were most attracted to lane 3, to α-casein. They also bound some bands in milk powder (lane 2) and κ-casein (lane 5). Rabbit-anti-α-casein recognized β-casein too (on the right side), IgY-anti-α-casein detection shows only very faint lines (on the left side). Both antibodies did not bind to α-lactalbumin (lane 6) and β-lactoglobulin (lane 7). The results confirmed the outcome from the cross reactivity studies.

4.13 Comparison of different extraction buffers for the extraction of α-casein

Different buffers were tested for their ability to extract α-casein from different matrices. The matrices were all blanks for α-casein: candy cone (Zuckertüte), cookie (Biskotten), soy milk and whey drink (Latella). The buffers tested were: coating buffer (carbonate buffer, pH 9.6), 50 mM PBS buffer (1 M NaCl, pH 7.5), 0.2 M PBS buffer (pH 7.5) and extraction buffer from an available test kit from R-Biopharm. The extraction was performed at room temperature and at 60 °C. The absorbances of the standards are listed in table 37. The absorbances of the blank materials are listed in table 38.

Table 37 Obtained absorbances for the α-casein standard.

	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.215	0.208	0.190	0.204	0.013		0.163	0.143	0.150	0.152	0.010
2000	0.300	0.302	0.320	0.307	0.011		0.392	0.251	0.255	0.299	0.080
1000	0.496	0.478	0.440	0.471	0.029		0.423	0.390	0.369	0.394	0.027
500	0.750	0.773	0.679	0.734	0.049		0.608	0.532	0.523	0.554	0.047
100	1.355	1.355	1.303	1.338	0.030		0.989	0.912	0.928	0.943	0.041
50	1.360	1.417	1.330	1.369	0.044		1.074	1.020	1.006	1.033	0.036
10	1.531	1.474	1.458	1.488	0.038		1.204	1.151	1.117	1.157	0.044
0.01	1.477	1.482	1.443	1.467	0.021		1.214	1.210	1.234	1.219	0.013

Table 38 Obtained absorbances for the blank matrices.

candy cone	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
A carbonate buffer RT	1.081	0.916	0.805	0.934	0.139		0.87	0.941	0.895	0.902	0.036
B 50 mM PBS RT	0.346	0.327	0.321	0.331	0.013		0.886	0.870	0.822	0.859	0.033
C 0.2 M PBS RT	0.405	0.404	0.387	0.399	0.010		0.963	0.942	0.890	0.932	0.038
D extraction buffer RT	1.649	1.634	1.639	1.641	0.008		0.901	1.103	1.164	1.056	0.138
E carbonate buffer 60°C	0.63	0.602	0.649	0.627	0.024		0.817	0.85	0.845	0.837	0.018
F 50 mM PBS 60°C	0.323	0.308	0.307	0.313	0.009		0.773	0.761	0.820	0.785	0.031
G 0.2 M PBS 60°C	0.400	0.401	0.374	0.392	0.015		0.995	0.976	0.968	0.980	0.014
H extraction buffer 60°C	1.590	1.550	1.426	1.522	0.086		1.245	1.214	1.225	1.228	0.016
cookie	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
A carbonate buffer RT	1.479	1.509	1.511	1.500	0.018		1.031	1.039	1.063	1.044	0.017
B 50 mM PBS RT	0.799	0.839	0.780	0.806	0.030		0.822	0.847	0.855	0.841	0.017
C 0.2 M PBS RT	1.016	1.03	0.988	1.011	0.021		0.893	0.934	0.964	0.930	0.036
D extraction buffer RT	1.814	1.786	1.784	1.795	0.017		1.079	1.118	1.131	1.109	0.027
E carbonate buffer 60°C	1.619	1.622	1.585	1.609	0.021		1.021	1.068	1.094	1.061	0.037
F 50 mM PBS 60°C	0.78	0.793	0.783	0.785	0.007		0.929	0.945	0.939	0.938	0.008
G 0.2 M PBS 60°C	0.962	0.921	0.943	0.942	0.021		0.963	0.965	0.969	0.966	0.003
H extraction buffer 60°C	1.602	1.612	1.630	1.615	0.014		1.146	1.172	1.178	1.165	0.017
soy milk	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
A carbonate buffer RT	1.402	1.441	1.433	1.425	0.021		1.031	1.145	1.079	1.085	0.057
B 50 mM PBS RT	0.787	0.852	0.801	0.813	0.034		1.003	1.036	1.012	1.017	0.017
C 0.2 M PBS RT	0.924	0.986	0.999	0.970	0.040		1.030	1.089	1.091	1.070	0.035
D extraction buffer RT	1.669	1.673	1.697	1.680	0.015		1.128	1.25	1.210	1.196	0.062
E carbonate buffer 60°C	1.454	1.492	1.477	1.474	0.019		1.052	1.075	1.087	1.071	0.018
F 50 mM PBS 60°C	0.913	0.925	0.939	0.926	0.013		1.021	1.025	1.042	1.029	0.011
G 0.2 M PBS 60°C	1.078	1.077	1.101	1.085	0.014		0.977	1.142	1.098	1.072	0.085
H extraction buffer 60°C	1.587	1.570	1.591	1.583	0.011		1.133	1.380	1.249	1.254	0.124
whey drink	IgY-anti-α-casein						rabbit-anti-α-casein				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
A carbonate buffer RT	1.006	1.054	1.010	1.023	0.027		0.553	0.573	0.568	0.565	0.010
B 50 mM PBS RT	0.618	0.555	0.566	0.580	0.034		0.630	0.601	0.601	0.611	0.017
C 0.2 M PBS RT	0.617	0.686	0.766	0.690	0.075		0.649	0.611	0.593	0.618	0.029
D extraction buffer RT	1.546	1.602	1.494	1.547	0.054		0.823	0.830	0.820	0.824	0.005
E carbonate buffer 60°C	1.021	0.999	1.108	1.043	0.058		0.557	0.558	0.581	0.565	0.014
F 50 mM PBS 60°C	0.564	0.554	0.537	0.552	0.014		0.625	0.592	0.585	0.601	0.021
G 0.2 M PBS 60°C	0.403	0.393	0.401	0.399	0.005		0.313	0.359	0.363	0.345	0.028
H extraction buffer 60°C	1.501	1.520	1.432	1.484	0.046		0.895	0.883	0.880	0.886	0.008

Magellan5 software calculated concentrations for the blank values (see table 38) based on the α-casein standard curve (see table 37). Table 39 shows the calculated concentrations of the blank samples.

Table 39 Calculated concentrations [ng mL^{-1}] for the blank samples.

	IgY-anti-α-casein	**rabbit-anti-α-casein**
	candy cone	**candy cone**
carbonate buffer RT	305.2	115.7
50 mM PBS RT	1852.1	141.9
0.2 M PBS RT	1431.5	99.3
extraction buffer RT	0	44.3
carbonate buffer 60°C	705.9	156.8
50 mM PBS 60°C	2003.5	197
0.2 M PBS 60°C	1468.1	75.7
extraction buffer 60°C	0	0
	cookie	**cookie**
carbonate buffer RT	4.8	48.7
50 mM PBS RT	433.9	154
0.2 M PBS RT	243.8	100
extraction buffer RT	0	26.2
carbonate buffer 60°C	0	42.5
50 mM PBS 60°C	458.8	96.2
0.2 M PBS 60°C	298.4	82.3
extraction buffer 60°C	0	10.4
	soy milk	**soy milk**
carbonate buffer RT	43.9	54.5
50 mM PBS RT	373.7	83.8
0.2 M PBS RT	239.9	60.4
extraction buffer RT	0	18.8
carbonate buffer 60°C	31.9	59.9
50 mM PBS 60°C	272.1	78.0
0.2 M PBS 60°C	170.5	59.5
extraction buffer 60°C	9.5	5.7
	whey drink	**whey drink**
carbonate buffer RT	205.3	619.2
50 mM PBS RT	751.4	510.3
0.2 M PBS RT	534.3	495.7
extraction buffer RT	16.2	212.1
carbonate buffer 60°C	193.9	617.4
50 mM PBS 60°C	824.5	531.9
0.2 M PBS 60°C	1474.3	1851.8
extraction buffer 60°C	29.6	161.9

It was very interesting that most buffers gave false positive results for the blank samples. The best results provided the extraction buffer from the Ridascreen test kit. The extraction

temperature of 60 °C was better for this buffer than room temperature, as can be seen from table 39. Figure 22 shows the results for this extraction buffer, extracting at 60 °C.

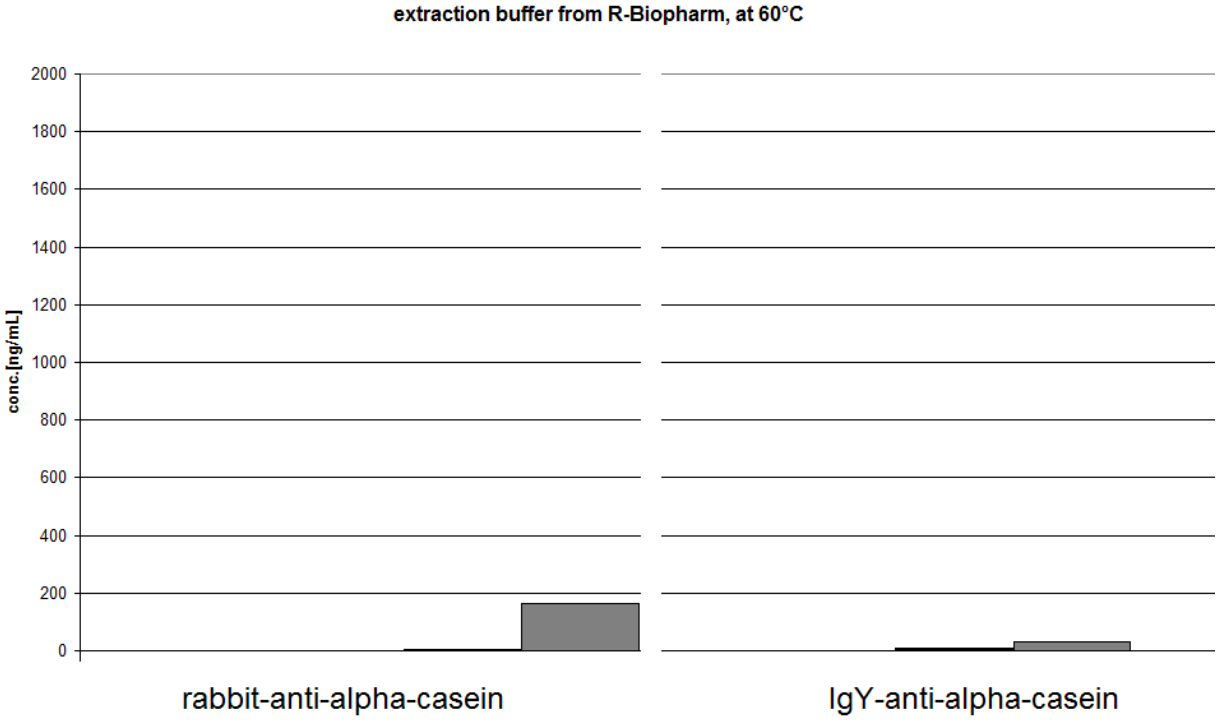

Figure 22 Results of the extraction with extraction buffer from Ridascreen test kit at 60 °C. The blank materials are: candy cone (□), cookie (▨), soy milk (■) and whey drink (■).

Though there seems to be a trend for higher extraction efficiencies at 60 °C, no statistically significant levels between room temperature and 60 °C could be observed. PBS buffer (in both molarities) and carbonate buffer did not resulted in highly false positive results (see figures 23-25) for the blank materials. Only the extraction buffer from the R-Biopharm shows blank values for the blank materials. This outcome leads to the assumption that the antibodies are very sensitive against different matrices. In general, this matrix sensitivity was higher for IgY-antibodies (see table 39).

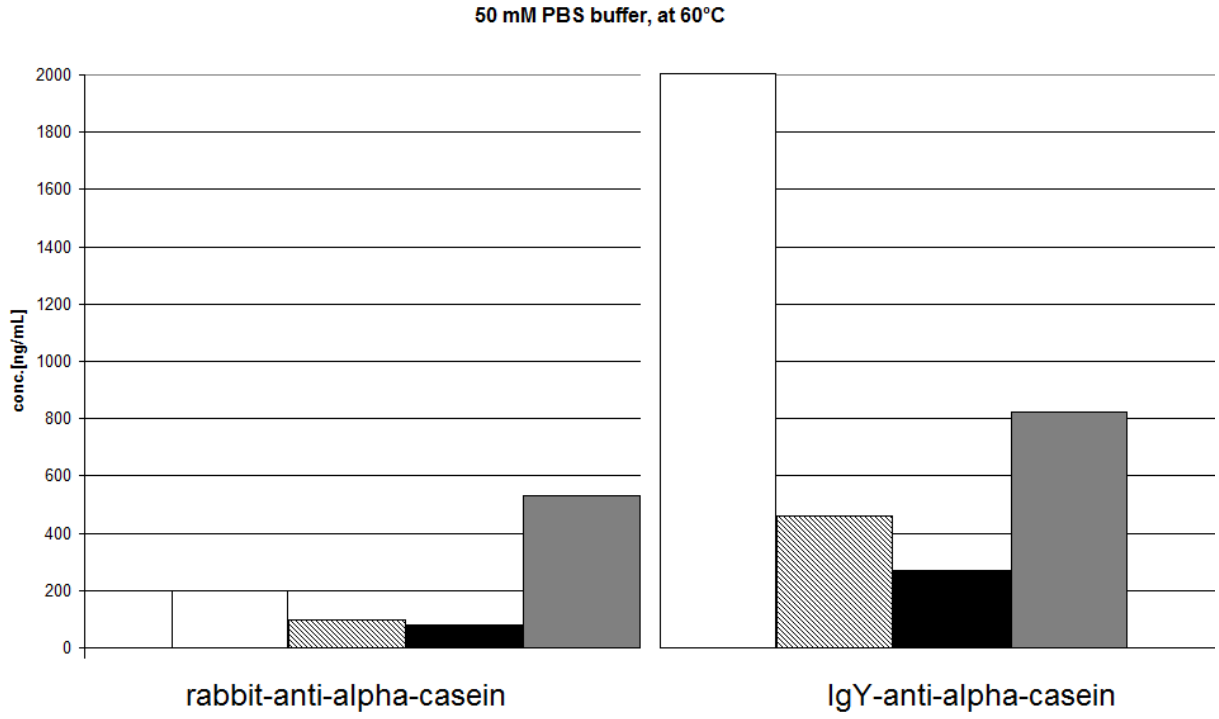

Figure 23 Results of 50 mM PBS buffer, extracting at 60 ℃. The blank materials are: candy cone (□), cookie (▨), soy milk (■) and whey drink (■).

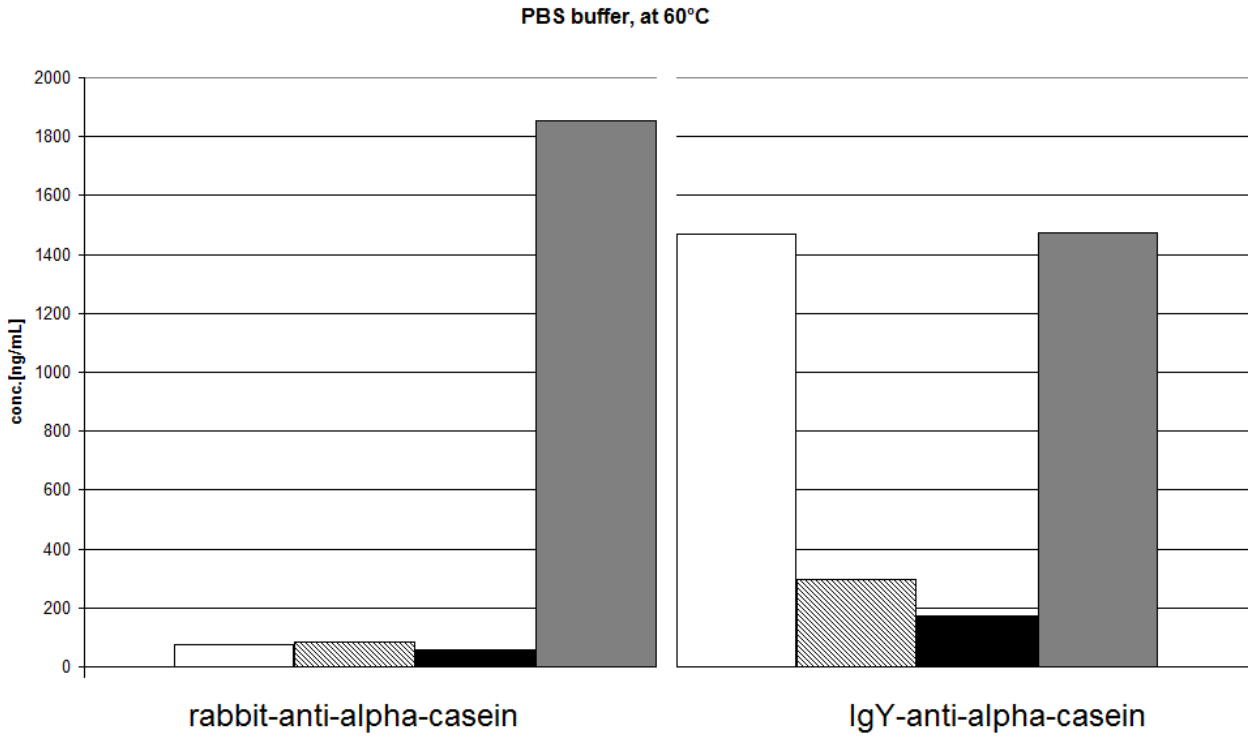

Figure 24 Results of 0.2 M PBS buffer, extracting at 60 ℃. The blank materials are: candy cone (□), cookie (▨), soy milk (■) and whey drink (■).

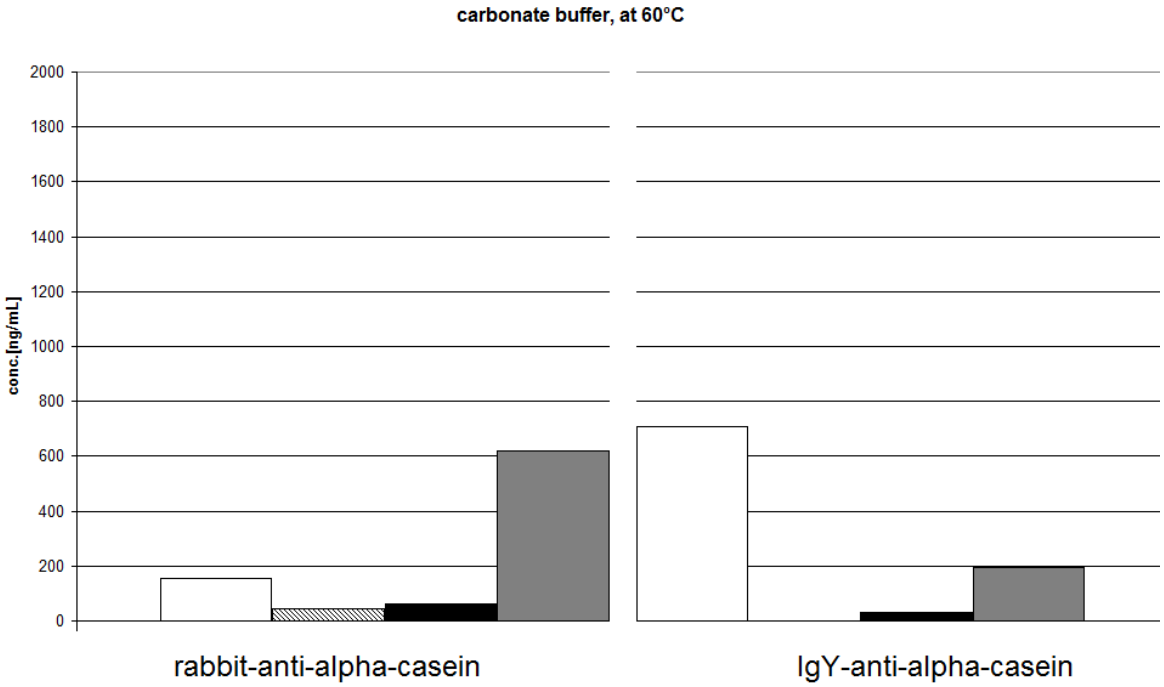

Figure 25 Results of carbonate buffer, extracting at 60 °C. The blank materials are: candy cone (□), cookie (▨), soy milk (■) and whey drink (■).

The search for an optimal extraction buffer must go on and matrix effects have to be tested for each matrix used within the ELISAs. The efficiency of 50 mM PBS buffer for the extraction of food allergens from solid samples was shown to be 52.7% for milk powder (see chapter 4.3).

4.14 Matrix effects in food samples, tested for the competitive immunoassay for α-casein

To find out about the behaviour of the synthetic and carbohydrate blocker in food matrices, one solid and one liquid sample were tested in the indirect competitive format. The experimental data for blocking with Ficoll in coating buffer are summarized in table 40, 41 and 42.

Table 40 Measured absorbances for two competitive formats in buffer solution. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	assay buffer; IgY-anti-α-casein; R=0.996						assay buffer; rabbit-anti-α-casein; R=0.997				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.141	0.158	0.175	0.158	0.017		0.203	0.203	0.191	0.199	0.007
2000	0.246	0.287	0.318	0.284	0.036		0.411	0.446	0.410	0.422	0.021
1000	0.411	0.421	0.412	0.415	0.006		0.595	0.654	0.634	0.628	0.030
500	0.453	0.503	0.485	0.480	0.025		0.827	0.835	0.729	0.797	0.059
100	0.775	0.754	0.732	0.754	0.022		1.243	1.211	1.226	1.227	0.016
50	0.754	0.847	0.742	0.781	0.057		1.375	1.317	1.302	1.331	0.039
10	0.984	0.939	0.893	0.939	0.046		1.495	1.500	1.496	1.497	0.003
0.01	1.026	0.995	0.945	0.989	0.041		1.538	1.589	1.561	1.563	0.026

Table 41 Measured absorbances for two competitive formats in cookie matrix solution. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	cookie matrix; IgY-anti-α-casein; R=0.734						cookie matrix; rabbit-anti-α-casein; R=0.996				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.317	0.299	0.290	0.302	0.014		0.376	0.310	0.331	0.339	0.034
2000	0.587	0.525	0.483	0.532	0.052		0.496	0.427	0.496	0.473	0.040
1000	0.833	0.819	0.774	0.809	0.031		0.635	0.705	0.591	0.644	0.057
500	1.135	1.076	1.091	1.101	0.031		0.740	0.805	0.707	0.751	0.050
100	1.310	1.484	1.721	1.505	0.206		1.133	1.135	1.144	1.137	0.006
50	1.626	1.772	1.627	1.775	0.084		1.226	1.211	1.151	1.196	0.040
10	1.298	1.475	1.484	1.552	0.105		1.358	1.295	1.305	1.319	0.034
0.01	1.174	1.496	1.212	1.294	0.176		1.504	1.426	1.493	1.474	0.042

Table 42 Measured absorbances for two competitive formats in soy milk matrix solution. The blocking was performed with 1% Ficoll in coating buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	soy milk matrix; IgY-anti-α-casein; R=0.821						soy milk matrix; rabbit-anti-α-casein; R=0.995				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.352	0.377	0.423	0.384	0.036		0.403	0.418	0.430	0.417	0.014
2000	0.565	0.539	0.549	0.551	0.013		0.624	0.570	0.556	0.583	0.036
1000	0.897	0.839	0.948	0.895	0.055		0.701	0.754	0.727	0.727	0.027
500	1.123	1.090	1.142	1.118	0.026		0.897	0.880	0.910	0.896	0.015
100	1.285	1.247	1.314	1.282	0.034		1.153	1.195	1.185	1.178	0.022
50	1.753	1.220	1.240	1.404	0.302		1.267	1.319	1.311	1.299	0.028
10	1.457	1.129	1.133	1.240	0.188		1.351	1.361	1.421	1.378	0.038
0.01	1.724	1.013	1.068	1.268	0.396		1.543	1.568	1.580	1.564	0.019

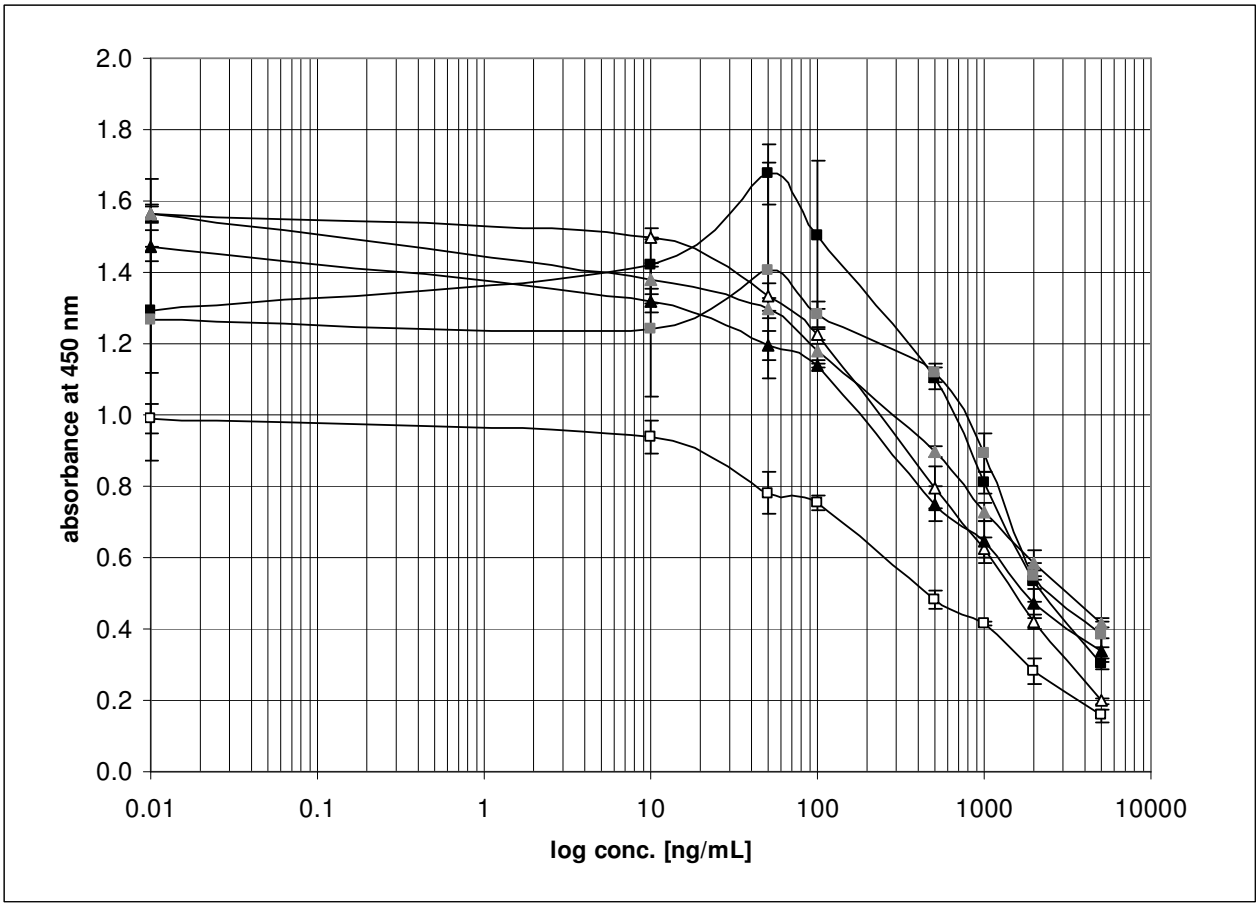

Figure 26 Obtained curves from the data measured. The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. 500 ng mL^{-1} of α-casein were coated on the microtiter plate and blocked with Ficoll at pH 9.6. Rabbit-anti-α-casein in buffer (Δ), rabbit-anti-α-casein in cookie matrix (▲) and rabbit-anti-α-casein in soy milk (▲) were diluted 1:10 000. IgY-anti-α-casein in buffer (□), IgY-anti-α-casein in cookie matrix (■) and IgY-anti-α-casein in soy milk (■) were diluted 1:1000.

Figure 26 shows the absorbances measured with the resulted standard deviation, linked for easy comparison.

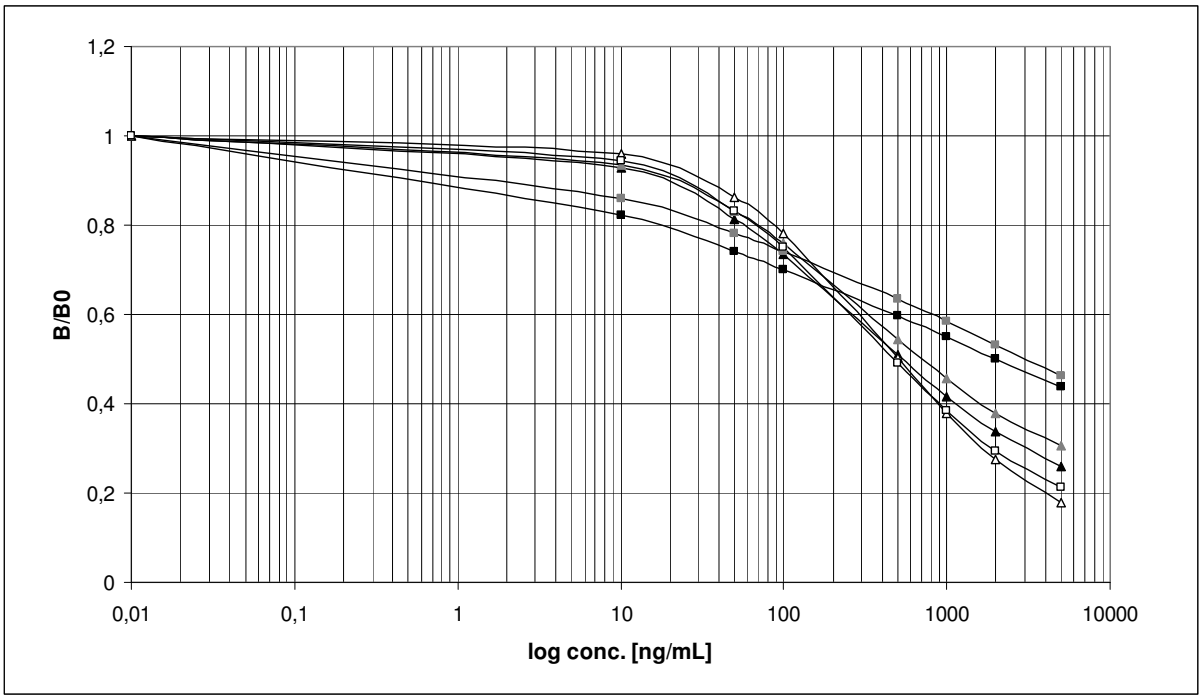

Figure 27 α-casein competitive ELISA: The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. 500 ng mL^{-1} of α-casein were coated on the microtiter plate and blocked with Ficoll at pH 9.6. Rabbit-anti-α-casein in buffer (Δ), rabbit-anti-α-casein in cookie matrix (▲)and rabbit-anti-α-casein in soy milk (▲) were diluted 1:10 000. IgY-anti-α-casein in buffer (□), IgY-anti-α-casein in cookie matrix (■) and IgY-anti-α-casein in soy milk (■) were diluted 1:1000.

Figure 27 shows the normalized results of B/B_0 of buffer and matrix standard curves blocked with Ficoll at pH 9.6. The rabbit antibody gave the same standard curves, not influenced by the different matrices used. In contrast, the chicken antibody was influenced by the cookie and the soy milk matrices, resulting in a different shape of the curve compared to the standard curve recorded in buffer solution. The original curves, obtained by the data measured, are shown in figure 26 and indicate that the antibodies are still working in matrix solutions, but the matrices lead to a loss in sensitivity. Rabbit-anti-α-casein antibody is able to overcome most of the matrix effects, in contrast to IgY-anti-α-casein antibody, which needs to be tested using higher standard concentrations in order to reduce the matrix effects. The importance of the validation of the matrices before use becomes obvious for ELISA assays.

Table 43 to 45 list the experimental data for blocking with PVA in PBS buffer.

Table 43 Measured absorbances for two competitive formats in buffer solution. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	assay buffer; IgY-anti-α-casein; R=0.996						assay buffer; rabbit-anti-α-casein; R=0.997				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.240	0.245	0.251	0.245	0.006		0.534	0.426	0.404	0.455	0.070
2000	0.497	0.489	0.504	0.497	0.008		0.502	0.481	0.545	0.509	0.033
1000	0.772	0.744	0.690	0.735	0.042		0.842	0.836	0.845	0.841	0.005
500	1.067	1.113	1.011	1.064	0.051		1.048	1.117	1.121	1.095	0.041
100	1.545	1.535	1.501	1.527	0.023		1.384	1.375	1.341	1.367	0.023
50	1.546	1.621	1.596	1.588	0.038		1.361	1.515	1.410	1.429	0.079
10	1.639	1.741	1.690	1.690	0.051		1.490	1.438	1.490	1.473	0.030
0.01	1.638	1.725	1.699	1.687	0.045		1.442	1.512	1.439	1.464	0.041

Table 44 Measured absorbances for two competitive formats in cookie matrix solution. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	cookie matrix; IgY-anti-α-casein						cookie matrix; rabbit-anti-α-casein; R=0.564				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.655	0.761	0.563	0.660	0.099		0.356	0.346	0.393	0.365	0.025
2000	1.149	1.685	1.169	1.334	0.304		0.637	0.640	0.639	0.639	0.002
1000	1.831	2.149	1.682	1.887	0.239		0.900	0.947	0.953	0.933	0.029
500	1.796	2.053	1.807	1.885	0.145		1.129	1.109	1.223	1.154	0.061
100	1.809	2.120	1.817	1.915	0.177		1.663	1.631	1.613	1.636	0.025
50	1.609	1.911	1.619	1.713	0.172		1.417	1.397	1.391	1.402	0.014
10	1.516	1.752	1.542	1.603	0.129		1.292	1.351	1.295	1.313	0.033
0.01	1.443	1.688	1.419	1.517	0.149		1.389	1.338	1.364	1.364	0.026

Table 45 Measured absorbances for two competitive formats in soy milk matrix solution. The blocking was performed with 1% PVA in 10 mM PBS buffer. The values on the left were received using IgY-anti-α-casein, the values on the right by using rabbit-anti-α-casein.

	soy milk matrix; IgY-anti-α-casein						soy milk matrix; rabbit-anti-α-casein; R=0.279				
standard conc. [ng/mL]	measured absorbances			average value	std.dev.		measured absorbances			average value	std.dev.
5000	0.776	0.787	0.809	0.791	0.017		0.433	0.461	0.438	0.444	0.015
2000	1.568	1.666	1.699	1.644	0.068		0.915	1.036	1.018	0.990	0.065
1000	2.100	2.157	2.079	2.112	0.040		1.227	1.304	1.322	1.284	0.050
500	1.893	1.947	1.920	1.920	0.027		1.527	1.599	1.528	1.551	0.041
100	1.877	1.816	1.789	1.827	0.045		1.916	1.874	1.850	1.880	0.033
50	1.673	1.795	1.674	1.714	0.070		1.588	1.587	1.610	1.595	0.013
10	1.584	1.640	1.669	1.631	0.043		1.551	1.585	1.596	1.577	0.023
0.01	1.383	1.406	1.383	1.391	0.013		1.457	1.536	1.489	1.494	0.040

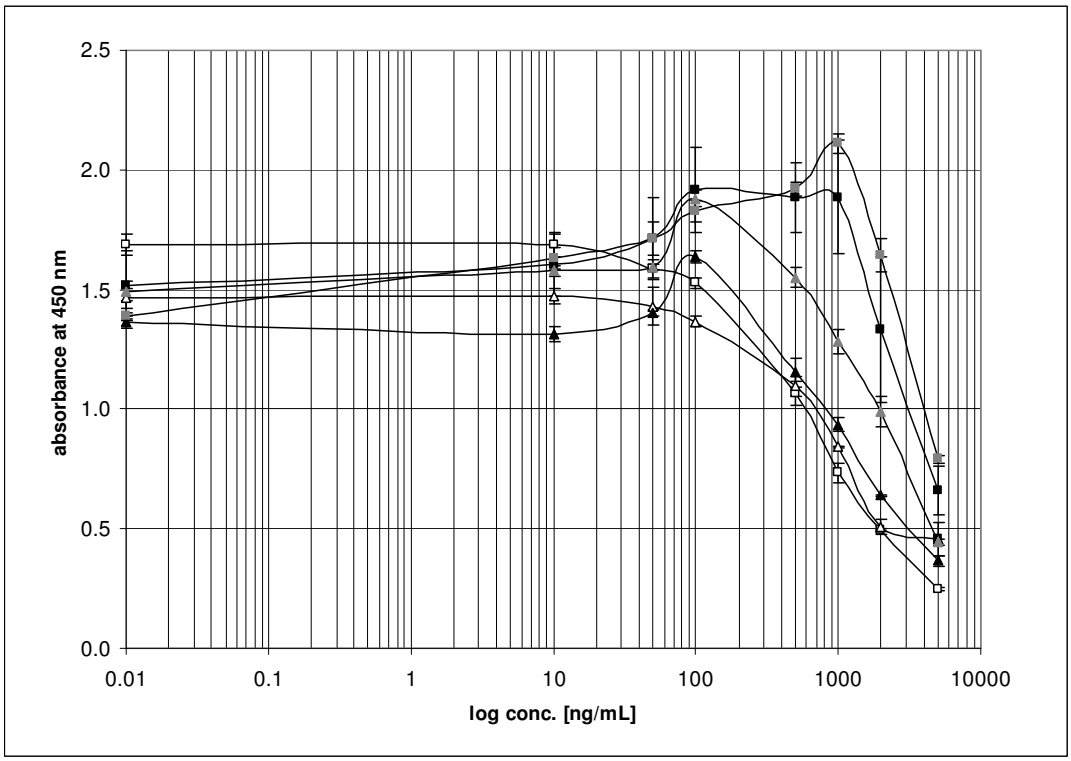

Figure 28 Obtained curves from the data measured. The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. 500 ng mL^{-1} of α-casein were coated on the microtiter plate and blocked with PVA at pH 7.6. Rabbit-anti-α-casein in buffer (Δ), rabbit-anti-α-casein in cookie matrix (▲)and rabbit-anti-α-casein in soy milk (▲) were diluted 1:10 000. IgY-anti-α-casein in buffer (□), IgY-anti-α-casein in cookie matrix (■) and IgY-anti-α-casein in soy milk (■) were diluted 1:1000.

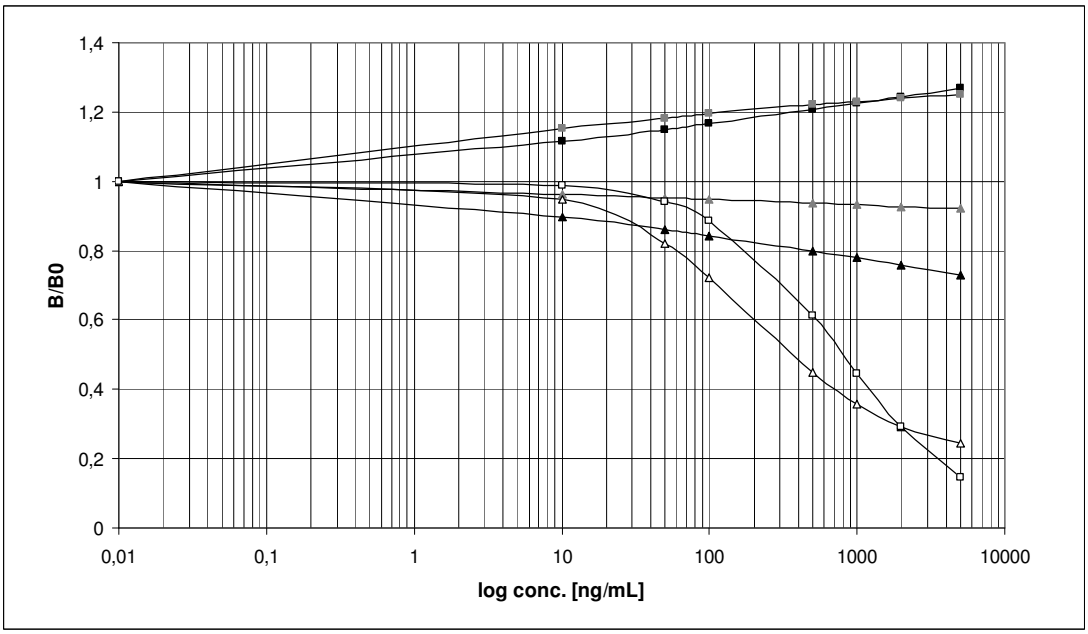

Figure 29 α-casein competitive ELISA: The assay was performed with rabbit-anti-α-casein and IgY-anti-α-casein. 500 ng mL^{-1} of α-casein were coated on the microtiter plate and blocked with PVA at pH 7.6. Rabbit-anti-α-casein in buffer (Δ), rabbit-anti-α-casein in cookie matrix (▲)and rabbit-anti-α-casein in soy milk (▲) were diluted 1:10 000. IgY-anti-α-casein in buffer (□), IgY-anti-α-casein in cookie matrix (■) and IgY-anti-α-casein in soy milk (■) were diluted 1:1000.

Although PVA blocking had proved satisfactory results in buffer solution, figure 28 and 29 demonstrate high matrix effects for both antibodies used. In figure 29 it looks like the antibodies are not working in matrix solutions, but figure 28 shows the opposite: The antibodies are still performing, but they are not sensitive enough for the low standard concentrations. For the concentration range selected, IgY-anti-α-casein can only react with the two highest concentrations of the standard (see figure 28). Hence, higher concentrations have to be considered to reveal good results in matrices. Additional studies are necessary to overcome matrix effects and to be able to use PVA as an alternative blocking solution in the development of ELISA assays.

5 Conclusion

This master thesis focused on the clean-up and performance characteristics of the antibodies. Different antibodies were compared, regarding their origin and the food allergen. Firstly, antibodies for different food allergens had to be characterized. To this end, some specific food allergens and their corresponding antibodies from rabbit and chicken were extracted, cleaned and the protein amount was determined. Further characterizations were obtained by means of electrophoresis. For soy, hazelnut, peanut and α-casein, ELISA assays in different formats were performed and partially optimized. The ELISA formats included indirect competitive and indirect Sandwich assays. During early tests with α-casein, the lack of blocking reagents and their effectiveness turned out to be indispensible to my research. Subsequent blocking studies revealed that non-specific binding on polystyrene microtiter plates during immunosorbent assays can be reduced by various blocking solutions. The most effective blockers remain the proteins, e.g. BSA and fish gelatine. However, for food allergens alternatives to proteins were required. PVA, PVP and PEG were tested, representing synthetic blockers, but only PVA was capable of inhibiting non-specific binding in buffer systems. Furthermore, carbohydrates were taken into account owing to their high molecular weight and their hydroxyl groups. Dextran 40, dextran 2000, Ficoll and trehalose were considered to represent a range of different carbohydrates. Only Ficoll showed the desired blocking efficiency.

Based on these findings, two feasible blockers other than proteins remained with their specific reaction conditions: 1% Ficoll at pH 9.6 for two hours at room temperature and 1% PVA at pH 7.6 at 4 °C overnight, the latter in buffer systems only.

The application of Ficoll and PVA for immunosorbent assays for food allergens was shown in two examples: α-casein and peanut detection. For each food allergen Sandwich and competitive formats were performed. Both Ficoll and PVA proved suitable for the different ELISA formats in buffer systems. PVA was not capable of dealing with the matrix, in contrast Ficoll gave comparable results in the buffer and the matrix. The only suitable extraction buffer used was from an available test kit from R-Biopharm. The extraction was performed at 60 °C, the extraction at room temperature did not vary significantly.

Cross-reactivity studies were performed for hazelnut, peanut, soy and α-casein with highly satisfying outcome. The anti-peanut antibodies were not cross-reactive with any of the food samples tested, yet anti-hazelnut antibodies reacted only slightly with peanut and soy bean flour. Rabbit-anti-soy antibody did not turn out to be cross-reactive at all and the anti-α-

casein antibodies recognized small amounts of β- and κ-casein as well as some of the α-casein in milk powder. These results were shown by means of Western Blot as well.
The comparison of various blocking results for different ELISA formats was published very recently [33].

6 References

[1] Peng Su, Defa Li et al. (2009) Food Chemistry 113:914

[2] Gibbs J. (2001) ELISA technical bulletin No.3. Corning Life Sciences.

http://www.corning.com/Lifesciences/technical_information/techDocs/elisa3.pdf. Accessed 08/08

[3] Bird Ch. R., Gearing A. J. H., Thorpe R. (1988) J. Immunol. Meth. 106:175

[4] Gardas A., Lewartowska A. (1988) J. Immunol. Meth. 106:251

[5] Graves H. C. B. (1988) J. Immunol. Meth. 111:157

[6] Roddha D. J., Yamazaki H. (1994) Immunol. Invest. 23:421

[7] Boyd S., Yamazaki H. (1995) Immunol. Invest. 24:795

[8] Kolosova A., Yu et al. (2006) Anal. Bioanal. Chem. 384:286

[9] Waterboer T., Sehr P., Pawlita M. (2006) J. Immunol. Meth. 309:200

[10] Haycock J. W. (1993) Anal. Chem. 208:397

[11] Studentsov Y. Y. et al. (2002) J. Clin. Microbiol. 40:1775

[12] Studentsov Y. Y.et al. (2003) J. Clin. Microbiol. 41:2827

[13] Nagasaki Y., Kobayashi H., Katsuyama Y., Jomura T., Sakura T. (2007) J. Coll. Inter. Sci. 309:524

[14] Vogt R. F., Phillips D. L., Henderson L. O., Whitfield W., Spietro F. W. (1987) J. Immunol. Meth. 101:43

[15] Dong D., Liu H., Xiao Q., Li R. (2008) J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 870:51

[16] Drs E. et al. (2004) Anal. chim. acta 520:223

[17] Polson A., Wechmar von M. B., Regenmortel van M. H. V. (1980) Immunol. Commun. 9:475

[18] Promega Corporation (2004) EGGstract IgY Purification System technical bulletin No. 188.

www.promega.com/tbs/tb188/tb188.pdf. Accessed 02/09

[19] http://wolfson.huji.ac.il/purification/PDF/affinity/PHARMACIA-AffinityManual.pdf. Accessed 02/09

[20] Ren C., Tang L., Zhang M., Guo S. (2009) “Structural Characterization of Heat-Induced Protein Particles in Soy Milk” to appear in J. Agric. Food Chem.

[21] Shapiro A. L., Viñuela E., Maizel J. V. (1967) Biochem. Biophys. Res. Commun. 28 (5): 815

[22] Hempelmann E., Schulze M., Götze O. (1984) Neuhof V Electrophoresis '84, Verlag Chemie Weinheim 1984: 328

[23] Crowther J. R. “The ELISA guidebook” Vol. 149. Humana Press Inc. 2001

[24] Raem A. M., Rauch P. Immunoassays. Elsevier GmbH 2007

[25] Holzhauser T., Stephan O., Vieths S. (2002) J. Agric. Food Chem. 50:5808

[26] Pederson M. H. et al. (2008) Mol. Nutr. Food Res. 52 (12): 1486

[27] Kaw C. H., Hefle S. L., Taylor S. L. (2008) J. Food Sci. 73:T135

[28] Holzhauser T. et al. (2009) J. Allergy Clin. Immunol. 123 (2): 452

[29] Rupa P., Hamilton K., Cirinna M., Wilkie B. N. (2008) Vet. Immunol Immunopathol 5;125(3-4):303

[30] Drs E. (2004) Dissertation: "Development of IgY-ELISAs for the detection of hidden peanut and hazelnut proteins in food"

[31] Fremy J. M., Usleber E. (2003) Journal of AOAC International Vol. 86, No. 4

[32] Berlitz, Grosch, Schieberle: "Lehrbuch der Lebensmittelchemie" Springer (2001) p.494, table 10.7.

[33] Huber D. et al. (2009) *"Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays"*, Anal. Bioanal. Chem. DOI 10.1007/s00216-009-2698-8 in press