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„Extraction of Egg White Allergens from Food Stuff and Determination of the Influence of Buffer Components in Indirect Competitive ELISA“

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ABBREVIATIONS

ab	antibody
AS	ammonium sulfate
BCA	bicinchoninic acid
BSA	bovine serum albumin
B/B0	absorbance standardized to absorbance of zero standard
cEW	crude egg white
CHAPS	3-[(3-chlolamidopropyl)dimethylammonio]-1-propanesulfonate
CMC	critical micelle concentration
Con	conalbumin
CTAB	cetyl-trimethylammonium bromide
Δabs	absorbance difference between zero standard and max. standard concentration
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EW	egg white
EY	egg yolk
FPLC	fast protein liquid chromatography
IC_{50}	inflection point on the calibration curve / 50% inhibition concentration
Ig	immunoglobuline
kDa	kilo Dalton
Lyz	lysozyme
MTP	microtiterplate
MW	molecular weight
Ova	ovalbumin
Ovm	ovomucoid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEY	egg yolk powder
ppm	parts per million (=mg/kg)
RT	room temperature
SEW	spray dried egg white
SDS	sodium dodecylsulfate
TBS	tris-HCl buffered saline
2-ME	ß-mercaptoethanol
WH	wheat

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1. THEORETICAL PART

1.1 Introduction and Objectives

Food products can be subjected to processing treatments such as heating, pressurization and sterilization, during which proteins contained in food are often denatured in various manners. When an antibody generated against a native protein is used to detect such denatured proteins, its reactivity may be altered as compared with its reactivity towards the native protein, because the denatured protein has undergone a change in 3D structure. In addition, it is necessary to extract the protein from the food commodity before enabling its detection check on the presence of allergenic substances by ELISA. Denatured or altered proteins are often less water-soluble than native proteins, which sometimes renders their extraction from food difficult. Surfactants and denaturants can be used to solubilize and extract insoluble proteins. However, measurement by ELISA with an extraction solution containing a denaturant cannot be made accurately because the antibody is affected by the denaturant. Furthermore, dilution of the extract to the extent that the denaturant does not influence the antibody sometimes makes detection of the extracted protein difficult [1].

When choosing the method, buffers and reagents to extract the proteins, three major requirements should be fulfilled. First, the antigenicity of the extracted proteins must be preserved in order to allow the specific antibody to recognize its epitope. Second, treatment with detergent should not cause ligands to bind non-specifically to the primary and secondary antibody. Third, the reagents used in this process should not hamper the spontaneous absorbance of the proteins to the surface of the ELISA microwells [2].

1.2 Food allergy

Food allergies are caused by abnormal immunological responses to certain foods, usually proteins. The most common types of food allergies are mediated by immunoglobulin E (IgE). IgE-mediated reactions (type I) are known as immediate hypersensitivity reactions or anaphylaxis because symptoms occur from <1 min to a few hours after the ingestion of the offending foods. Examples of symptoms include asthma, atopic dermatitis, rhinitis and urticaria. It is estimated that IgE-mediated food allergies afflict ~1-2% of adults and 5-8% of infants.

Type II or antibody-dependent cytotoxic hypersensitivity is both IgG and IgM mediated. The antibody binds to cell-bound antigen, leading to phagocytosis. The type III hypersensitivity is mainly IgG mediated. The antibody-antigen complexes are formed in large quantities, leading to tissue injuries. Type IV hypersensitivity or delayed hypersensitivity is not mediated by antibodies but primarily by T-cell lymphocytes and macrophages, causing a number of inflammatory responses after a long delay (>8 h after ingestion of the offending foods).

The relatively high prevalence of food allergies in infants is due to an immature gastrointestinal epithelial membrane barrier, which allows more proteins through the barrier and into the circulatory system [3].

1.3 Mucosal immune response to food allergens

Currently, it remains unclear how the mucosal immune system is oriented toward sensitisation versus immune tolerance when exposed to innocuous dietary antigens. However, it is suggested that upon ingestion, food proteins are capable of crossing the intestinal epithelial barrier and being captured by the underlying immune system. Food proteins are then processed into peptidic fragments by a class of specialized immune cells, known as antigen-presenting cells (APC). The peptidic fragments are displayed on their surface in association with major histocompatibility (MHC) class II molecules. The peptide-MHC II complexes can in turn be recognized by specific T-cell receptors (TCR) and potentially lead to the development of a specific immune response.

An allergic immune response is believed to be orchestrated by a class of $CD4^+$ T-lymphocytes or T-helper (Th) cells. Indeed, cytokines produced by $CD4^+$ T-lymphocytes mediate a wide range of pro-inflammatory and anti-inflammatory responses. Most $CD4^+$ T-cells belong to either a Th type 1 (Th 1) or type 2 (Th 2) subgroup, producing type 1 or type 2 cytokines, respectively. Interferon (IFN)-γ is the archetypal type 1 cytokine, whereas type 2 cells typically produce a range of cytokines including interleukin (IL)-4, IL-5, IL-9, and IL-13, contributing to the differentation of B-cells into IgE-producing plasma cells and the recruitment of effector cells such as eosinophils, basophils, and mast cells, as its shown in Figure 1 [4].

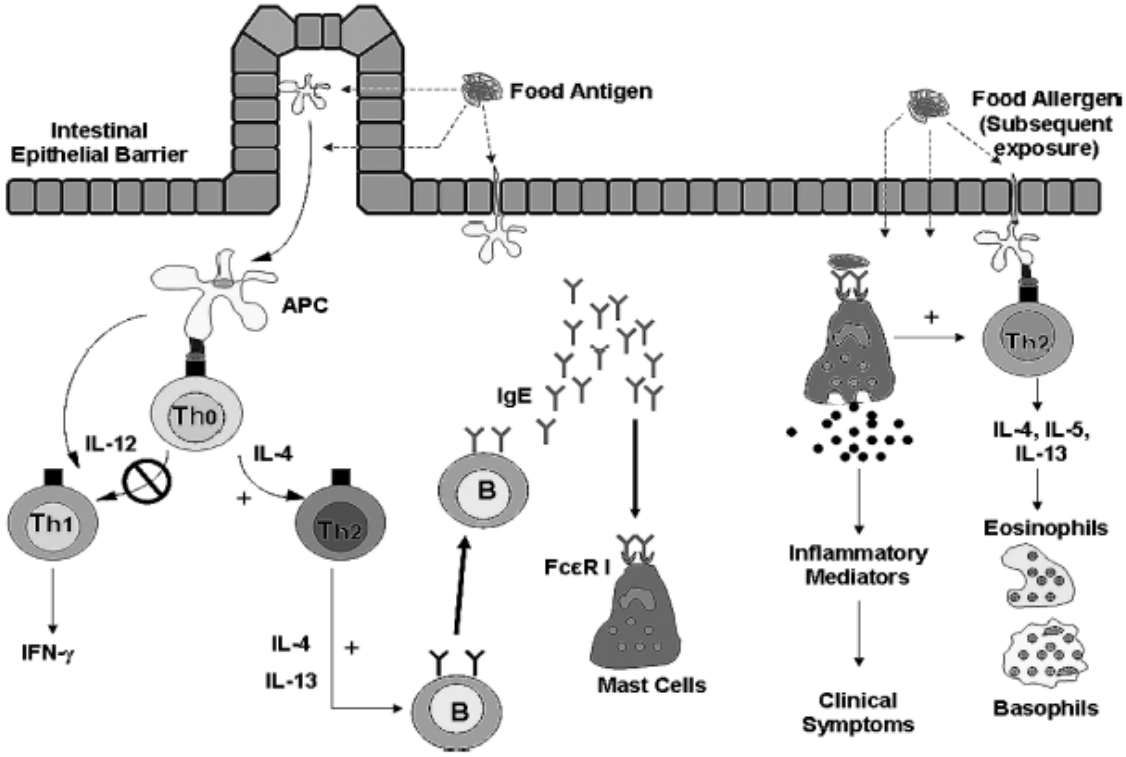


Figure 1. Schematic representation of cellular and molecular events underlying an allergic response [4].

Allergic individuals will preferentially develop an interleukin (IL)-4-rich microenvironment, which drives the immune response toward a Th2 bias to the detriment of a Th1-biased response. The activation of Th2 cells will lead to the production of molecules including IL-4 and IL-13, which both promote immunoglobulin E production by B-cells. Allergen-specific IgE will then bind to high-affinity receptors (FcεRI) present at the surface of mast cells. Upon subsequent ingestion of the food allergen, antigen presentation will lead to a rapid activation of Th2 cells, followed by the recruitment and activation of effector cells such as eosinophils and basophils. In the meantime, allergenic fragments (epitopes) may also bind to receptor-bound IgE present on mast cells, triggering the aggregation of the receptors and the subsequent release of inflammatory and vasoactive mediators such as histamine, directly responsible for the clinical signs and symptoms of food allergy (Copyright 2005 Blackwell).

1.4 Antibodies

Antibodies can be divided into five classes: IgG, IgM, IgA, IgD and IgE, based on the number of Y units and the type of heavy chain. The basic structure of an antibody (immunoglobulin) was discovered by using the enzyme papain, which causes digestion of the antibody into Fc, for “fragment crystallisable” and two remaining fragments were designated Fab, or “fragment antibody binding”. Each Y contains two identical copies of a “heavy chain” (about 50 kDa each), and two identical copies of a “light chain” (about 25 kDa each). The four chains are held together by non-covalent forces and disulfide bonds. Both heavy and light chains contain peptide sequences which show little dissimilarity between antibodies, and which are termed the “constant” regions; other regions vary considerably among individual immunoglobulins, and thus are called the “variable” regions (see Figure 2).

Antigen-antibody interactions occur through multiple non-covalent bonds between the antigen and the amino acids in the binding site of the antibody, including van der Waals forces, electrostatic attractions, and hydrophobic and hydrogen bonds. Because of the varied nature of the forces involved in antigen-antibody bonding, electrostatic forces are very dependent on the pH. Outside the range of pH 6-8, binding can be affected adversely. Temperature and ionic strength can also affect binding in a negative way.

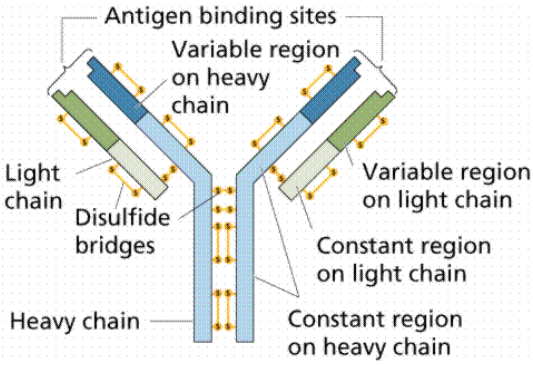


Figure 2. Structure of an antibody monomer (Image from Purves et al., Life: The Science of Biology, 4th edition by Sinauer Associates)

To differentiate molecules that make an immune response and those that are antibody-binding targets, “immunogen” is used for the former and “antigen” for the latter. Antibodies do not recognize the whole antigen, merely portions of it. These portions are called “epitopes” or “antigenic determinants”. Each individual antibody is specific for a particular antigenic determinant of the antigen.

For allergens, antibodies can be produced against any protein or peptide that has at least one antigenic determinant (generally 4-6 amino acids). Once an immune response to an antigen is made, the plasma B cells make large amounts of antibody [5].

1.5 Food labelling and hidden allergens

Food labelling plays a crucial role in food production by providing the consumer access to the information that he or she requires to implement a successful avoidance therapy. Legislation has been put into place in the USA and European Union (EU) aimed at achieving a high level of health protection for allergic consumers. Within the EU, Directives 2000/13/EC and 2003/89/EC require a mandatory declaration of allergenic foods, which includes milk, eggs, fish, crustacean shell fish, peanuts, soybeans, wheat, tree nuts and in addition to these celery, mustard, sesame seeds and sulphites. Recently, Commission Directive 2006/142/EC announced the inclusion of lupines and molluscs to the list of the 12 allergenic foods.

The above mentioned legislation on food labelling only concerns allergenic ingredients that are knowingly and deliberately introduced into food products, in contrast to traces of such allergens whose presence in a food product is unintentional. The presence of such "hidden allergens" can affect the safety of the food product, since it can pose a threat to the health of consumers.
Precautionary labelling has been introduced as a means of manufacturers and retailers to voluntarily provide information to consumers by indicating the possible presence of such "hidden allergens". It is based on the fact that it can be hard for the food industry to guarantee that food products are not contaminated with food allergens, since many different types of food products are stored and produced in the same facilities and often different products are manufactured on the same machinery [6].

1.6 Sensitivity and Selectivity

In order to prevent food allergy reactions from occurring it is necessary to know how much, or how little, of an allergenic food is capable of triggering an allergic reaction. A major complication here is the fact that not only the clinical reactions, but also the eliciting doses (EDs) vary widely between individuals. Moreover, symptoms and EDs can change over time for individual allergic patients. An individual`s sensitivity can only reliably be determined using food challenge studies.
The analytical detection of trace amounts of allergenic ingredients can be complicated by difficulties with their extraction, or the presence of other, often very abundant components of the food matrix that can mask the allergen, while the type of food matrix is known to impact the recovery of food allergens.
The design of methods for the detection of allergenic ingredients in food products requires the identification and selection of target analytes. The selection of a single allergen for detection of the allergenic food has a clinical relevance restricted to a subset of allergic patients.
In order to achieve good sensitivity, the abundance of the target analyte within the allergenic food should be taken into account. Theoretically, high sensitivity can be achieved by the selection of multiple target analytes. Alternatively, that do not possess any allergenic potential can be used as markers for the detection of allergenic ingredient in food products, justified by the fact that they are highly abundant in the allergenic commodity. A major requirement for the selection of target analytes or markers for allergenic commodities concerns their specificity. Is the marker representative for the allergenic food only, or can the same marker be detected in other foods [6]?

1.7 Methods for allergen analysis in food

Protein-based methods usually involve immunochemical detection protocols such as the radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), rocket immuno-electrophoresis (RIE), immunoblotting, and enzyme-linked immunosorbent assay (ELISA). Whereas RIE and immunoblotting render only qualitative or semiquantitative results, RAST, EAST and ELISA are quantitative methods.
Methods operating on the DNA level are based on an amplification of a specific DNA fragment by the polymerase chain reaction (PCR). With real-time PCR highly accurate quantitative results can be obtained.
The choice of method is mainly dependent on the food concerned (availability of specific antibodies/ DNA primers and the achievable detection limit) and on the history of processing involved during food production. However, the employment of DNA analysis in allergen detection is discussed controversially, since proteins are the allergenic component and processing may differentially affect nucleic acids and proteins [7].

1.7.1 RAST/EAST inhibition

RAST and EAST assays are *in-vitro* tests, which are mainly used in clinical diagnosis of food allergy. RAST and EAST inhibition represent competitive IgE binding assays. In principle, an antigen/allergen bound to a solid phase binds specific human IgE. Antigens in a sample solution inhibit IgE binding to the antigen immobilized on the solid phase. An anti-IgE antibody labelled with an isotope (RAST) or an enzyme (EAST), followed by addition of a substrate that changes colour or emits light, is used to detect the bound human IgE antibodies.
Commercial applications of RAST and EAST for allergen quantification are limited due to the reliance on human sera from appropriately allergic subjects and difficulties in standardizing these assays [7].

1.7.2 SDS-PAGE/immunoblotting

In most cases, immunobotting is used in conjunction with sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Separation of proteins prior to electrophoretic transfer to nitrocellulose or polyvinylidene difluoride membranes can also be two-dimensional, in which case SDS-PAGE is preceded by isoelectric focusing.
In food allergen research, immunoblotting has been used for many different purposes. Most frequently, the technique has been applied for qualitative purposes, i.e. the identification of allergenic IgE-binding molecules and the establishment of IgE cross-reactivity between foods. The major disadvantage of SDS-PAGE and immunoblotting with IgE for routine food analysis are the elaborate and time-consuming procedures and the reliance on appropriate human sera [7, 5].

1.7.3 Rocket immuno-electrophoresis (RIE)

Rocket immuno-electrophoresis employs an antibody containing gel. Antigens to be analysed migrate according to their electrophoretic mobility until antigen-antibody complexes precipitate in the gel. Rocket-shaped precipitates are formed at a constant antigen/antibody ratio. The height of the rockets is proportional to the amount of antigen applied.

However, rocket immuno-electrophoresis is not widely used for allergen determination due its low sensitivity, laborious gel preparation and immuno-staining procedures [7].

1.7.4 Polymerase chain reaction (PCR)

PCR technology has already been established as the DNA-based method for the identification of genetically modified organisms, pathogens and food-related plant and animal species. Since the mid 1990s this methodology has attracted increasing attention for the detection of allergen traces in food products.

The basis for PCR is a thermo-stable DNA polymerase which is able to amplify a specific DNA fragment that is flanked by two specific oligonucleotides (primers). In general, the PCR is a temperature-dependent reaction that consists of a series of 25-45 cycles with each cycle consisting of three phases, the denaturation, the annealing, and the extension phase. All phases are performed in one reaction vial at defined temperatures. With a well-optimized and highly efficient PCR, the amount of the generated PCR product is ideally doubled within each cycle which results in an exponential amplification. The detection of PCR products by sequence verification can be done either by Southern blotting with sequence-specific hybridisation probes, by nucleotide sequencing, or with endonuclease restriction cuts. However, these methods are very time-consuming and can be overcome by the use of a real-time PCR or a PCR-ELISA [5].

Despite that the allergen is not detected, DNA-based methods offer many advantages, primarily that the target DNA is efficiently extracted under harsh denaturing conditions and is less effected that the extraction of proteins from food matrices, or the DNA stability against geographical and seasonal variations, which may vary protein composition [7].

1.7.5 PCR-ELISA

After PCR amplification, the products are detected sequence-specific with an ELISA-technique. This method requires a modification of the PCR protocol: biotinylated primers are used in the PCR which leads to biotinylated PCR products that are coupled onto a streptavidin-coated microtiterplate. Subsequently, the PCR product is denatured resulting in a surface-bound single-stranded DNA that is accessible for the binding of a sequence-specific DNA probe. The probe, which is linked to a label is detected by an enzyme-conjugated antibody that is directed against the label.

The PCR-ELISA can only be used as a qualitative test for the absence or presence of the allergenic food within the validated range of detectability. The reason for this is the qualitative nature of the PCR as an endpoint method [5].

1.7.6 Real-time PCR

The principle of this state-of-the-art method for quantitative detection of amplifiable DNA is based on the usage of target-specific oligonucleotide probe with a reporter dye and a quencher dye attached. The probe anneals to the single-stranded amplified segment within the region flanked by the two oligonucleotides priming the enzyme reaction. Due to the proximity of the quencher to the reporter fluorescence is suppressed. During amplification the 5´exonuclease activity of the polymerase cleaves the hybridised probe and separates the dyes, which are displaced by the synthesized new copy strand and become soluble, with the intensity of the fluorescence of the free reporter dye producing a measurable signal. The resulting increase in fluorescence is proportional to the amount of specific PCR product. In the case of the quantitative analysis of allergenic foods in composed food products, the availability of standard reference materials is essential to cover the influence of matrix and processing effects.

1.7.7 Biosensors and SPR technology

Biosensors are analytical devices consisting of a biological recognition element (e.g. cells, proteins or oligonucleotides) in direct contact with a transducer that produces a signal, which is further processed to give an output that is proportional to the concentration of a specific analyte.

Surface plasmon resonance is an affinity-based optical transduction principle that detects the binding between molecules through local changes in the refractive index close to a surface. Opposite the flow cell side of the sensor surface, a prism is optically coupled to the sensor surface. Polarized light from a light-emitting diode is reflected in the glass support and detected by a charge-coupled diode (CCD) array. At a specified resonance wavelength and angle, surface plasmons interact with the photons resulting in a drop in the reflected light detected by the CCD. The resonance angle is sensitive to the refractive index close to the surface. Attractive features of this technology are the short analysis time and a high degree of automation [7, 5].

1.7.8 Lateral flow devices (LFDs)

The more sensitive, quantitative immunochemical tests take a trained person to carry out; the desire to improve on speed and easy-to-use, especially in non-research settings, has prompted researchers to look for faster and simpler procedures. Thus, new rapid test platforms have been developed, such as the chromatographic test strip (lateral flow test). The success of these tests is based on the fast flow of fluids running through the membrane enabling the application of various immunoreactants at different locations along the membrane.

In general, the sample runs through the sample filter and conjugate pad. This conjugate pa contains the affinity-purified and labelled (gold/latex/carbon) antibody, specific for the analyte under test. The analyte, if present, will form a complex with the conjugate and migrate further along the membrane to the test zone. This test zone contains an immobilised antibody, specific for the analyte, but preferably not competing with the conjugated antibody for the same epitopes. This test line will thus capture the migrating analyte-conjugate complex. The intensity of the test line correlates well with the amount of analyte in the sample. In contrast to ELISA, in this test system all components are acting simultaneously and within a very short time-frame.

Thus, selecting antibodies for a one-step test requires their affinities to be sufficient and tolerant so that they will allow each other to participate in forming a visible complex. In addition, gold labelling has a greater interference with antibodies, where binding affects overall charge and secondary and tertiary structure more than labelling with enzymes [5].

1.8 Enzyme-linked immunosorbent assay (ELISA)

Currently, the ELISA technique is the most commonly method used in laboratories of the food industry and official food control agencies to detect and quantify hidden allergens in food. ELISA tests are based on the use of an enzyme linked to an antibody to detect the binding of antigen and antibody. The enzyme converts a colourless substrate to a coloured product, indicating the presence of antigen-antibody complex. Additionally, qualitative results can be visualized against the standard used. Standard curves are generally plotted as the standard protein concentration versus the corresponding mean optical density (OD) value of replicates. The concentrations of the analyte-containing samples can be interpolated from the standard curve. This process is made easier by using a computer software curve-fitting program, which is part of the ELISA reader operating software. There are two techniques for antigen measurement, the “sandwich technique” and the “competitive technique”. Almost all commercial allergen ELISA test kits use the sandwich format (see Figure 3).

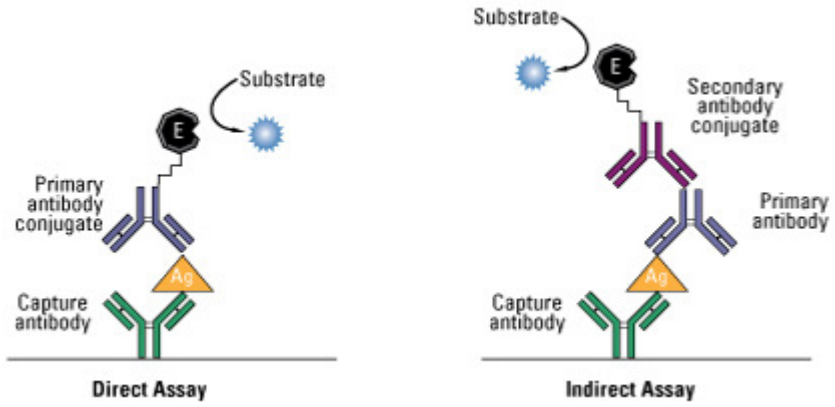


Figure 3. Sandwich ELISA format. In the assay, the antigen of interest is immobilized to the assay plate by first attaching a capture antibody to the plate surface. Detection of the antigen can then be performed using an enzyme-conjugated primary antibody (direct detection) or using a matched set of unlabeled primary antibody and conjugated secondary antibody (indirect detection, image from www.piercenet.com/proteomics/browse.cfm?fldID=f88adec9-1b43-4585-922e-836fe09d8403).

1.8.1 Sandwich ELISA

A sandwich ELISA can be more specific because antibodies directed against two or more distinct epitopes are required. The basic sandwich ELISA method uses excess of highly purified, specific antibodies (capture antibodies), which are absorbed or "coated" onto plastic microwell plates. The immobilized antibodies serve to specifically capture their corresponding antigens, such as food allergens, that are present in samples. After washing away unbound material, the captured antigens are detected using enzyme-conjugated antibodies (detector antibodies). While sandwich ELISA results can be quantified against a standard curve, the intensity of the colour change is roughly proportional to the concentration of allergen in the sample.

1.8.2 Competitive ELISA

The competitive inhibition ELISA is a technique that uses a one-epitope approach for the antibody to recognize allergenic residue in a sample. In a competitive assay, the antigen is coated on the wells, and a solution containing a limited amount of first antibody along with the antigen or analyte is added. The assay is based on the principle that an antigen in the sample will bind to an antibody and then compete for the binding of the antigen coated on the wells. After the unbound antibody is washed off, a second antibody-enzyme conjugate is used to detect the bound antigen-antibody complex in the wells. Then a substrate of the enzyme is added. In this format, the colour produced is inversely proportional to the concentration of the analyte.

1.8.3 Advantages of ELISA

- ease of use, simple, fast, and can be automated
- convenient and standardized 96-well format, which may come in strips of microwells
- sensitive (in low ppm range)
- selective to the allergenic residues
- availability of labelled reagents
- rapid data reduction
- low initial cost
- portability

1.8.4 Disadvantages of ELISA

- lengthy developmental time
- cross-reactivity possible
- matrix effects
- potential false positives from noise or matrix
- confirmation requirement for positives
- no multi-residue analysis yet
- difficult to diagnose problems when assay does not meet the quality assurance specifications

1.9 Egg allergy

Hen eggs are one of the most frequent causes of adverse reactions to food. Elucidation of allergic reactions has shown that they are more frequently caused by egg white proteins than egg yolk [3].
The estimated prevalence of egg allergy varies between 1.6 and 3.2% and thus makes it the second most common cause of food allergies in children. In egg-allergic patients, the clinical signs and symptoms involve various organs such as the skin, the respiratory system and/or the gastrointestinal system. However, in infancy, atopic dermatitis represents the main clinical manifestation. Hypersensitivity to egg proteins is mostly known to develop upon ingestion of eggs or egg-containing food products, but reports of sensitisation to egg after inhalation have also emerged. The diagnosis of egg allergy is often determined by skin prick tests or radio-allergosorbent (RAST) assays [4].

Currently, the most efficient approach for egg allergy is total avoidance of the offending compound. However, the omnipresence of egg-derived components in cooked or manufactured food products renders the approach difficult [4]. Modern food contains an increasing number of additives or ingredients. These products are generally used for specific applications, such as jellification (meat products), foaming action (biscuit), thickening (sauces), and emulsifying and they can be derived from egg white and yolk.

1.10 Allergenic components of the egg

The chemical composition of hen`s egg has been extensively investigated (Table 1). The major egg allergens were found in the egg white. Despite a large number of studies into egg white allergy, no clear consensus has been reached as to relative antigenicity and allergenicity of egg white proteins. The contradictory results on egg white allergenicity may, therefore, be attributable to several aspects:

a) differing degrees of purity of individual protein fractions and antibody sources,
b) using sera from either humans with egg allergy or antibodies raised from experimental animals, or
c) different routes of administration (injection or oral administration) [3].

Table 1. Chemical Composition of Hen`s Egg [Adapted from Kovacs-Nolan et al.]

constituent	% (w/v)	major components (rel %, w/w)
egg shell	9.5	inorganic salts (91.87) proteins (6.4) water (1.7) lipids (0.03)
egg yolk	27.5	proteins (15.7-16.6) lipids (32-35) carbohydrates (0.2-1) ash (1.1)
egg white	63.0	proteins (9.7-10.6): ovalbumin (54) conalbumin (12) ovomucoid (11) ovomucin (3.5) lysozyme (3.4) G2 globulin (4?) G3 globulin (4?) ovoinhibitor (1.5) ovoglycoprotein (1.0) ovoflavoprotein (0.8) ovostatin (0.5) cystatin (0.05) avidin (0.05) lipids (0.03) carbohydrates (0.4-0.9) ash (0.5-0.6)

On the basis of RAST assays and cross-radio-immunoelectrophoresis (CRIE), studies established that ovomucoid, ovalbumin and conalbumin are the major egg allergens and, later, lysozyme is also demonstrated to be a significant egg allergen. In vitro analyses based on RAST and Western blot assays revealed minor egg allergens represented by ovomucin, another egg white protein and furthermore phosvitin, α-livetin and apovitellenins I and VI present in the egg yolk. The molecular and biological characteristics of the most common egg allergens are presented in table 2.

Table 2. Molecular and biological properties of identified egg allergens

protein name	MW (kDa)	protein family	biological function
ovomucoid (Gal d 1)	28	Kazal-type serine protease inhibitor	serine protease inhibition activity
ovalbumin (Gal d 2)	45	serine protease inhibitor	storage protein?
conalbumin (Gal d 3)	76-77	transferrin	iron-binding capacity with antimicrobial activity
egg lysozyme (Gal d 4)	14.3	glycoside hydrolase family 22	antibacterial activity
ovomucin	165	contains trypsin inhibitor-like domains	heavily glycosylated protein with potent antiviral activities

1.11 Biochemical properties of major egg allergens

Ovomucoid (OVM) consists of 186 amino acid residues, 25% carbohydrate and exhibits an isoelectric point (pI) of 4.1. It is very stable being a serine protease inhibitor with 9 disulfide bonds and no free –SH groups. The molecule consists of three structurally independent tandem homologous domains. The carbohydrate chains are penta-antennary, heterogeneous and partially sialylated, resulting in substantial mass and charge heterogeneity of native ovomucoid.

Ovalbumin (OVA) consists of 385 amino acid residues, 3% carbohydrate and shows a pI of 4.5. It has one disulfide bond and four free –SH groups, which result in some dimerization. Native ovalbumin displays considerable charge heterogeneity because of sequence variations, and two potential phosphorylation sites. Finally, during storage in atmospheric air, ovalbumin rearranges to S-albumin, a conformationally different form, exposing an additional carboxylate group. In total, the post-translational modifications increase the sequence-derived MW of 42.7 to 44-45 kDa. Besides its role as a major source of amino acid in hen`s egg white, no biological function has yet been attributed to this protein.

Conalbumin (CON), also called Ovotransferrin is a monomeric glycoprotein consisting of 686 amino acids with a theoretical pI of 6.1. It contains a single glycan chain (3% by weight), 15 disulfide bonds and is divided into two domains, an N domain and a C domain, with a short linkage region. Charge heterogeneity arises from sequence variations and variations of Fe^{3+}. Conalbumin can bind two Fe^{3+} in association with binding of an anion and its main function is commonly accepted as iron transport molecule, and it belongs to the transferrin protein family. Its antimicrobial activities have been well investigated on the basis of its iron-scavenging properties, as well as its antioxidant properties.

Lysozyme (LYZ) is a glycosidase consisting of 129 amino acid residues with 4 disulfide bonds and no free-SH groups. It is an elementary protein with the isoelectric point of 10-11 and has no post-translational modifications. Offering bacteriolytic activity against prokaryotic organisms, it is a good example of naturally occurring enzymes used in the food industry to maintain product quality and reduce the incidence of spoilage [4, 8, 9].

1.12 Alteration of Antigenicity & Allergenicity

There is variability in the stability of food allergens to heat, proteolysis and digestion. Food allergens may be altered by heat and acidity. An epitope is that portion of the antigen which binds with antibody. Many epitopes on proteins involve amino acids from regions of the polypeptides that are distant from one another in the primary structure. These are called conformational epitopes and denaturation of the secondary structure of a protein by heat or acidity can destroy the allergenicity, e.g., boiling egg noodles may reduce the amount of detectable egg white protein by greater than 99% [10].
In the study of Mine et al., 2002 [3] urea-treated ovalbumin, lysozyme and conalbumin led to an increase in human IgG-binding activity, whereas thermal treatment of ovomucoid and conalbumin led to a significant drop.

Ovomucoid is characterized by its high heat stability and can also be resistant to other forms of denaturation, possibly related to the presence of its strong disulfide bonds. Ovalbumin is easily denaturated by urea and furthermore, the protein is relatively heat labile, compared to ovomucoid.
Conalbumin is a heat-labile allergen, but it was reported that when coupled to bi- or trivalent metal ions, it could form heat-stable complexes [4]. Lysozyme is a very stable enzyme. Two of the four disulfide bonds are responsible for the thermal stability of the enzyme, which still exists at 100°C and in acidic solution pH 3-4. However, thiol compounds rapidly inactivate lysozyme, and thus enzyme stability in hen egg white does not exist when heated at 60°C because of the presence of free SH groups of ovalbumin at that temperature [9].

1.13 Detection of egg allergens in food products

Ideally, analytical methods aimed at the detection of egg and other food allergens should provide specific (e.g., reliable detection in a wide range of matrices), sensitive (e.g., at levels relevant to thresholds reported in allergic populations), and rapid (e.g., suitable for routine testing and large volume production) analyses [4].
Diffusion-in-Gel methods like RIE allowed the investigation of the clinical importance of ovalbumin and ovomucoid as major egg allergens, but they lack practicality for the food industry to quantify allergens.
Performing RIE the level of detection varies somewhat with antigen and with the titer of antiserum but lies normally at mg levels per 100 g food samples. This level of detection is generally not low enough, as it is usually recommended that methods have detection limits of 1-10 ppm for allergenic residues (Morisset *et al.*, 2003). Electrophoretic and blotting methods may be useful for the detection of egg components in raw or slightly cooked products, but may fail in the analyses of highly cooked or sterilized products because of the denaturation and protein aggregation processes undergone during thermal treatment. Also the protein unfolding caused by the use of SDS in PAGE may lead to the loss of conformational epitopes being preserved. However, electrophoretic and blotting methods are most commonly used to study the IgE- and IgG-binding abilities of modified or native egg allergens and have been rarely used for the quantitative determination of egg allergen content. The use of PCR for the detection of egg allergens has not been reported as yet. Unless combined with protein-based specific detection methods, a PCR-based approach may not allow chicken-derived DNA to be distinguished from egg-derived DNA and may not therefore represent the best alternative [4, 5].

Currently, the methods mostly employed in the detection of egg allergens are based on immunological methods such as ELISA. Currently, commercial ELISA kits for the detection of egg allergens are mostly based on the detection of ovalbumin and ovomucoid. It seems, however, that most immunoassays have so far been based on the binding properties of specific IgG isotypes, thus detecting antigenic components rather than providing a reliable indication of allergenicity [4].
Limits of detection in processed food products depend on various parameters, such as fat content, severity of heat processing, state of meat maturation, etc. From a theoretical point of view, ELISA methods are quantitative but, for the same reasons as the detection limit, results can be only semi-quantitative or qualitative unless validated appropriately [5].

1.14 Extraction of egg white allergens from food stuffs

Since a lot of different food sources are studied for their allergenic properties the first requirement for correct research procedure is to obtain good protein extracts that is starting material with a sufficiently high protein concentration and suitably low lipid content so as to allow for quantitative determination studies. In the case of animal foods good extracts have often been achieved with simple incubation of the food in a buffer solution to extract proteins. As reported by Langeland (1982) hen's egg antigen solution was prepared by stirring crude egg with an equal volume of physiological saline (0.15 M) for 4 h at RT. The method was substantially confirmed by the study of Bernhisel-Broadbent et al. (1994), in which egg was extracted by overnight incubation at room temperature with PBS. A protocol from Wittemann et al. (1994) included the extraction at RT in water maintained at pH 8 for 4 h. A further method for protein extraction was described by Berkelmann and Stenstedt (1998). For this, proteins were extracted with a urea solution (8 M) at <37 °C and equal volumes of the sample and acetate buffer (0.1 M) were used in a study by Hirose et al. (2004) for the protein extraction. Fernández et al. (1999) described a protein extraction method using Tween 20 (0.2%) as the extraction solution [11].
There are obstacles to the quantification of egg contaminants in food matrices. The efficiency of egg protein extraction has a direct correlation on the interpretation of a test, and a low extraction yield can lead to false results.

2. MATERIALS AND METHODS

2.1 Antibodies

Polyclonal antibodies are achieved from sera of animals, which were immunized with the corresponding antigen for several times. In contrast to monoclonal antibodies, polyclonal antibodies are produced by more B-lymphocyte clones without the same origin. Therefore, polyclonal antibodies may react with different structure and sequence motifs of one antigen. Consequently, the probability to recognize a modified or processed antigen, as it appears in food stuffs, increases, but also the risk for cross reactivity. The advantage of polyclonal antibodies is the faster, cheaper and less complicated production; otherwise the amount of serum from one animal is limited. Before usage, the sera are purified via affinity chromatography or ammonium sulphate precipitation to remove unspecific proteins and to concentrate the antibodies.

In this work four types of polyclonal rabbit antibodies were used to study the extraction efficiency of egg white proteins from processed food samples and the effect of extraction buffer components in indirect competitive ELISA (Table 3). The antigens for immunization were prepared in 0.2M PBS with defined concentration (100 µg/100 µL). The spray dried egg white (SEW) and the egg yolk powder (PEY) was extracted at 60 °C for 15 min and centrifuged. For crude egg white (cEW) and ovomucoid (OVM) preparation the proteins were only solved in buffer. To receive denatured ovomucoid the commercial standard was heated up to 100 °C for 10 min.

Table 3. Characterisation of antibodies

nomenclature	origin	immunogen	supplier
anti - SEW	rabbit - polyclonal	spray dried egg white	Assoc. Prof. Marcela Hermann Department of Medical Biochemistry Max F. Perutz Laboratories Campus Vienna Biocenter
anti - nativeOVM	rabbit - polyclonal	purified ovomucoid (Sigma: T2011-5G)	
anti - heatedOVM	rabbit - polyclonal	heated purified ovomucoid	
anti - cEW	rabbit - polyclonal	crude chicken egg white	
anti - PEY	rabbit - polyclonal	indian egg yolk powder	

2.2 Antibody Purification

Several methods for antibody purification from serum or culture supernatant exist depending on how the antibody will be used for various assay and detection methods. Three levels of purification specificity include the following approaches:

- *Precipitation with ammonium sulphate:* This simple technique provides crude purification of total immunoglobulin from other serum proteins.
- *Affinity purification with immobilized Protein A or G:* These proteins bind to most species and subclasses of IgG, the most abundant type of immunoglobulin produced by mammals in response to immunogens.
- *Affinity purification with immobilized antigen:* Covalently immobilized purified antigen (i.e., the peptide or hapten used as the immunogen to induce production of antibody by the host animal) on an affinity support allows the specific antibody to be purified from crude samples

2.2.1 Affinity Chromatography

Affinity purification makes use of a specific native or added property of the target molecule to isolate it from all contaminants in the sample.
Binding sites of receptors and antibodies or active sites of enzymes are examples of very specific properties that can be used for affinity chromatography. Common for all types of affinity chromatography is that a ligand (affinity ligand) specific for the binding site of the target molecule, is coupled to an inert chromatography matrix. Under binding conditions this specific ligand on the chromatography matrix will bind molecules according to its specificity only. All other sample components will pass through the chromatography medium unbound. After a wash step the bound molecules are released and eluted by changing the conditions.
Different classes of affinity targets, as well as different purification goals, require consideration of different priorities (e.g., high purity vs. high yield), technical limitations and buffer conditions for development of a successful procedure.

Most affinity purification procedures involving protein-ligand interactions use binding buffers at physiologic pH and ionic strength, such as phosphate buffered saline (PBS). This is especially true when antibody-antigen or native protein-protein interactions are the basis for the affinity purification. Once the binding interaction occurs, the support is washed with additional buffer to remove non-bound components of the sample. Nonspecific (e.g., simple ionic) binding interactions can be minimized by adding low levels of detergent or by moderate adjustments to salt concentration in the binding and/or wash buffer. Finally, elution buffer is added to break the binding interaction and release the target molecule, which is then collected in its purified form. Elution buffer can dissociate binding partners by extremes of pH (low or high), high salt (ionic strength), the use of detergents or chaotropic agents that denature one or both of the molecules, removal of a binding factor or competition with a counter ligand.

The most widely used elution buffer for affinity purification of proteins is 0.1M glycine-HCl, pH 2.5-3.0. This buffer effectively dissociates most protein-protein and antibody-antigen binding interactions without permanently affecting protein structure. However, some antibodies and proteins are damaged by low pH, so eluted protein fractions should be neutralized immediately.

The most used affinity ligands for antibody purification are the bacterial proteins Protein A and G, which demonstrate specific binding to the Fc (non-antigen binding) portion of many classes of immunoglobulins. Protein A resins have historically been popular for most potential applications, however it has been demonstrated that Protein G resin can enhance and broaden the scope of application. The binding characteristics of the two proteins for various types of immunoglobulins vary and may be used to good advantage.

2.2.2 Ammonium sulphate precipitation

Ammonium sulphate precipitation is a simple and effective means of fractionating proteins. It is based on the fact that at high salt concentrations inter- and intramolecular charges of a protein are influenced and the stabilizing effect of the solvation shell is also reduced. This charge neutralization means that proteins will tend to bind together and aggregate. Since each protein will start to aggregate at a characteristic salt concentration, this approach provides a simple way of enriching for particular proteins in a mixture.
Ammonium sulphate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed and because its non-denaturating effect to proteins.

2.3 Protein determination by Bicinchoninic acid (BCA)

The BCA protein assay kit is purchased from Thermo Scientific Pierce and is used for total protein quantification. The assay combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an environment containing sodium potassium tartrate.

In the second step of the colour development reaction, bicinchoninic acid reacts with the reduced cuprous cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The reaction that leads to BCA colour formation is strongly influenced by four amino acid residues (cysteine or cystine, tyrosine, and tryptophan) in the sequence of the protein. However, the universal peptide backbone also contributes to colour formation, helping to minimize variability caused by protein compositional differences.

Protein concentrations generally are determined and reported with reference to standards of a common protein (in this kit BSA). A series of dilutions of known concentrations are prepared from the protein and assayed alongside the unknown samples before the concentration of each unknown is determined based on the standard curve.

The following is a short list of compatible substances and their concentrations that the BCA protein assay can tolerate and which were used in this study for allergen extraction (Table 4).

Table 4. Interfering substances in BCA protein determination

Substance	compatible conc.
PBS	undiluted
TBS	undiluted
Brij-35	5 %
CHAPS, CHAPSO	5 %
SDS	5 %
Triton X-100	5 %
Tween 20	5 %
EDTA	10 mM
Dithiothreitol (DTT)	1 mM
Ethanol/Methanol	10 %
Urea	3 M

2.4 Gel Electrophoresis

SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis, is a technique widely used for the separation of proteins according to their electrophoretic mobility, which depends on the length of polypeptide chain or the molecular weight. In native PAGE also the charge of the protein influences the electrophoretic mobility in an electric field. Therefore, one protein may cause more bands according to various charged isoforms. This effect is avoided by SDS, an anionic detergent which denatures the folding state of a protein and masked all intrinsic charges applying a total negative charge in proportion to the mass of the protein. To ensure complete denaturation of the proteins DTT (Dithiothreitol) is used as reducing agent to disrupt disulfide bonds.

The proteins migrate through a synthetic gel of a crosslinked polymer with well defined pore sizes, in which smaller proteins run faster. For evaluation of the samples, a commercial marker consisting of a mixture of proteins with known molecular weight is also loaded on the gel. To visualize the running front of the proteins in the gel a dye is added, which facilitates loading procedure of the samples, too. Staining of the gel can either be performed with Coomassie Brilliant Blue or silver nitrate, which includes a longer and more complex staining procedure, but also higher sensitivity.

2.5 Western Blot

The Western Blot is an analytical technique to detect specific proteins, which were previously separated by SDS-PAGE. The proteins from the gel are transferred to a nitrocellulose membrane (Figure 4).

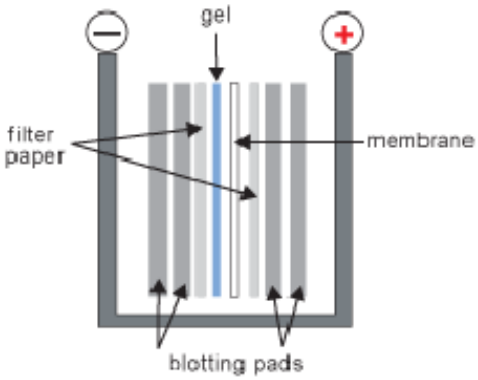


Figure 4. Assembling of the blot chamber

(Copyright 2001, Georgia Institute of Technology)

The nitrocellulose membrane provides a surface with high affinity for proteins. Therefore, also immunoglobulins would be immobilized on the membrane, so that detection of a specific protein is impossible. To block unspecific binding, the membrane is incubated in a solution with 2% BSA (bovine serum albumin) before probing with antibodies. The secondary antibody is labelled with the enzyme HRP (horseradish peroxidase), which oxidizes TMB (Tetramethyl-benzidine) in presence of H_2O_2, which causes a blue colour reaction (Figure 5).

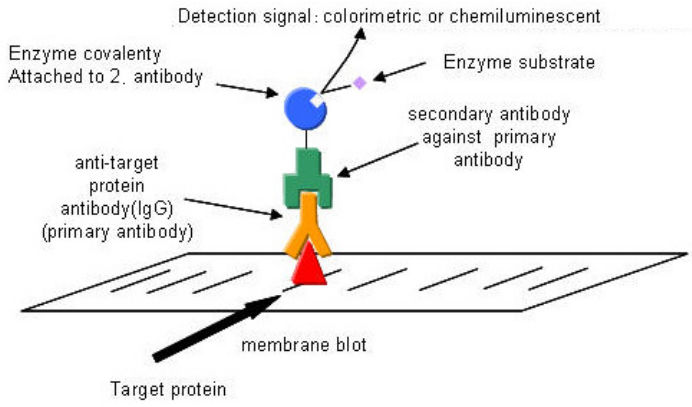


Figure 5. Detection of the target protein by a specific antibody

(image from: employees.csbsju.edu/…/TechElectrophoresis.htm)

2.6 Indirect competitive ELISA

2.6.1 ELISA procedure

In indirect competitive ELISAs the polystyrene plates are only unsaturated coated with the antigen, which is in most cases the protein used for immunization. In the next step, the assay is incubated with the sample and the specific antibody against the allergen, whereat the sample has to be added first. After the addition of the 1st antibody the competition between the antigen coated onto the plate and the antigen contained in the sample for antibody binding starts. After removing unbound molecules, a second antibody labelled with HRP is added, this detects the 1st antibody independent of the present antigen. After a final washing step the substrate solution containing TMB is incubated for max. 30 min and stopped (see scheme below).

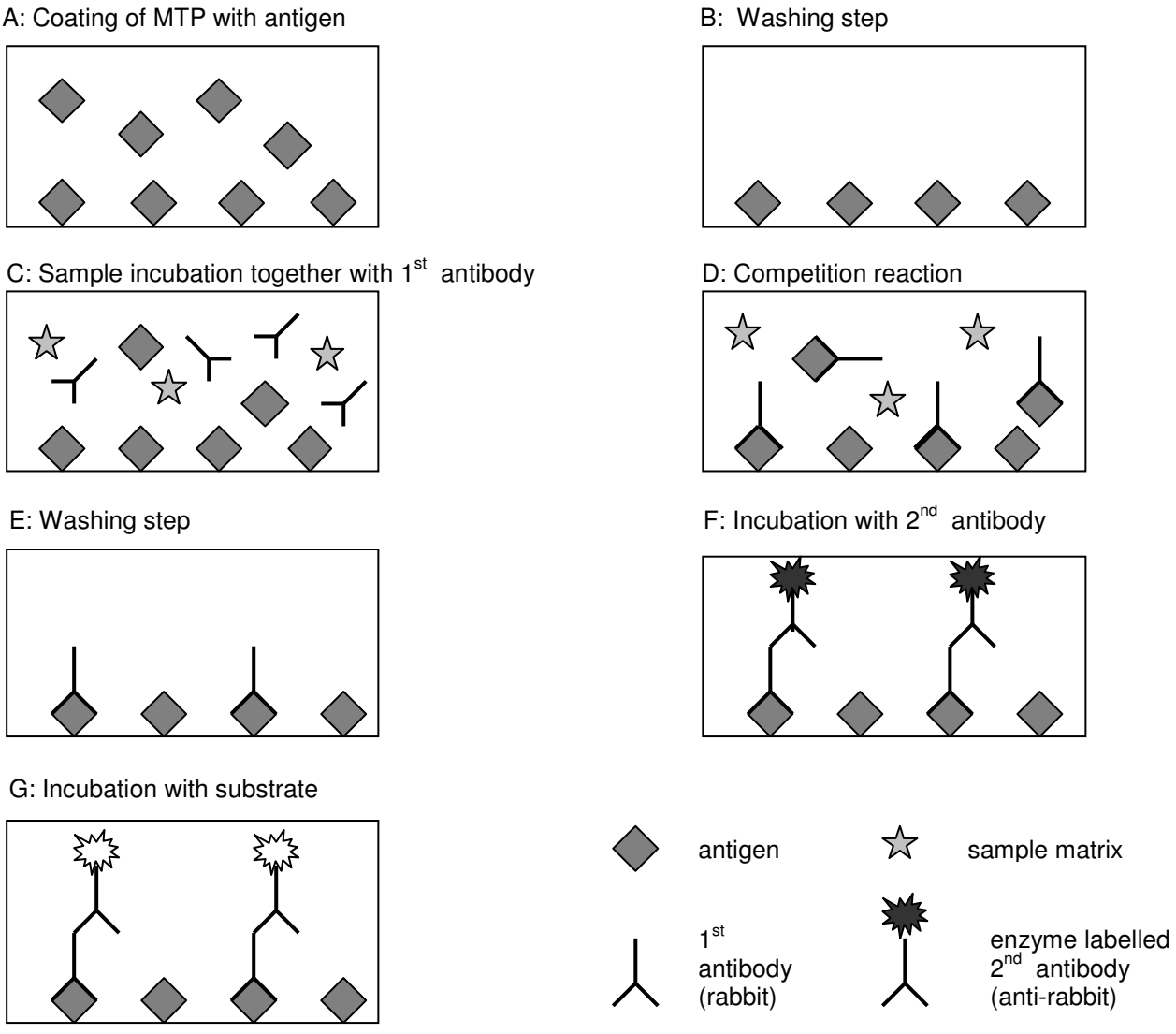


Figure 6. Schematic setup of an indirect competitive ELISA. The primary antibodies used in this work are polyclonal and produced in rabbits, the secondary antibody is labelled with the enzyme HRP (horse radish peroxidase) and purchased from Sigma.

2.6.2 Data validation

The results of the ELISA analysis were obtained with the Tecan Reader and the four-parameter curve derived by Magellan5 software. The standard curves of indirect competitive immunoassays show a characteristic sigmoid shape, with a lower boundary (asymptote) near the background response (non-specific binding) and an upper asymptote near the maximum response. The 4-parameter logistic model is generally acknowledged to be the reference model of choice for fitting calibration curves of this shape. This function provides an accurate depiction of the sigmoidal relationship between the measured response and the analyte concentration. The equation describing the model is as follows:

$$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^{B}}$$

in which Y is the response, D is the response at infinite analyte concentration, A is the response at zero analyte concentration, x is the analyte concentration, C is the inflection point on the calibration curve (IC_{50}), and B is a slope factor. This model has several useful characteristics (Figure 6):

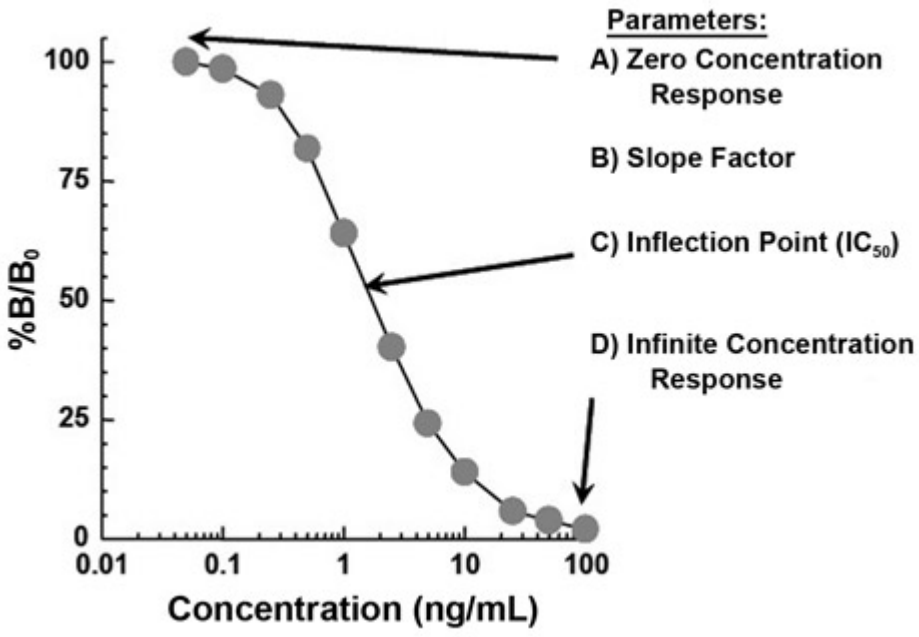


Figure 7. Signal curve of an indirect competitive ELISA according to Rodbard`s four-parameter equation (copyright by the American Association of Pharmaceutical Scientists).

The calibration curve is symmetric around the IC_{50} concentration C, with a response at that concentration of (A+D)/2. The slope parameter B defines the steepness of the curve. Since the curve is sigmoid in shape, the slope is changing throughout, but at the IC_{50} the slope is given by B*(D-A)/4C.

If comparability between several graphs is needed, the curves have to be standardized by uniting the maximum absorbance of each curve into one point. This standardization is performed by the equation $y = B/B_0$, in which B is the respective resulting absorbance and B_0 represents the absorbance of the zero concentration, which is indicated by the maximum absorbance A. The B/B_0 standardization allows a clear comparison of the horizontal curve shift and helps to recognize sensitivity improvement after optimisation efforts. In contrast, non-standardized graphs were used to visualize great differences in steepness of the curves and signal inhibition [12].

3. EXPERIMENTAL

3.1 Antibody purification

3.1.1 Affinity chromatography

Buffers and reagents:

- Binding buffer (A): 20 mM Na_2HPO_4, pH 7 with H_3PO_4
- Elution buffer ProtG (B): 0.1 M Glycine, pH 2.7 with HCl
- 1 M Tris-HCl, pH 9
- 20 % ethanol

Equipment :

- FPLC system: Pharmacia LKB
- Software: FPLCdirector™ version 1.03
- Protein G columns: 1mL Hi Trap HP columns from Amersham Biosciences
- UV/VIS spectrometer: Lambda 2S Perkin Elmer
- NuPAGE™ 3-8% Tris-Acetate Gel (1 mm x 15 well) from Invitrogen
- HiMark™ Pre-stained HMW protein standard from Invitrogen
- NuPAGE™ LDS sample buffer (4x)

Procedure :

First of all a 1.9 mL aliquot of serum was filled up with buffer A to 10 mL and the peristaltic pump was rinsed with buffer A. After attaching the ProtG column to the pump it was equilibrated with 10 mL of buffer A. At least 1 mL/min of the sample was applied to the column via the peristaltic pump. In the meantime the FPLC system was rinsed with buffer A increasing the flow rate stepwise. Afterwards the fraction collector was turned on to rinse the sample collection part of the tube system, too. Finally, the system was rinsed with buffer B and the column was connected with the FPLC. The clean-up was performed with a flow rate of 1 mL/min starting with buffer A and switch to buffer B to elute the sample in a fraction size of 2 mL. The collection tubes were prepared with 50 µL Tris-HCl to neutralize the acidic elution buffer. After detaching the column the FPLC system was rinsed with 20 % ethanol. Again the column was attached to the peristaltic pump and rinsed first with buffer A, then with ethanol and was storaged at 4 °C.

3.1.2 Ammonium sulphate precipitation

Buffers and reagents:

- 0.05 M PBS, pH 7.5
- 0.01 M PBS, pH 7.5
- Ammonium sulphate/$(NH_4)_2SO_4$

Equipment:

- Spectra/Por™ Dialysis membrane from Spectrumlabs
- Allegra™ X-22R centrifuge from Beckman

Procedure:

First a 1.9 mL aliquot of serum was filled up with cold ddH_2O to 10 mL. The clean-up was carried out by stepwise precipitation. First 1.66 g $(NH_4)_2SO_4$ were added, resulting in a 30% solution to remove other serum components. The precipitation took place for 1 h at 4 °C while gently stirring. After centrifugation for 30 min at 4 °C with 9500 rpm the supernatant was precipitated again under the same conditions at 40% ammonium sulphate adding 0.57 g $(NH_4)_2SO_4$.The pellet of the second precipitation step was resuspended in 1.5 mL of 0.05 M PBS. To remove the $(NH_4)_2SO_4$ the suspension was dialyzed against 0.01 M PBS at 4 °C overnight.

3.2 Protein determination by Bicinchoninic acid (BCA)

Buffers and Equipment:

- 0.2 M PBS, pH 7.5
- BCA™ protein assay kit from Pierce Thermo Scientific
- MTPs non-binding from Greiner bio-one
- ELISA reader: Sunrise™ from Tecan Austria GmbH

Procedure:

The samples were measured in different dilutions to ensure being in the linear range of 25-2000 µg/mL. Generally, the dilutions were prepared with 0.2 M PBS, but some food stuff extracts were diluted only with ddH_2O to avoid precipitation caused by higher ionic strength. 20 µl of each sample and of the ready-to-use BSA standards were put into the wells and 200 µl of the colouring reagent were added and mixed by pipetting up and down and avoiding air bubbles. The colouring reagent consisted of 25 mL of reagent A and 500 µL of reagent B. The assay was incubated for 30 min at 37 °C in the dark and afterwards measured with the Tecan reader. The results were calculated with help of the ValiData software using the BSA standard curve.

3.3 Gel Electrophoresis

Buffers and equipment:

- MES SDS running buffer: 50 mM MES, 50 mM Tris base, 0.1% SDS, 1mM EDTA
- Tris-Acetate SDS running buffer: 50 mM Tricine, 50 mM Tris base, 0.1% SDS
- NuPAGE™ 3-8% Tris-Acetate Gel (1 mm x 15 well) from Invitrogen
- NuPAGE™ 12% Bis-Tris Gel (1 mm x 15 well) from Invitrogen
- HiMark™ Pre-stained HMW protein standard from Invitrogen
- SeeBlue™ Plus2 prestained standard
- NuPAGE™ LDS sample buffer (4x)
- Xcell SureLock™ Mini-Cell

Procedure:

The amount of loaded sample was depended on its protein concentration and averaged about 5 µg. The loading volume was 10 µl including 2.5 µl of 4x sample buffer, the sample and the rest was filled up with ddH_2O. To ensure complete denaturation the samples were heated up to 70 °C for 10 min.

The ready-to-use gels were rinsed with ddH_2O and the protection stripe was removed to allow buffer circulation. The chamber assembling was performed according manufacturer's instructions, filled up with running buffer and after removing the comb the samples were loaded into the slots. As standard for the MW estimation a protein marker was also loaded. For protein extracts the 12% Bis-Tris gels were used to separate proteins over a molecular weight range of 1-200 kDa. To check antibody purification Tris-acetate gels, which resolve proteins of 36-400 kDa, and the high molecular weight (HMW) marker were used. The Bis-Tris gels needed the MES SDS running buffer and the run was performed at constant 200 V with max. 125 mA for 1h. The conditions for electrophoresis with Tris-acetate gels included the Tris-acetate SDS running buffer and 150 V constant, max. 55 mA for 1h.

After the run the gels were washed 3x for 5 min with ddH_2O to remove the running buffer salts and stained with Coomassie Blue.

3.4 Western-Blot and Immunoblotting

Buffers and reagents:

- Transfer buffer stock (20x): 25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA
- Transfer buffer (1x): 50 mL stock, 200 ml methanol filled up to 1000 mL with ddH_2O
- Blocking buffer: 2% (w/v) BSA in 0.05 M PBS
- Washing buffer: 0.2 M PBS + 0.1% Tween 20
- Antibody dilution buffer: 0.2 M PBS
- Preparation buffer: 0.01 M Tris-HCl, pH 6
- Substrate buffer: 0.15 M citric acid, pH 5 with NaOH
- Substrate reagent: 24 mg TMB + 80 mg DONS dissolved in 10 mL EtOH
- Substrate solution: substrate diluted in substrate buffer 1:4 + 5 µl H_2O_2/10 mL

Equipment:

- Xcell SureLock™ Mini-Cell
- Xcell II™ Blot module
- Nitrocellulose membrane (NC): Protran BA 85 from Schleicher&Schull
- Filter paper: Gel-blotting paper GB 002 from Roth

Procedure:

After the electrophoresis the gel was rinsed in ddH_2O and the sandwich was assembled with 4 sponges, 2 filter papers and the NC membrane, which were already soaked in transfer buffer. The sandwich was carefully but also rapidly placed into the transfer cassette and the inner buffer chamber was filled up with buffer. To remove air bubbles the whole cell chamber was gently tapped several times on the working bench. Finally, the outer buffer chamber was filled with ddH_2O and the blot was performed with 30 V constant and max. 170 mA for 1 h. After the blot was finished the protein transfer to the NC membrane could be controlled with Ponceau Red stain, a water-soluble dye. To block unspecific binding sites the membrane was incubated with 2 % BSA for min. 2 h at RT or overnight at 4 °C. Afterwards the membrane was rinsed with ddH_2O and washed 3 x 5min with washing buffer. Next step was the incubation for 1 h shaking with the first antibody, whereat the dilution factor was antibody dependent (from 1:1000 to 1:5000). After a further washing step as mentioned before the first antibody was detected with the second antibody, in this work with anti-rabbit HRP purchased from Sigma diluted 1:10000. After 1 h incubation with the 2nd antibody the membrane was washed a third time and the antibody-antigen binding was visualized by the HRP substrate TMB. For this purpose, the membrane was incubated in the preparation buffer for 1 min and finally the substrate solution, which was mixed a short time before, was added. The reaction was stopped before the development of the blue colour signal was too intensive by washing the membrane in ddH_2O and drying. The blot was scanned immediately to document the results because the blue colour decreases after some time.

3.5 Indirect competitive ELISA

Buffers and reagents:

- Coating buffer (1 L): 1.22 g Na_2CO_3, 3.25 g $NaHCO_3$, 0.1 g NaN_3 (store at 4 °C)
- Blocking solution: 1% Ficoll in coating buffer
- Washing buffer: 0.01 M PBS with or without 0.1% Tween20
- Assay buffer: 0.05 M PBS + 0.1% Tween20
- Substrate buffer (1 L): 42 g citric acid, 100 mg sorbic acid, pH 4 with NaOH
- TMB stock solution: 375 mg TMB dissolved in 5 mL DMSO in 25 mL MeOH
- Substrate solution (for 1 MTP): 12.5 mL substrate buffer, 100 µL TMB stock, 2.5 µL 30% H_2O_2

Equipment:

- MTPs high binding from Greiner Bio-one
- MTP washer : Tecan 96PW™ from Tecan Austria GmbH
- ELISA reader: Sunrise™ from Tecan Austria GmbH
- ELISA software Magellan5 Tecan Austria GmbH

Procedure:

First the MTPs were coated with 100 µL/well of an unsaturated concentration of 500 ng antigen, which had also been used for immunization, per mL coating buffer overnight at 4 °C. Next day the MTPs were washed 2 x with 0.01 M PBS without Tween and blocked with 1 % Ficoll for 2 h at 37 °C. Afterwards the plates were washed 2 x with buffer containing 0.1 % Tween and the remained washing buffer in the wells was removed by tapping the plate upside down on towels. 75 µL of the samples in dilution series were put into the wells and 25 µl of the 1st antigen-specific antibody diluted in assay buffer were added. The competition reaction took place for 1 h at RT on a shaker before the MTPs were washed again 2 x with buffer containing Tween. The detection of the 1st antibody was performed with 100 µL/well of the 2nd antibody (anti-rabbit HRP) diluted 1:50000 in assay buffer, shaking for 1 h. After a final washing step 100 µL of the fresh substrate solution was added into the wells and incubated on the shaker, covered with a box to protect the reaction from light. The reaction was resulting in the development of a deep blue colour and stopped by adding 25 µL of 5% sulphuric acid. Finally, the signal was determined with the Tecan reader at 450 nm.

3.6 Sample preparation and extraction

The food stuff samples were milled with an Osterizer Blender and stored at 4 °C or -20 °C if the sample was perishable (e.g. cooked noodles). The extraction was performed under various conditions, see different extraction approaches. To achieve higher extraction temperature a water bath from Müller-Scherr or an incubator from Heraeus were used. In the case of extraction in the incubator, at RT or at 4 °C the samples were shaken with the horizontal-shaker from VWR or with the overhead-shaker from Labor-Brand.

4. ANTIBODY PURIFICATION

The antibodies used for the following immunoblots and ELISA analyses were cleaned up according to chapter 3.1., with purifying every IgG by ammonium sulphate precipitation and by Protein A or G. The concentration was measured via BCA protein determination and the purity of the single fractions was controlled with SDS-PAGE.

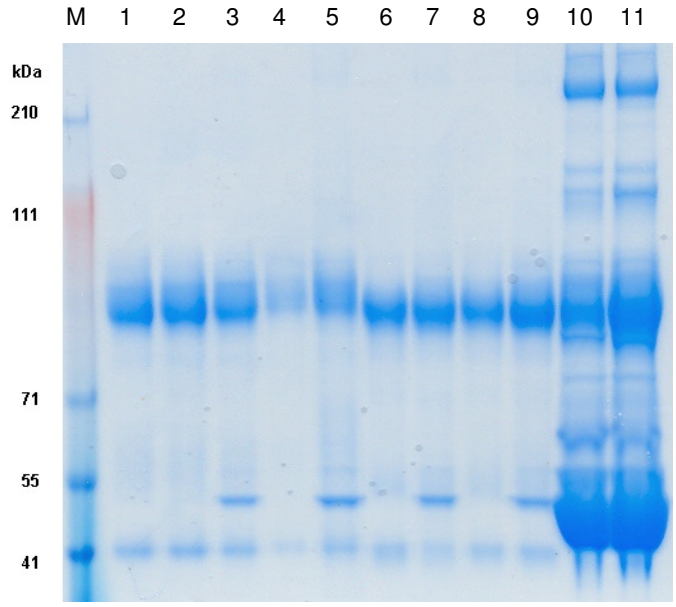
4.1 Results

BCA protein determination:

Table 5. IgG yield after purification of rabbit's sera

polyclonal rabbit antibody	purification method	protein [mg/mL]
anti - SEW	Protein A	4.2
	Protein G	5.3
	AS precipitation	5.0
anti - cEW	Protein G	3.7
	AS precipitation	8.5
anti - nativeOVM	Protein G	5.7
	AS precipitation	9.9
anti - heatedOVM	Protein G	7.4
	AS precipitation	11.7

SDS-PAGE:



M = marker
1 = anti-SEW ab, ProtA purified
2 = anti-SEW ab, ProtG purified
3 = anti-SEW ab, AS purified
4 = anti-cEW ab, ProtG purified
5 = anti-cEW ab, AS purified
6 = anti-nativeOVM ab, ProtG purified
7 = anti-nativeOVM ab, AS purified
8 = anti-heatedOVM ab, ProtG purified
9 = anti-heatedOVM ab, AS purified
10 = pre-serum (before immunization)
11 = serum (after immunization)

Figure 8. SDS-PAGE of antibodies to check purity after different clean-up methods

In most cases the ammonium sulphate precipitation caused the highest purification yield (table 5), but also less purity according to the band pattern in the gel. The whole rabbit IgG offers 158 kDa, but the corresponding band was located between 71 and 111 kDa on SDS-PAGE (see figure 8). The high amount of protein in the sera fractions around 50 kDa might be serum albumin (lane 10, 11).

4.2 Discussion

The AS fractions always showed an additional band at 50 kDa. This band could correspond to serum albumin (lane 3, 5, 7 and 9). In some cases the contaminations in AS purified antibodies enhance the signal in ELISA, thus Protein A or G purified antibodies are used for immunoassays. However, to ensure the specificity of the purified antibodies and their functionality after the purification step, they were compared in indirect competitive ELISA (data not shown). Every antibody was able to detect egg white proteins and the signal curves of the various purified fractions were very similar. Finally the Protein A purified ab against SEW, the Protein G purified ab against native OVM and heated OVM and the AS purified ab against cEW were used for further analyses.

5. ALLERGEN EXTRACTION

5.1 First extraction approach

At the beginning of this work the most common protein extraction buffers from literature (see chapter 1.14) were used to extract samples like crude egg, cooked egg and egg powder. For comparison also four commercial egg white standards purchased from Sigma were treated in the same way. The results were obtained by quantitative BCA protein determination and to some extent by SDS-PAGE.

5.1.1 Experimental

Sample preparation:

The fresh crude egg white and yolk were separated and the egg white was gently stirred to homogenize the viscous substance. The crude egg yolk with undamaged membrane was moved gently on a filter paper to remove lipids on the surface. Afterwards the yolk sack was pricked with a needle and the liquid was collected. The egg white and yolk from cooked egg was easier to be separated and the dried powder forms of egg white and egg yolk were a gift from Fa. Inovo Food Ingredients Handels-GmbH (Innsbruck, Tyrol, Austria).

Extraction buffers:

- Buffer A1: saline solution with 0.15 M NaCl
- Buffer A2: 50 mM PBS, 1% Tween 20, 0.4% Triton X100, pH 7.5 with HCl
- Buffer A3: 50 mM TBS, 0.1% Tween 20, pH 8.4 with HCl
- Buffer A4: 25 mM PBS, 0.1% Tween 20, pH 7.2 with HCl
- Buffer A5: 0.1 M sodium acetate buffer, pH 4.5
- Buffer A6: 0.2 M sodium carbonate-bicarbonate buffer, pH 9.5
- Buffer A7: 0.075 M K-acetate, 0.3 M NaCl, 0.01 M EDTA, 0.25% Triton X100, pH 7.4
- Buffer A8: 0.06 M Tris-HCl, 2% SDS, 1 mM DTT, 2% glycerol, pH 7
- Buffer A9: 0.05M Tris-HCl, 1% NaCl, 0.1% Tween 20, 5% Urea, pH 7.5

Egg white standards:

- Trypsin inhibitor, type III-O: Chicken egg white purified ovomucoid (Sigma T2011-5G)
- Albumin from chicken egg white, grade V, min. 98% GE (Sigma A5503-10G)
- Lysozyme from Chicken egg white, >90% (Sigma L-6876)
- Conalbumin from Chicken egg white (Sigma C-0755)

Extraction procedures:

The samples were weighed in, the various extraction buffers were added and the suspensions were mixed with the vortexer to homogenize. The extraction was performed for 4 h at RT rotating with 20 rpm. Finally the extracts were centrifuged with 3500 rpm for 30 min at 20 °C and the supernatants were transferred into new tubes.

- 20 mg egg white standard in 1 mL extraction buffer
- 1 g crude egg white (EW), egg yolk (EY) and egg mix (EM) in 10 mL extraction buffer
- 1 g cooked egg white and egg yolk in 10 mL extraction buffer
- 1 g spray dried egg white (SEW) and Indian egg yolk powder (PEY) in 10 mL extraction buffer
- 50 mg defatted egg yolk powder (def.PEY) in 1 mL extraction buffer

5.1.2 Results

BCA protein determination:

Table 6. Protein yield of egg white and egg yolk samples extracted with the buffers A1-A9

Extraction buffers	**protein content [mg/ml] of the extracts**							
	crude EW	crude EY	crude EM	cooked EW	cooked EY	SEW	PEY	def.PEY
Buffer A1	9.12	10.88	6.77	1.02	0.32	69.07	7.92	7.31
Buffer A2	9.72	16.61	10.32	0.90	0.71	64.29	17.54	20.63
Buffer A3	10.08	12.92	9.07	1.02	0.36	65.29	9.39	10.71
Buffer A4	11.30	16.33	10.10	0.98	0.40	66.36	17.54	14.04
Buffer A5	6.30	10.67	7.75	0.81	0.26	52.72	6.16	5.72
Buffer A6	9.30	15.84	8.33	1.01	0.50	62.99	15.14	13.84
Buffer A7	10.92	14.72	9.75	0.93	0.51	72.04	12.54	12.60
Buffer A8	9.40	15.54	9.90	1.09	1.27	54.13	17.33	22.32
Buffer A9	9.46	3.88	n.d.	0.96	0.44	71.36	13.91	15.57

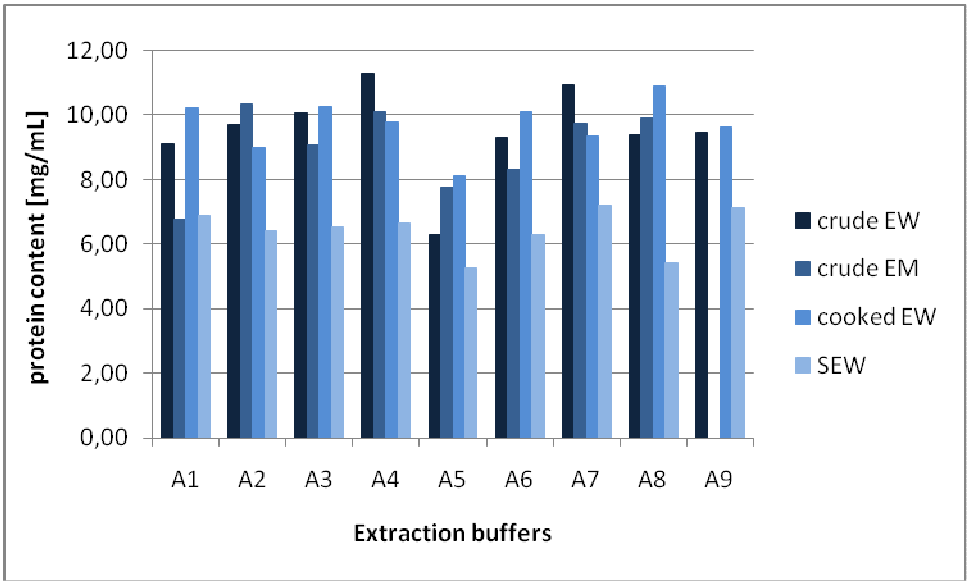
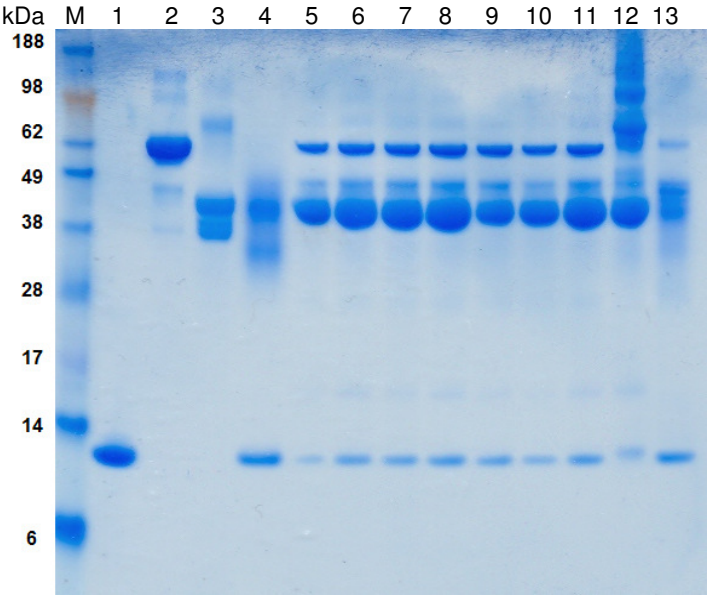


Figure 9. Comparison of protein yield after extraction with the buffers A1-A9. The values for SEW were divided and the values for cooked EW were multiplied by the factor 10 to allow the illustration of the extracts in the same diagram

SDS-PAGE:



M = Marker
1 = lysozyme
2 = conalbumin
3 = ovalbumin
4 = ovomucoid
5 = cEW in buffer A1
6 = cEW in buffer A2
7 = cEW in buffer A3
8 = cEW in buffer A4
9 = cEW in buffer A5
10 = cEW in buffer A6
11 = cEW in buffer A7
12 = cEW in buffer A8
13 = cEW in buffer A9

Figure 10. SDS-PAGE of crude egg white extracts with the buffers A1-A9 and egg white standards purchased from Sigma

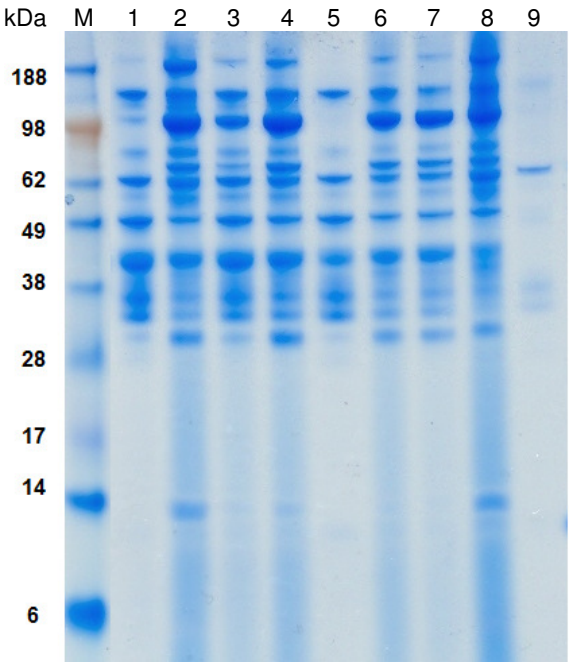


Figure 11. SDS-PAGE of egg yolk powder extracts with the buffers A1-A9

The egg white standards purchased from Sigma showed some contaminations (see figure 10), especially conalbumin (lane 2) and ovomucoid (lane 4) in the upper gel offered additional bands. Only the crude EW extract of buffer A8 (lane 12) caused a different band pattern than the others. Furthermore, higher band intensity was obtained for egg yolk powder extracted with the buffers A2, A3 and A8 (see figure 11: lanes 2, 4 and 8). In contrast, the extracts of buffer A1 and A5 (lane 1, 5) lacked a few bands, in particular the band about 98 kDa, which was pronounced in other samples. Buffer A9, the second one with denaturing capacity, exhibited the disadvantage of irreversible protein precipitation, if the extract had been stored at -20 °C. Therefore, the protein pattern could not be visualized on the gel (lane 9).

5.1.3 Discussion

The quantified protein yield of the extracts showed that each sample favoured different extraction buffers (see table 6). However, buffer A5 with pH 4.5 caused less extraction yield than the others, also buffer A6 with alkaline pH offered only for crude egg yolk good results. High protein yield was observed with the buffers A2, A3, A4 and A7 for crude egg and for the dried powder forms. Importantly, buffer A8, containing denaturing and reducing agents, reached the highest protein amounts at the extraction of cooked egg white and egg yolk. The band patterns in SDS-PAGE were very similar between the different extracts, only the buffer A8 extracts showed a more compact protein pattern in the upper range of the gel.

The gap observed between theoretical (28 kDa) and experimental (38 kDa) value of ovomucoid could be explained by the high glycosylation degree of this protein. Indeed, it has been highlighted that glycoproteins migrate more slowly in SDS electrophoresis because the sugar moieties do not bind SDS, thus lowering the SDS:protein ratio. The broad shape of ovomucoid bands in SDS-PAGE could be also caused by incomplete reduction of the nine disulfide bridges hindering formation of unique ellipsoid SDS-protein complexes [13, 14].

5.2 Second extraction approach

5.2.1 Experimental

The best extraction buffers for egg white of the first approach were selected and used once more for extracting crude and cooked egg white, spray dried egg white and crude egg mix, but extraction time (1 h, 4 h and overnight) and temperature (4 °C, RT and 37 °C) were varied. The sample preparation and the amounts of sample and buffer were the same as in the first extraction approach except for SEW, where only 0.5 g were resuspended in 10 mL buffer.

5.2.2 Results

BCA protein determination:

Only the results of the cooked EW extraction are shown, because the optimal extraction buffer for food stuff should be found in this work and cooked EW reflected processed food more than the other samples.

Table 7. Extraction of cooked egg white for 1 h at different temperatures

Extraction buffer	protein content [mg/mL]		
	4 °C	RT	37 °C
A3	0.86	0.75	1.13
A4	0.79	0.60	0.92
A7	0.93	0.69	0.99
A8	1.07	0.92	1.04
A9	0.95	0.70	0.99

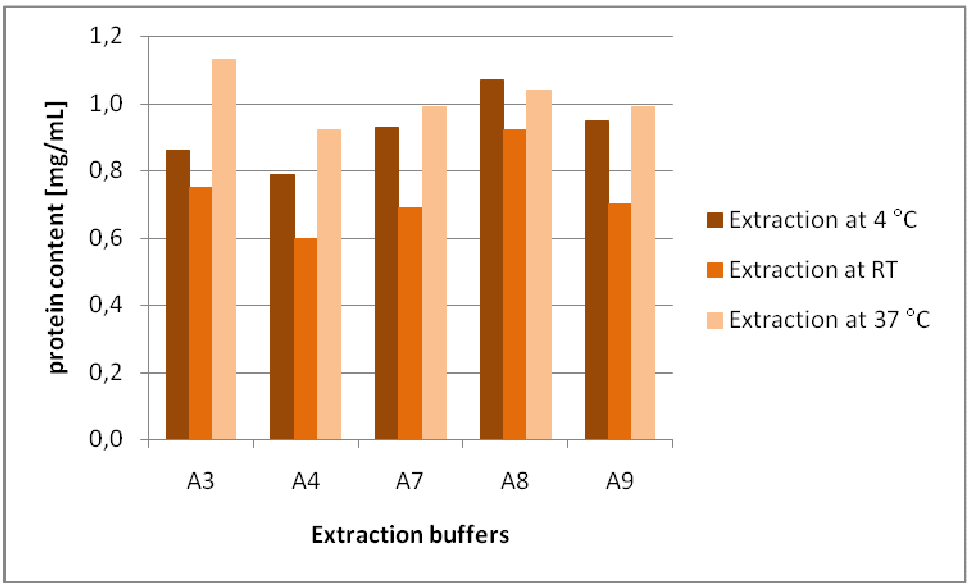


Figure 12. Comparison of cooked egg white extracted for 1 h at different temperatures

Table 8. Extraction of cooked egg white for 4 h at different temperatures

Extraction buffer	protein content [mg/mL]		
	4 °C	RT	37 °C
A3	1.21	1.08	1.13
A4	1.06	0.88	1.16
A7	1.05	0.97	1.16
A8	1.04	1.05	1.53
A9	1.14	0.83	1.33

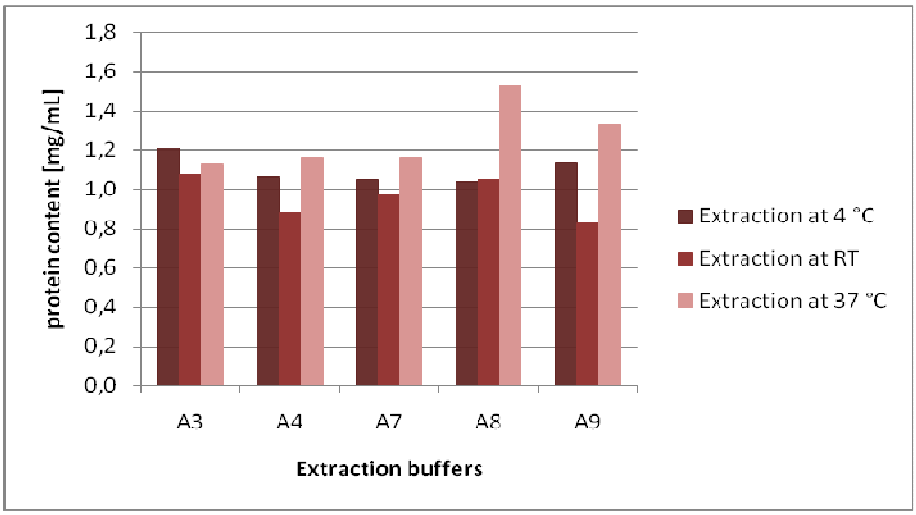


Figure 13. Comparison of cooked egg white extracted for 4 h at different temperatures

Table 9. Extraction of cooked egg white overnight at different temperatures

Extraction buffer	protein content [mg/mL]		
	4 °C	RT	37 °C
A3	1.46	1.12	1.56
A4	1.45	1.11	1.60
A7	1.45	1.12	1.66
A8	1.57	2.79	6.77
A9	1.52	1.16	1.52

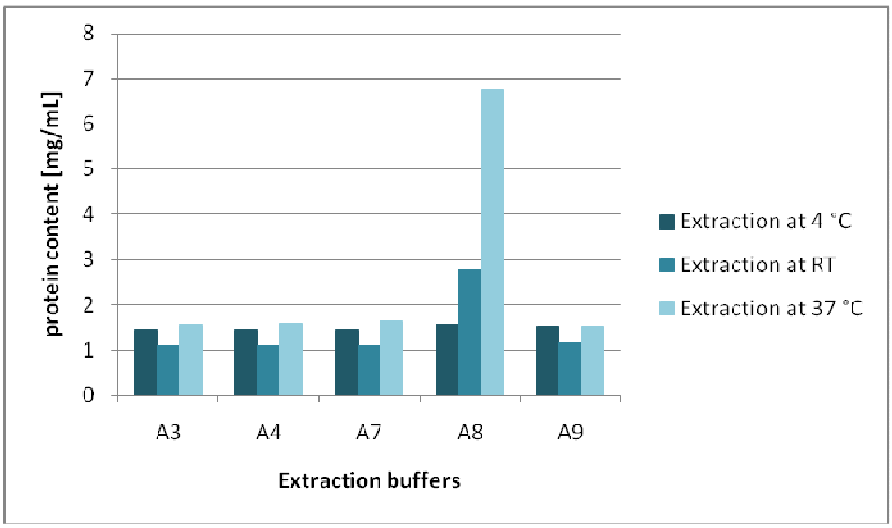


Figure 14. Comparison of cooked egg extracted overnight white at different temperatures

SDS-PAGE:

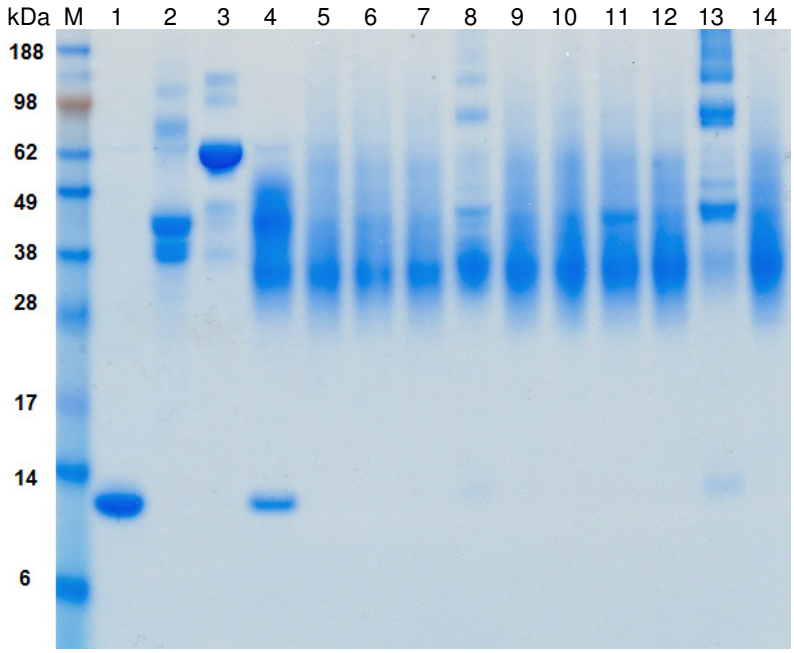


Figure 15. SDS-PAGE of cooked egg white extracted at 37 °C with the buffers A3, A4 and A7-A9

5.2.3 Discussion

The crude samples egg white and egg mix did not require longer extraction time and showed highest protein yield at 4 °C. Spray dried egg white offered good extraction results at 4 °C and at room temperature, but the protein yield was similar for 1 h and 4 h extraction time. However, overnight extraction increased the protein content of the extracts a little bit. Interestingly, extraction of cooked egg white at 4 °C and at 37 °C caused higher protein yield than at RT (see tables 7-9). The accelerated extraction efficiency of buffer A8 was visible at the extraction overnight at 37 °C and in SDS-Page analysis (figure 15). Only the extract in the buffer including SDS and DTT showed a clear band pattern and therefore, buffer A8 is suitable for the extraction of processed food stuff.

5.3 Third extraction approach

To determine the influence of matrix effects on the extraction efficiency of some buffers, self-made food samples were prepared containing egg, wheat flour and oil in various combinations. Fat acids and carbohydrates may avoid the extraction of egg white proteins dependent on the used buffer. For this approach only two extraction buffers were chosen: a salt buffer (buffer A4) and a buffer containing a detergent (buffer A8).

5.3.1 Experimental

Sample preparation:

First of all the egg white and egg yolk were separated and the egg white was beaten until it was stiffed. Afterwards egg yolk and/or wheat flour and/or oil were added and mixed gently. The samples were processed at 100 °C for 40 min and at 200°C for 20 min in the baking oven. The baked samples were air-dried to become hardened and were grinded by crushing them between to towels on the working bench.

Extraction procedure:

0.5 g of each sample were resuspended in 5 mL of buffer and extracted for 4 h at 37 °C on the overhead-shaker. After centrifugation at 9000 rpm for 30 min the extracts were filtrated, but the samples containing oil showed strong turbidity.

5.3.2 Results

BCA protein determination:

Table 10. Extraction of self-made baked samples with buffer A4 at 37 °C for 4 h

sample	protein content [mg/ml]	
	processed at 100 °C	processed at 200 °C
EW	7.19	2.46
EW + WH	7.91	1.67
EW + oil	6.91	1.22
EW + WH + oil	7.63	0.70
Egg	5.07	1.22
Egg + WH	11.52	2.47
Egg + oil	3.66	1.65
Egg + WH + oil	4.31	1.17

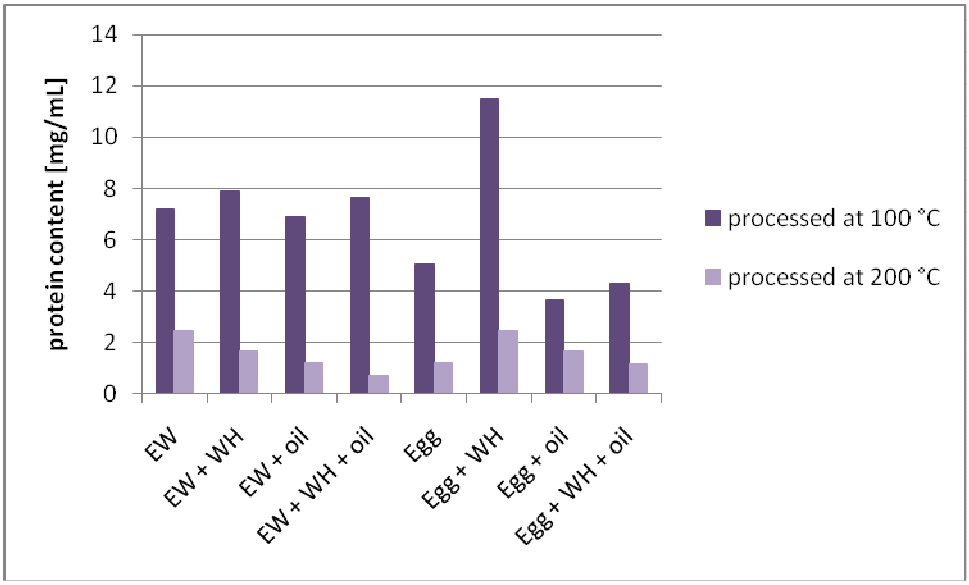


Figure 16. Comparison of extraction capability of samples processed at different temperatures using buffer A4

Table 11. Extraction of self-made baked samples with buffer A8 at 37 °C for 4 h

sample	protein content [mg/ml]	
	processed at 100 °C	processed at 200 °C
EW	10.55	6.18
EW + WH	9.81	6.23
EW + oil	9.77	4.64
EW + WH + oil	7.57	3.81
Egg	14.45	10.88
Egg + WH	15.41	14.72
Egg + oil	11.14	7.07
Egg + WH + oil	11.54	7.96

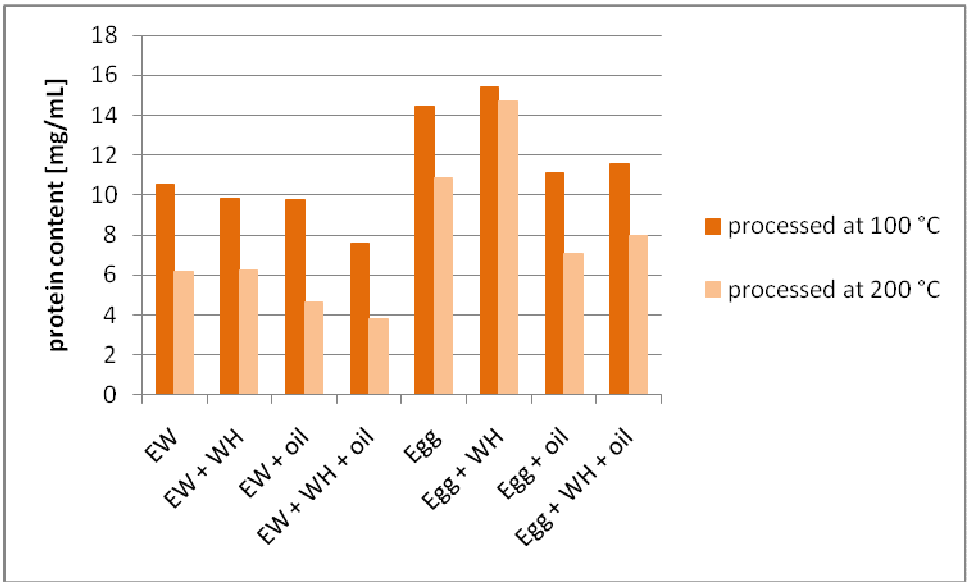
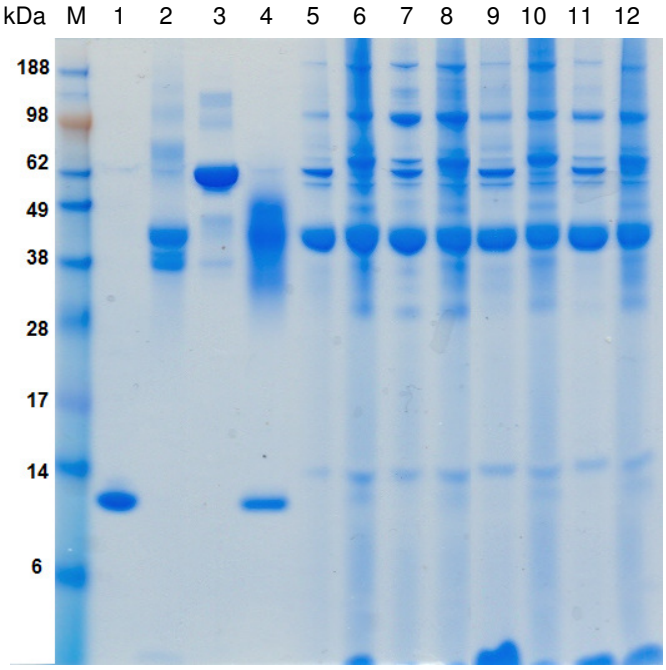


Figure 17. Comparison of extraction capability of samples processed at different temperatures using buffer A8

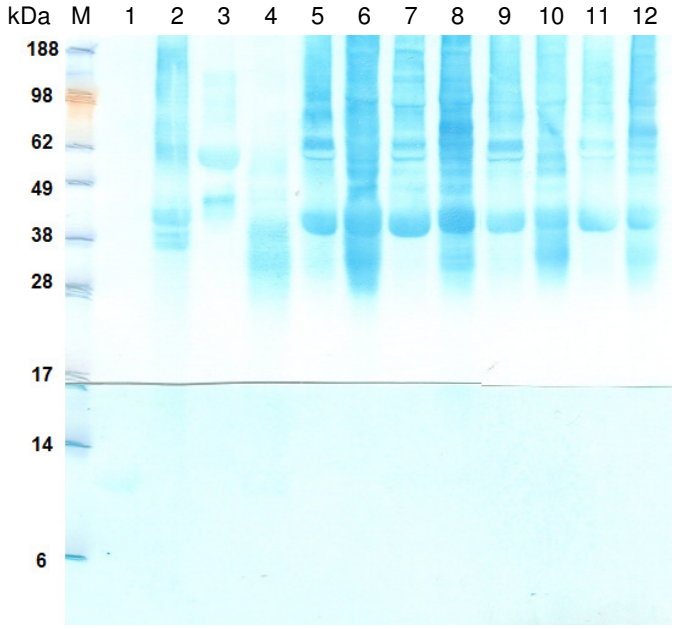
SDS-PAGE & Western Blot:

The samples baked with 200 °C were not analysed by gel electrophoresis and immunoblot, because the complete denaturation of proteins would lead to a diffuse band pattern. Therefore, only the samples processed at 100 °C are shown below. The egg white was detected with rabbit anti-SEW antibodies in the Western Blot. The lower part of the immunoblot was longer incubated with the substrate solution to detect also lysozyme, if it´s not destroyed by the heating process.



M = marker
1 = lysozyme
2 = ovalbumin
3 = conalbumin
4 = ovomucoid
5 = egg in buffer A4
6 = egg in buffer A8
7 = egg + WH in buffer A4
8 = egg + WH in buffer A8
9 = egg + oil in buffer A4
10 = egg + oil in buffer A8
11 = egg + WH + oil in buffer A4
12 = egg + WH + oil in buffer A8

Figure 18. SDS-PAGE of self-made baked samples extracted with buffer A4 and A8



M = marker
1 = lysozyme
2 = ovalbumin
3 = conalbumin
4 = ovomucoid
5 = egg in buffer A4
6 = egg in buffer A8
7 = egg + WHin buffer A4
8 = egg + WH in buffer A8
9 = egg + oil in buffer A4
10 = egg + oil in buffer A8
11 = egg + WH + oil in buffer A4
12 = egg + WH + oil in buffer A8

Figure 19. Western Blot of self-made baked samples extracted with buffer A4 and A8

5.3.3 Discussion

A comparison of the total protein content of the extracts was not meaningful, because wheat flour contains protein, too. Also the decreased extraction yield of samples including oil (table 10) might be caused by the initial weight without considerating the relative egg proportion in the samples. However, buffer A8 showed again the best extraction results (table 11) and the difference in extraction ability of samples processed at 100 °C and 200 °C is not as huge as with buffer A4 (see figure 16 and 17). The SDS-Page offered no big differences in the protein content of samples containig egg alone or egg and wheat flour (see figure 18). Furthermore, the band patterns in the Western Blot looked also the same (see figure 19).

5.4 Comparison of different extraction temperatures

In earlier experiments the extraction was performed at max. 37 °C. In this approach higher temperatures should be tested to improve the extraction yield. Samples in different processing states were extracted with 3 buffers for 1 h.

5.4.1 Experimental

Extraction buffers:

- Buffer A7: 0.075 M K-acetate, 0.3 M NaCl, 0.01 M EDTA, 0.25% Triton X100, pH 7.4
- Buffer A8: 0.06 M Tris-HCl, 2% SDS, 1 mM DTT, 2% glycerol, pH 7
- Buffer A9: 0.05M Tris-HCl, 1% NaCl, 0.1% Tween 20, 5% Urea, pH 7.5

Extraction procedure:

For the extraction 0.2 g spray dried egg white, 0.1 g baked egg white, 2 g cooked egg white, 0.5 g cookies and 1 g noodles were resuspended in 5 mL buffer. The extraction was performed for 1 h at 37 °C, 58 °C and 70 °C with the overhead-shaker in an incubator. After centrifugation at 9500 rpm for 10 min, the extracts were filtered if necessary.

5.4.2 Results

BCA protein determination:

Table 12. Extraction yield of samples with different processing level at 37 °C, 58 °C and 70 °C

Sample	Buffer	protein yield [mg/ml]		
		37 °C	58 °C	70 °C
cooked EW	A7	2.87	2.99	3.78
	A8	2.50	5.34	6.91
	A9	2.86	3.16	3.39
SEW	A7	42.10	36.47	36.17
	A8	35.02	37.49	36.31
	A9	38.06	36.69	33.77
baked EW	A7	2.04	4.21	3.08
	A8	6.97	16.52	11.41
	A9	2.41	4.74	3.04
cookies	A7	2.44	2.60	5.80
	A8	4.01	3.84	9.56
	A9	2.49	2.45	5.05
noodles	A7	2.42	2.89	3.69
	A8	12.01	19.20	21.18
	A9	3.76	5.76	5.47

As expected the higher processed food samples showed the best results at 70 °C. However, the extraction at 37 °C and 58 °C was more efficient for SEW and baked EW. Again the buffer containing SDS and DTT caused the highest protein yield for all samples extracted at 58 °C and 70 °C (see table 12).

5.4.3 Discussion

Higher extraction temperature may be helpful for increasing the protein yield, but it is only true for processed food stuff. The extraction of crude ingredients would be inhibited by too high temperatures, like crude egg in mayonnaise or beaten egg white in desserts. However, the decrease of extracted protein from crude samples is not as remarkable as the increase of extraction yield from processed samples at high temperatures. Therefore, an extraction temperature of 60 °C will be efficient for food stuff analysis and this temperature is also very common in literature.

5.5 Determination of denaturing agent concentration for protein extraction

Some chemicals are known to have the ability to denature protein structures. Denaturation of allergens affects their conformational structure, which may destroy or lay open immunoglobulin-binding epitopes and thus alter the antigenic potential of the egg white proteins. Although the determination of the antigenic potential of the extracted proteins was the primary aim, it was also a goal of this study to isolate the whole egg protein fraction with a sufficiently high protein concentration, so that it could be used as the starting material for further research. In the study of Hildebrandt (2008), an extraction buffer including 8 M urea provided twofold protein yield than the usual PBS buffer [15].
Furthermore, a denaturing agent would compensate matrix effects and protein aggregation during processing of food samples.

5.5.1 Experimental

Extraction buffers:

- B0: 0.1 M TBS, pH 7.5
- B1: B0 + 1% SDS/1 mM DTT
- B2: B0 + 2% SDS/1 mM DTT
- B3: B0 + 5% SDS/1 mM DTT
- B4: B0 + 5% urea
- B5: B0 + 5% urea/1 mM DTT
- B6: B0 + 10% urea
- B7: B0 + 15% urea
- B8: B0 + 20% urea
- B9: B0 + 20% urea/1 mM DTT

Extraction procedure:

Noodles, egg biscuit and egg puffs were used for extraction with SDS and urea in different concentrations. The samples were minced with the retsch mill and 0.5 g was mixed with 5 mL buffer. The extraction was performed for 1 h at 60 °C on the horizontal shaker. After centrifugation at 9500 rpm for 15 min the extracts were filtered using syringe filters with 0.45 µm pore size.

5.5.2 Results

BCA protein determination:

Table 13. Extraction of food stuff with buffers including denaturants

extraction buffer	buffer components	protein content [mg/mL]		
		noodles	biscuit	egg puffs
B0	0.1 M TBS, pH 7.5	1.52	1.08	0.58
B1	B0 + 1% SDS/1 mM DTT	6.08	7.72	3.90
B2	B0 + 2% SDS/1 mM DTT	8.05	6.50	6.72
B3	B0 + 5% SDS/1 mM DTT	7.71	6.24	6.70
B4	B0 + 5% urea	2.10	1.99	0.64
B5	B0 + 5% urea/1 mM DTT	2.58	2.24	1.60
B6	B0 + 10% urea	2.72	2.33	0.74
B7	B0 + 15% urea	3.31	2.58	0.80
B8	B0 + 20% urea	4.79	2.72	0.70
B9	B0 + 20% urea/1 mM DTT	5.21	4.38	2.70

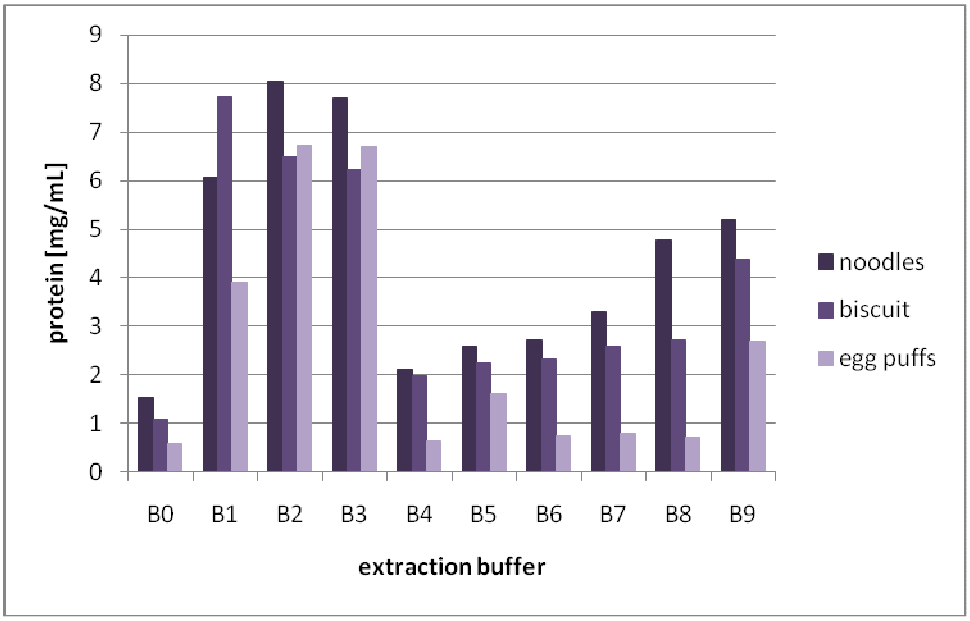


Figure 20. Comparison of extraction cabability of buffers with increasing denaturating agent concentration

5.5.3 Discussion

Already Watanabe (2008) noticed that increasing SDS concentration in the extraction buffer do not ensure higher protein yield (see table 13). The working group tested buffers containing max. 1% SDS and the best results were achieved with even 0.5% SDS for a few samples [1]. Also in this approach the highest protein yield offered extracts produced with 1% or 2% SDS. Furthermore, the protein yield accelerated with increasing urea content in the extraction buffers (see figure 20). However, 20% urea could not achieve the same efficiency as 1% SDS. Finally the use of a reducing agent (DTT) during extraction will be necessary to handle with processed food, which includes a higher risk for formation of disulfide bridges between ovomucoid or ovalbumin and other ingredients.

5.6 Detergent comparison

Because of the high extraction efficiency of SDS, other detergents were determined and combined with urea to look for increasing protein yield. Tween 20 and Brij35 are non-ionic detergents, CHAPS is zwitterionic and CTAB (Hexadecyltrimethyl ammonium bromide) is a common cationic detergent used for DNA extraction (details in chapter 11.1.).

5.6.1 Experimental

Extraction buffers:

- C0: 0.1 M TBS, pH 7.5
- C1: C0 + 1% SDS/1 mM DTT
- C2: C0 + 2% SDS/1 mM DTT
- C3: C0 + 1% CTAB/1 mM DTT
- C4: C0 + 2% CTAB/1 mM DTT
- C5: C0 + 20% urea/1 mM DTT
- C6: C0 + 30% urea/1 mM DTT
- C7: C0 + 5% Tween 20/1 mM DTT
- C8: C0 + 2% CHAPS/1 mM DTT
- C9: C0 + 5% Brij 35/1 mM DTT
- C10: C0 + 20% urea/1 mM DTT + 1% Triton X-100
- C11: C0 + 20% urea/1 mM DTT + 1% SDS
- C12: C0 + 20% urea/1 mM DTT + 1% CTAB

Extraction procedure:

0.1 g SEW, 2 g cooked EW, 1 g waffle, 1 g biscuit and 0.5 g dried noodles were mixed with 5 mL buffer and extracted for 30 min at 60 °C. After centrifugation at 9500 rpm for 15 min the extracts were filtrated with syringe filters.

5.6.2 Results

BCA protein determination:

Table 14. Extraction of food samples with buffers containing denaturants and detergents

Extraction buffer	buffer components	protein yield [mg/mL]				
		SEW	cooked EW	noodles	biscuit	waffle
C0	0.1 M TBS, pH 7.5	8.17	3.42	2.18	1.88	1.96
C1	C0 + 1% SDS/DTT	8.17	7.17	5.39	3.02	5.86
C2	C0 + 2% SDS/DTT	8.02	7.79	4.05	2.42	7.15
C3	C0 + 1% CTAB/DTT	2.64	4.10	4.48	5.40	2.63
C4	C0 + 2% CTAB/DTT	4.07	7.25	6.01	5.94	4.65
C5	C0 + 20% urea/DTT	5.59	2.95	6.60	3.16	3.79
C6	C0 + 30% urea/DTT	4.14	3.32	7.62	4.40	4.57
C7	C0 + 5% Tween 20/DTT	12.18	3.20	3.10	2.46	2.24
C8	C0 + 2% CHAPS/DTT	10,56	3.21	4.16	2.52	2.71
C9	C0 + 5% Brij35/DTT	10.80	3.27	3.31	2.08	2.31
C10	C0 + 20% urea/DTT + 1% Triton X-100	7.45	3.21	9.31	5.06	4.26
C11	C0 + 20% urea/DTT + 1% SDS	7.72	11.09	7.17	4.28	8.52
C12	C0 + 20% urea/DTT + 1% CTAB	2.47	5.14	7.44	7.42	5.28

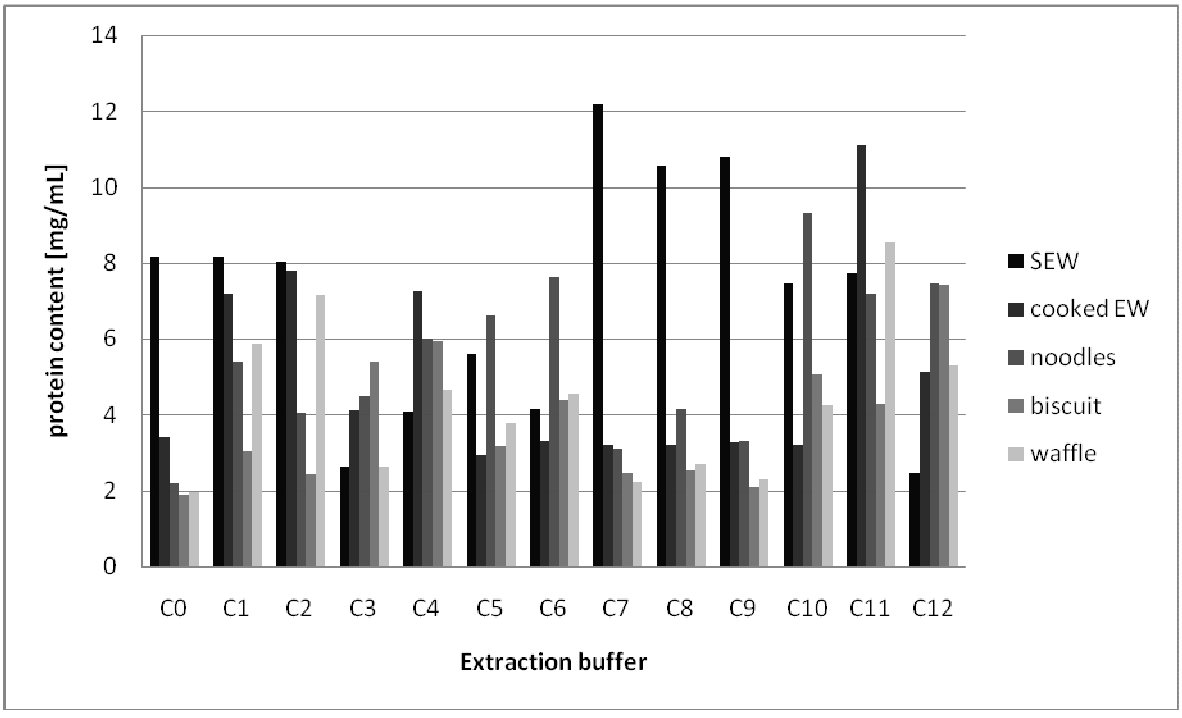
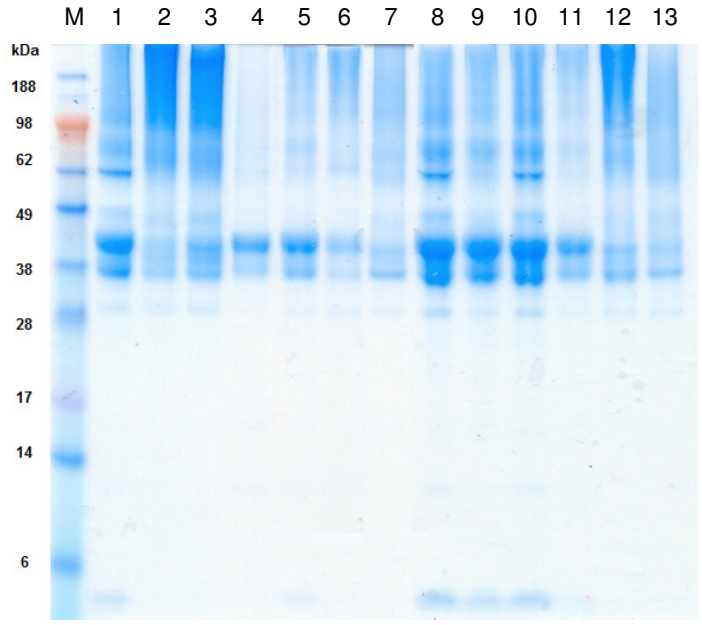


Figure 21. Comparison of extraction capability of buffers containing various detergents

Interestingly, each sample prefered another buffer for efficient protein extraction. Spray dried egg white showed the highest protein yield with buffers using nonionic detergents (C7, C8 and C9), but all other samples offered lowest protein content using this extraction buffers (see figure 21). Optimal efficiency for food stuff extraction was reached with the buffers containing 20% urea and a detergent, whether nonionic, cationic or anionic depended on the sample (see buffer C10-C12 in table 14).

SDS-PAGE:



M = marker
1 = SEW in C0
2 = SEW in C1
3 = SEW in C2
4 = SEW in C3
5 = SEW in C4
6 = SEW in C5
7 = SEW in C6
8 = SEW in C7
9 = SEW in C8
10 = SEW in C9
11 = SEW in C10
12 = SEW in C11
13 = SEW in C12

Figure 22. SDS-PAGE of SEW extracted with buffers including various detergents

The BCA results of the SEW extracts fitted well to the band pattern of the SDS-Page (figure 22), which showed higher intensity with the buffers C7-C9 (lane 8-10) and with the buffers C0-C2 (lane 1-3).

Indirect competitive ELISA:

The BCA determination and SDS-Page analysis revealed only the quantitative protein content of the extracts, but ignored the actual part of egg white. High extraction efficiency of a buffer for total protein does not mean necessarily high extraction yield for egg white proteins. Therefore, the dried noodles, which also contain wheat flour proteins, were analysed by ELISA using the same volume of each extract and the signals were compared with the results of BCA protein determination.

Table 15. ELISA results of the noodle extracts produced with the buffers C1-C12

extract	abs_{max}	abs_{min}	Δabs	IC_{50}
noodles in C1	1.369	0.197	1.172	1239
noodles in C2	1.295	0.178	1.117	2607
noodles in C3	1.254	0.144	1.110	1932
noodles in C4	1.232	0.121	1.111	1206
noodles in C5	1.233	0.242	0.991	2320
noodles in C6	1.223	0.129	1.094	1234
noodles in C7	1.305	0.831	0.474	504
noodles in C8	1.626	0.726	0.900	1560
noodles in C9	1.630	0.616	1.014	3562
noodles in C10	1.550	0.176	1.374	1966
noodles in C11	1.630	0.252	1.378	2298
noodles in C12	1.579	0.247	1.332	3229

The best signal curve in ELISA showed the extract of buffer C10, which contained 20% urea and 1% Triton X-100, and this result was approved by BCA determination.

5.6.3 Discussion

In the ELISA analysis the noodle extract of buffer C1 generated a better signal than the extract of buffer C6 (table 15), which was not evident in the BCA results (table 14). The reason for this might be a higher extraction capability for egg white proteins with SDS than with urea or an inhibition effect of urea in anitody-antigen binding in ELISA. However, the negative influence of 30% urea in ELISA was compensated by higher extraction yield compared to the sample extracted with 20% urea. Worst results in BCA protein determination of the noodle extracts and in ELISA were obtained with the extraction buffers containing only nonionic detergents in contrast to SEW extraction.

It can be assumed, that a denaturating agent is necessary to cope with highly processed samples and with complex background matrices.

6. CROSS-REACTIVITY

Cross-reactivity refers to the ability of an individual antibody combining site to react with more than one antigenic determinant or the ability of a population of antibody molecules to react with more than one antigen. Cross reactions arise because the cross reacting antigen shares an epitope in common with the immunizing antigen or because it has an epitope which is structurally similar to one on the immunizing antigen (multispecificity).

Cross-reactivity studies were performed by immunoblotting and ELISA analysis to ensure the specificity of the used antibodies. Other bird eggs were not determined because only chicken eggs are common in Austria and eggs from other species were not available. After the analysis of some food samples, negative controls like toast bread and noodles without egg showed a signal in Western blot depending on the used extraction buffer. Therefore, egg yolk and also cereals were determined with indirect competitive ELISA.

6.1 Determination of cross-reactivity of egg yolk

6.1.1 Experimental

Extraction buffers:

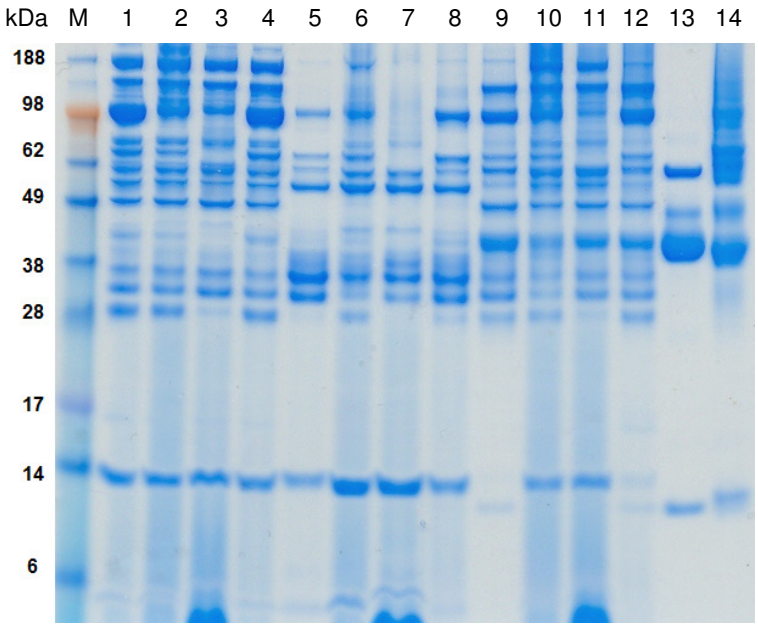
- Buffer D1: 0.1 M TBS + 0.1% Tween
- Buffer D2: 0.1 M TBS + 1% SDS/1 mM DTT
- Buffer D3: 0.1 M TBS + 1% CTAB
- Buffer D4: 0.1 M TBS + 20% Urea

Extraction procedure:

Three egg yolk (EY) samples with different processing grade were used in this approach. For extraction 0.5 g crude egg yolk, 1 g cooked egg yolk and 0.25 g indian egg yolk powder were resuspended with 5 mL extraction buffer and shaken at 37 °C for 15 min except the cooked EY samples were extracted for 30 min. After centrifugation with 9500 rpm for 10 min, the protein yield of the extracts was measured with BCA protein determination (data not shown).

6.1.2 *Results*

SDS-PAGE:



M = marker
1-4 = crude EY in buffer D1-D4
5-8 = cooked EY in buffer D1-D4
9-12 = indian EY powder in buffer D1-D4
13 = crude egg white in buffer D1
14 = crude egg white in buffer D2

Figure 23. SDS-PAGE of egg yolk samples extracted with different buffers

The band patterns on SDS-gel of the crude EY samples showed no great differences, only the band intensity varied (figure 23). The extraction of cooked EY with SDS (lane 6) or urea (lane 8) caused more protein bands, whereas the band pattern of the EY powder looked again very similar. A protein band appeared between 38 and 49 kDa in the EY powder extracts (lane 9-12), which is on the same level as the ovalbumin band from crude EW (lane 13).

Western Blot:

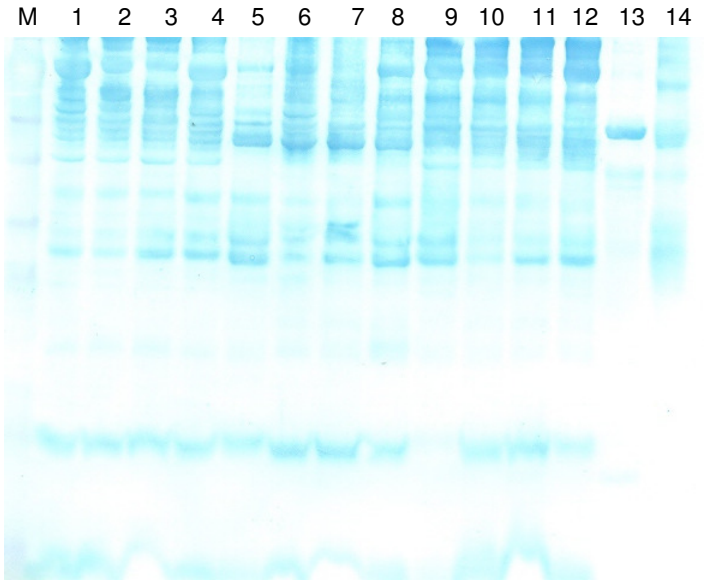


Figure 24. Western Blot of egg yolk and egg white samples detected with rabbit anti-PEY antibody

The rabbit anti-PEY antibody (diluted 1:1000) was able to detect nearly all protein bands of the EY samples independent of their processing level. However, also conalbumin of crude egg white showed a signal (lane 13), which is provided at 62 kDa.

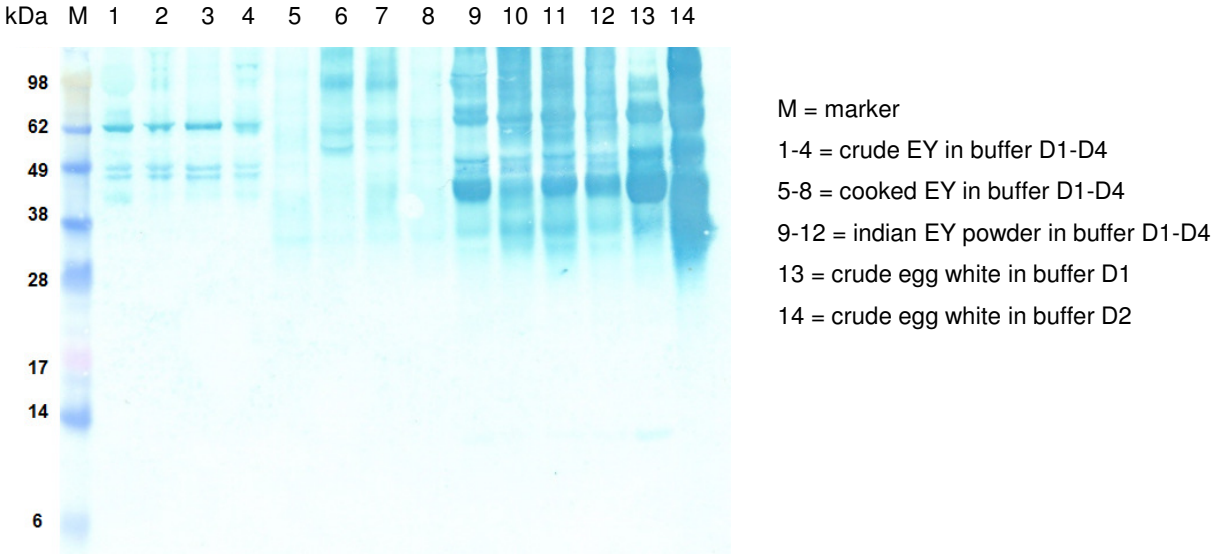


Figure 25. Western Blot of egg yolk and egg white samples detected with rabbit anti-SEW antibody

The detection of the EY samples with rabbit anti-SEW antibody (diluted 1:2500) offered strong signals for the EY powder (lane 9-12). The crude EY showed a band at 62 kDa (lane 1-4) and the cooked EY caused only a signal with the extraction buffers B and C (lane 6 & 7).

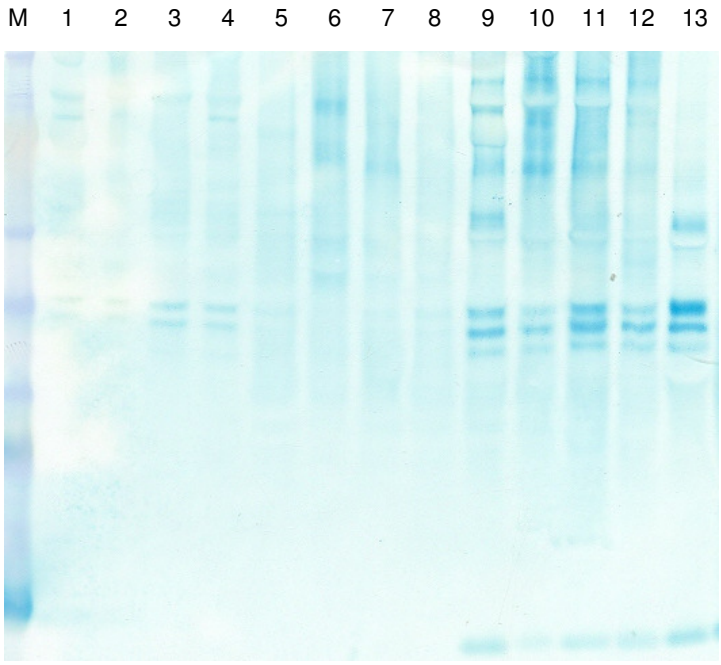


Figure 26. Western Blot of egg yolk and egg white samples detected with rabbit anti-native OVM antibody

The rabbit anti-native OVM antibody (diluted 1:5000) only detected protein bands of the EY powder extracts (lane 9-12), which corresponded to ovomucoid or ovalbumin like the band pattern of crude egg white showed.

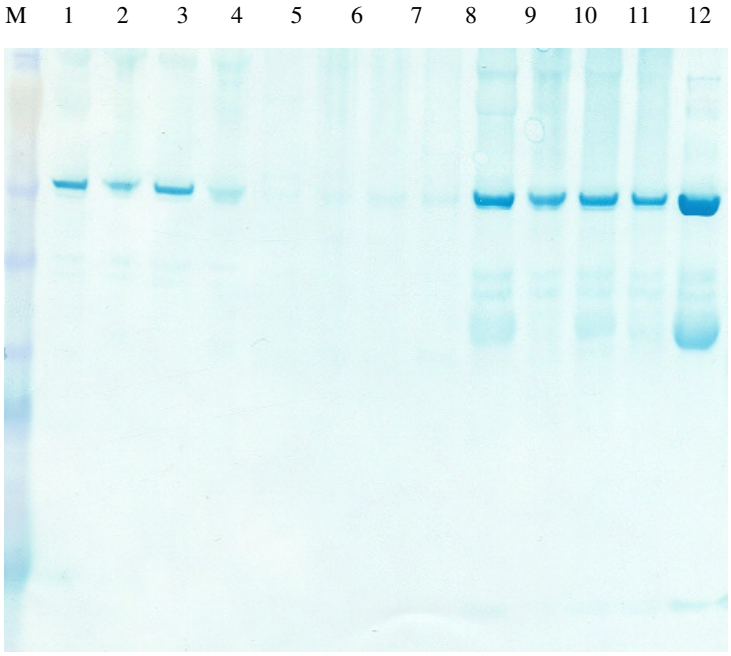


Figure 27. Western Blot of egg yolk and egg white samples detected with rabbit anti-cEW antibody

The rabbit anti-crude EW antibody (diluted 1:2000) caused a signal at 62 kDa at the crude EY (lane 1-3) and EY powder samples (lane 9-12). The bands were identical with conalbumin from crude EW (lane 13).

Indirect competitive ELISA:

The MTPs were coated with 500 ng/mL SEW extracted with 0.1 M TBS and the sample extracts of cooked EY and EY powder were serially diluted with assay buffer (0.05 M PBS + 0.1% Tween). The concentrations of the cross-reacting substances were adapted to the antigen standards for comparison. The detection was performed with rabbit anti-SEW antibody diluted 1:5000 in assay buffer.

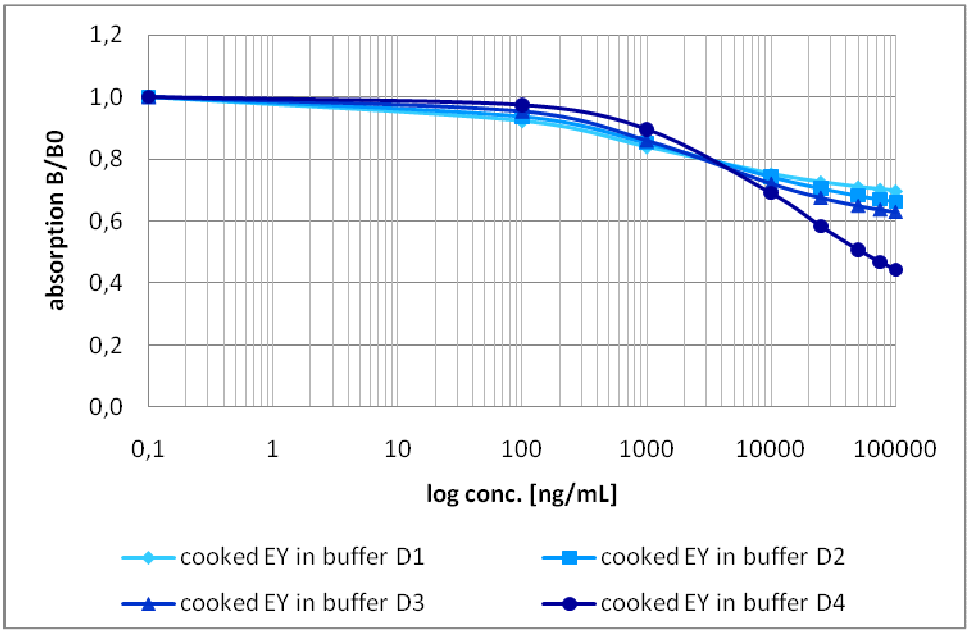


Figure 28. Signal curves of cooked egg yolk extracts in ELISA analyses detected with anti-SEW antibody

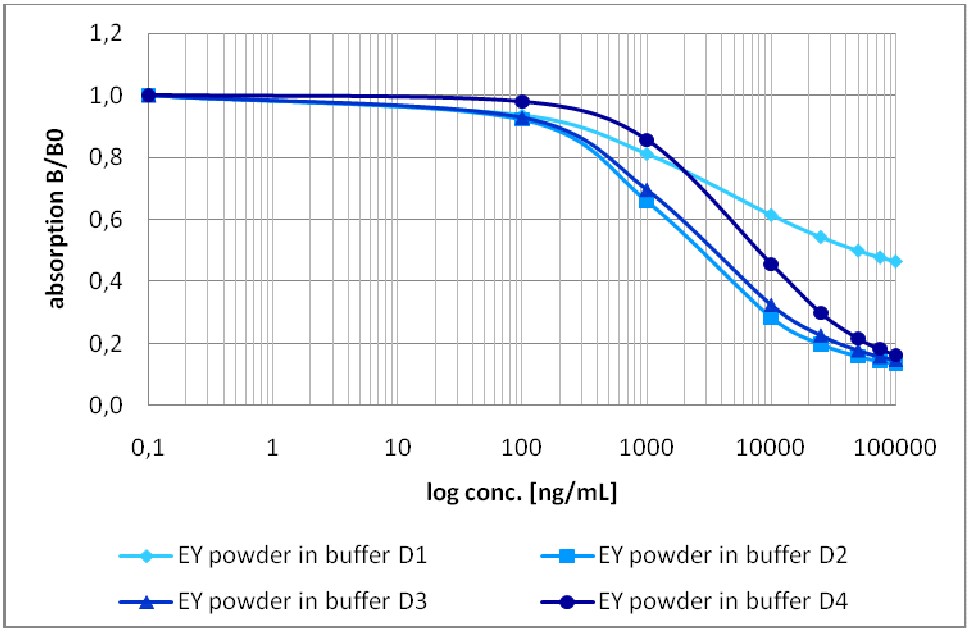


Figure 29. Signal curves of egg yolk powder extracts in ELISA analyses detected with anti-SEW antibody

Calculation of cross-reactivity:

The polyclonal rabbit antibodies against cEW, native OVM and heated OVM were also tested, because every antibody recognizes a special epitope, which influences its cross-reactivity properties for other proteins. The values for cross-reactivity were calculated according to the formula:

$$\text{Relative cross-reactivity [\%]} = \frac{[IC_{50}] \text{ of antigen}}{[IC_{50}] \text{ of cross-reacting substance}} \times 100$$

Table 16. Cross-reactivity of egg yolk samples using antibodies against egg white proteins

Antibody	Sample	buffer	IC_{50}	cross-reactivity
rabbit anti – crude EW	crude EW standard	0.2 M PBS	116	-
	cooked EY	Buffer D1	4456	2.6%
		Buffer D2	12741	0.9%
		Buffer D3	12203	1.0%
		Buffer D4	16972	0.7%
	EY powder	Buffer D1	509	22.8%
		Buffer D2	704	16.5%
		Buffer D3	791	14.7%
		Buffer D4	704	16.5%
rabbit anti – native OVM	native OVM standard	0.2 M PBS	119	-
	EY powder	Buffer D1	3037	3.9%
		Buffer D2	4803	2.5%
		Buffer D3	2941	4.0%
		Buffer D4	3211	3.7%
rabbit anti - heated OVM	heated OVM standard	0.2 M PBS	61	-
	EY powder	Buffer D1	1701	3.6%
		Buffer D2	2505	2.4%
		Buffer D3	1792	3.4%
		Buffer D4	1668	3.7%

6.1.3 Discussion

The results showed that cross-reactivity depends to some extent on the extraction buffer and it is essential to use the same buffer for extraction of the antigen and of the cross reacting protein. The high cross-reactivity of the EY powder in the immunoblots (see figures 25-27) and also in ELISA (figure 29) compared to crude and cooked EY may be caused by incomplete separation of egg white and yolk before the drying process started. Furthermore, the identical bands of crude EW and of the EY powder in SDS-PAGE, which were detected with the antibodies against egg white proteins, indicate the contamination of the EY powder with conalbumin, ovalbumin and ovomucoid. The self-made samples (crude and cooked EY) showed nearly no cross reactivity in ELISA in contrast to the commercial EY powder (see table 16).

6.2 Determination of cross-reactivity of cereals

As already mentioned before, food stuff containing no egg but wheat flour showed a signal in Western blots. To characterize the cross-reacting protein, the extraction of some cereal species was performed in fractions according to Osborne (1907). Afterwards the protein yield was determined with BCA and only the fractions, which contained enough protein were analysed with immunoblotting and ELISA.

6.2.1 Experimental

Extraction buffers:

- E1: ddH_2O
- E2: 0.5 N NaCl
- E3: 0.2 M PBS
- E4: 70% EtOH
- E5: 0.1% Acetic Acid
- E6: 0.2 M NaOH
- E7: 50% 2-ProOH + 1% 2-ME
- E8: 75% 2-ProOH + 1% 2-ME
- E9: 100% 2-ProOH + 1% 2-ME
- E10: 0.1 M TBS + 2% SDS/1 mM DTT

Samples:

Various flour types from wheat, barley, spelt, rye, oat, millet, rice and maize were analysed. Additionally, flour of lupines, soy and buckwheat was chosen as negative control. The commercial proteins gliadin and gluten from Sigma were also tested.

Extraction procedure:

The complete Osborne fractionation [17] was only performed with wheat and started with 1 g wheat flour in 10 mL ddH_2O. After shaking for 1 h at RT the extract was centrifuged at 9500 rpm for 15 min, the supernatant was collected in a new tube and the pellet was extracted with 0.5 N NaCl and so on.

1 g of rice and maize flour, 2.5 g of barley, 0.75 g of rye, spelt, oat, millet and buckwheat, and 0.5 g of lupin and soja flour were extracted with 10 mL ddH_2O. The second fraction was performed with PBS, the third with 70% EtOH, the fourth with 0.02 M NaOH and the last with buffer E10 containing SDS/DTT.

6.2.2 *Results*

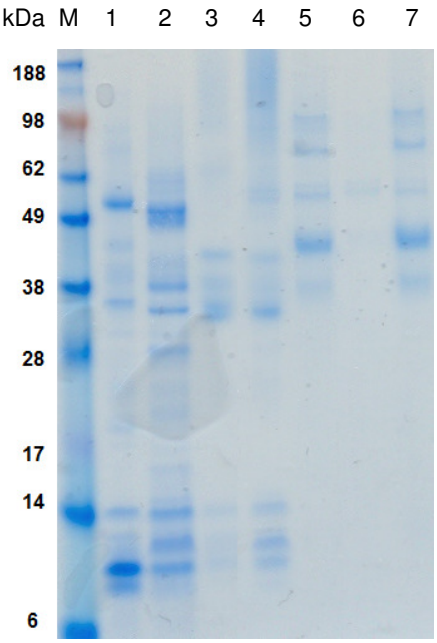
BCA determination of the wheat flour extracts:

Table 17. Extraction yield of the wheat fractions according to Osborne

Extract	buffer	selected protein	protein [mg/ml]
fraction 1	E1	albumins	1.45
fraction 2	E2	globulins	0.78
fraction 3	E4	gliadin	1.36
fraction 4A	E5	soluble glutenins	2.35
fraction 4B	E7	unsoluble glutenins	1.17
fraction 5A	E8	HMW glutenins	0.00
fraction 5B	E9	LMW glutenins	0.49

The supernatant of the fraction 4B was separated and 100% 2-ProOH + 1% 2-ME was added to one part until a final concentration of 75% 2-ProOH was reached. The resulting small pellet should include the high molecular weight (HMW) glutenins and was solubilized in 50% 2-ProOH. Finally, the proteins, which were still in solution in 75%-100% 2-ProOH were identified as low molecular weight (LMW) glutenins (see table 17).

SDS-PAGE:



M = Marker
1 = albumin
2 = globulin
3 = gliadin
4 = soluble glutenin
5 = unsoluble glutenin
6 = HMW glutenin
7 = LMW glutenin

Figure 30. SDS-PAGE of wheat fractions extracted according to Osborne

Fraction 1 and 2 containing the albumins and globulins (lane 1 & 2) showed a band pattern with high intensity, whereas the other fractions offered less bands. The ethanol and acetic acid fractions (lane 3 & 4) looked very similar, and should contain gliadin and soluble glutenin. The last fractions revealed the unsoluble HMW and LMW glutenins (see figure 30).

Western Blot:

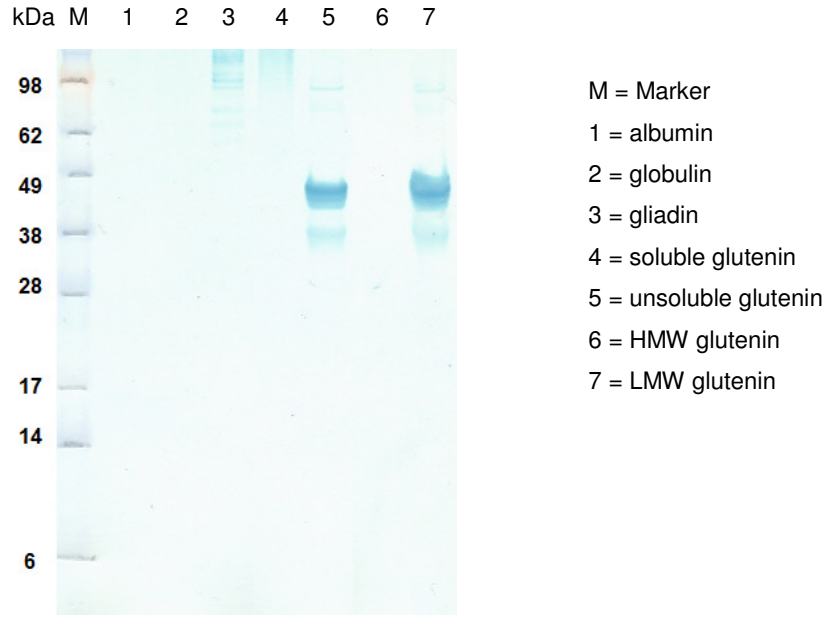


Figure 31. Western Blot of wheat fractions detected with rabbit anti-SEW antibody

The rabbit anti-SEW antibody detected the fractions containing gliadin (lane 3, 4) and glutenin (lane 5-7), whereas the albumins and globulins of wheat showed no cross-reactivity.

SDS-PAGE & Western Blot of other flour types:

- Fraction 1 (F1): extracted with E1 (ddH_2O)
- Fraction 2 (F2): extracted with E3 (0.2 M PBS)
- Fraction 3 (F3): extracted with E4 (70% EtOH)
- Fraction 4 (F4): extracted with E6 (0.02 M NaOH)
- Fraction 5 (F5): extracted with E10 (SDS/DTT buffer)

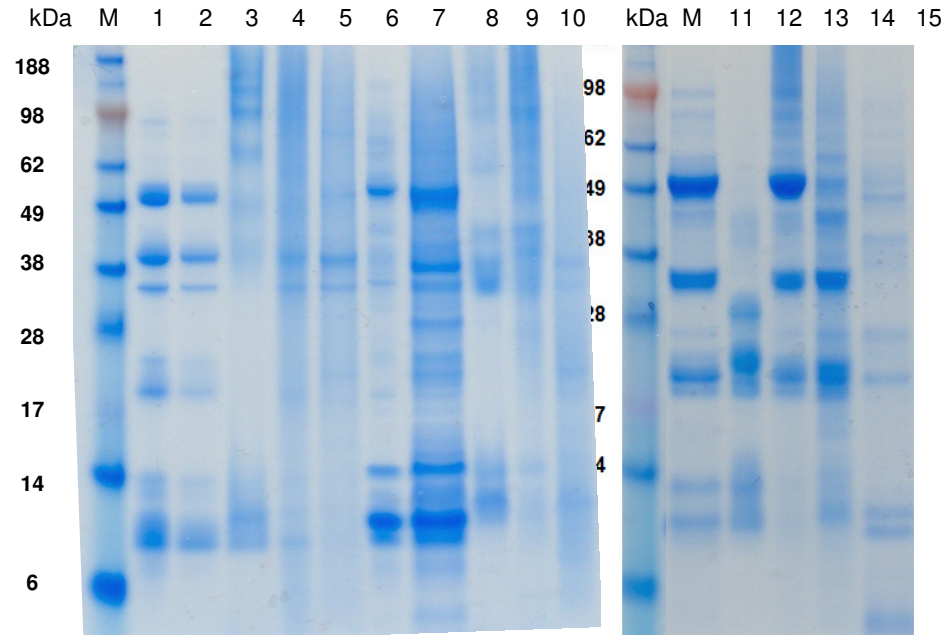


Figure 32. SDS-PAGE of Osborne fractions extracted from various cereals

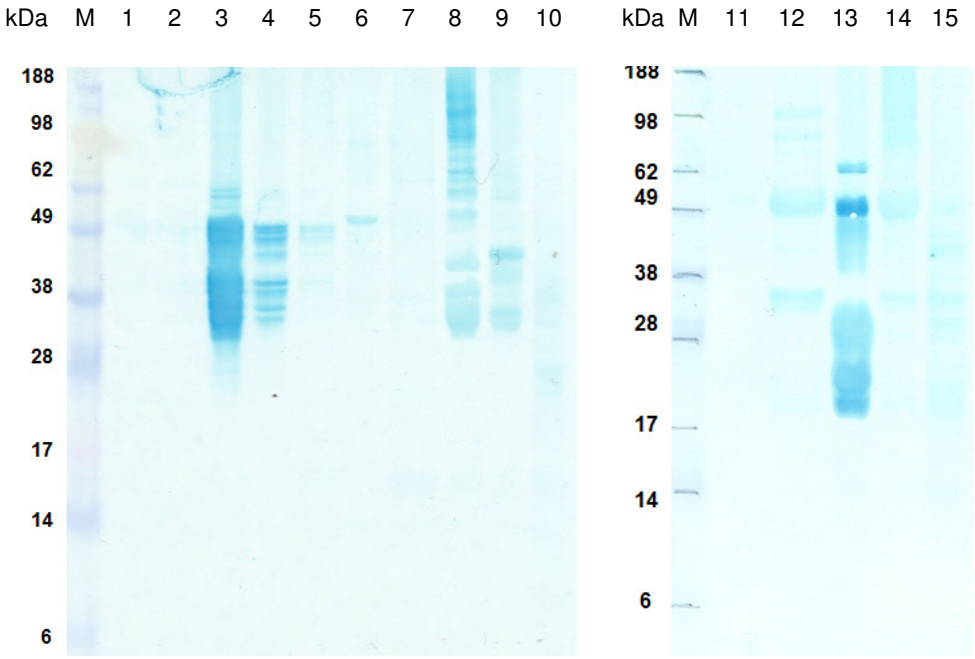
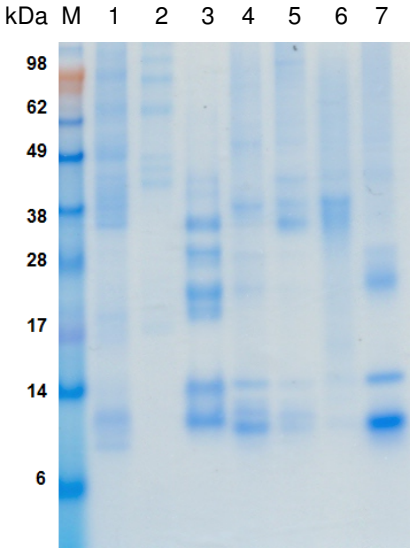


Figure 33. Western Blot of Osborne fractions extracted from various cereals, detected with rabbit anti-SEW antibody

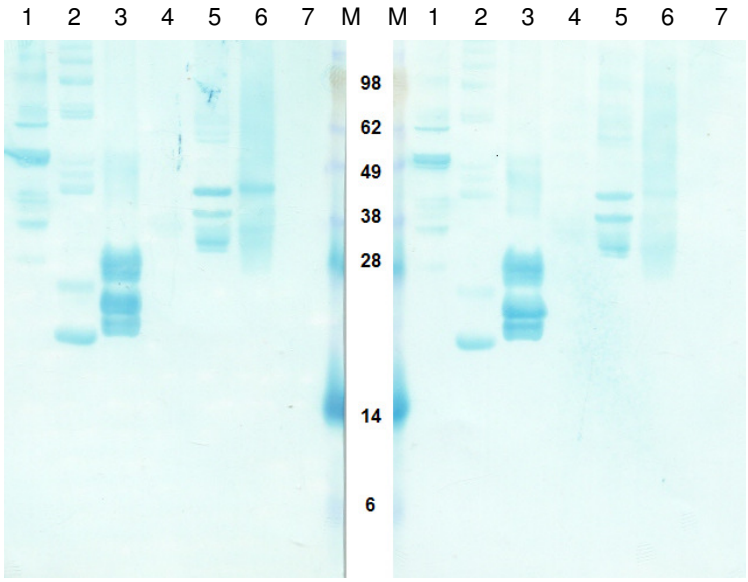
As already mentioned for wheat flour, the fractions of barley, spelt and oat, which were extracted with PBS buffer showed no signal. The proteins included in the alcohol fraction offered the highest cross-reactivity (see figure 32). Additionally, positive results with rabbit anti-SEW antibody were achieved for rye and maize, whereas buckwheat, lupines and soy showed no signal (data not shown). The fractions, which caused the strongest signal, were also determined with the other antibodies.

Detection of cross-reacting fractions with other antibodies:



M = marker
1 = barley F3
2 = maize F5
3 = oat F3
4 = spelt F4
5 = wheat F5
6 = gliadin in 70% EtOH
7 = gluten in SDS/DTT buffer

Figure 34. SDS-PAGE of cereal fractions and wheat standards (gliadin, gluten) purchased from Sigma



M = marker
1 = barley F3
2 = maize F5
3 = oat F3
4 = spelt F4
5 = wheat F5
6 = gliadin in 70% EtOH
7 = gluten in SDS/DTT buffer

Figure 35. Western Blot of cereal fractions and wheat standards. The left blot was performed with anti-native OVM antibodies and the right blot with anti-crude EW antibodies.

Every antibody against egg white proteins caused a signal with certain cereals (figure 35). Surprisingly, the band pattern caused by different antibodies looked the same. Although the immunogens used for antibody production exhibited different processing levels (crude EW versus spray dried EW), the antibodies showed the same cross-reactivity for cereals.

Indirect competitive ELISA:

The cereal fractions, which caused a signal in the immunoblots, were also determined by ELISA with all available antibodies against egg white proteins (data not shown). However, no signal curve was obtained for cereal samples although high concentrations were used.

6.2.3 Discussion

The different results in immunoblotting and ELISA clarified that cross-reactivity depends on the used method for determination and the corresponding circumstances. An explanation for the strong signals in immunoblots may be the high unspecific affinity of the cellulose membrane or the denatured state of the proteins. Otherwise, in the ELISA analysis a competition between antigen and cross-reacting protein took place and if the antigen-antibody binding was too strong, no cross-reactivity could be detected.
Finally, fractions extracted with 70% ethanol caused the strongest signal in Western blots, but no alcohol was included in the extraction buffers for food stuff in this work. Therefore, the risk for possible cross-reactions in the subsequent food analysis by ELISA did not exist.

7. Determination of the inhibiting effect of urea in ELISA

Hildebrandt et al. (2008) and Ochiai et al. (2003) found that using an extraction solution containing 8 M urea, allowed high protein yield from food products. As already shown in an earlier extraction approach (see chapter 5.5) the protein yield of the extracts could be increased with increasing urea concentration. However, the extraction efficiency of 1% or 2% SDS was not even reached with 20% urea. Therefore, higher concentrations of urea should be tested for total protein extraction and also for inhibiting influences in indirect competitive ELISA.

7.1 Experimental

Extraction buffers:

- G0: 0.1 M TBS + 0.1% Tween 20, pH 7.5
- G1: P0 + 5% urea
- G2: P0 + 10% urea
- G3: P0 + 15% urea
- G4: P0 + 20% urea
- G5: P0 + 30% urea
- G6: P0 + 40% urea

Extraction procedure:

Spray dried egg white and cooked pasta were analysed for their extractability with urea. 50 mg of SEW were mixed with 1.5 mL buffer and 2 g of cooked pasta with 5 mL buffer. The extraction was performed for 15 min at 60 °C, which is usual for commercial ELISA kits. After centrifugation the extracts were filtrated if it was required.

7.2 Results

BCA protein determination:

Table 18. Changes in extraction yield with increasing urea concentration

Extraction buffer	protein yield [mg/mL]	
	SEW	pasta
G0	24.38	0.53
G1	29.95	0.65
G2	24.41	0.61
G3	20.54	0.78
G4	20.63	0.75
G5	13.60	0.78
G6	10.33	0.84

The correlation between urea concentration and protein yield of the extracts was not straight proportional, but in general urea increased the extraction capabilty of processed samples and had a negative effect for extraction of native proteins (see table 18).

Indirect competitive ELISA:

To visualize only the effect of urea on antibody-antigen binding independently of the extraction yield, the SEW extract of buffer G0 without urea was used to prepare dilution series with the buffers G1-G6 containing the same SEW concentration. The same experiment was performed with G0 extracts of crude EW and OVM and the corresponding antibodies (data not shown).

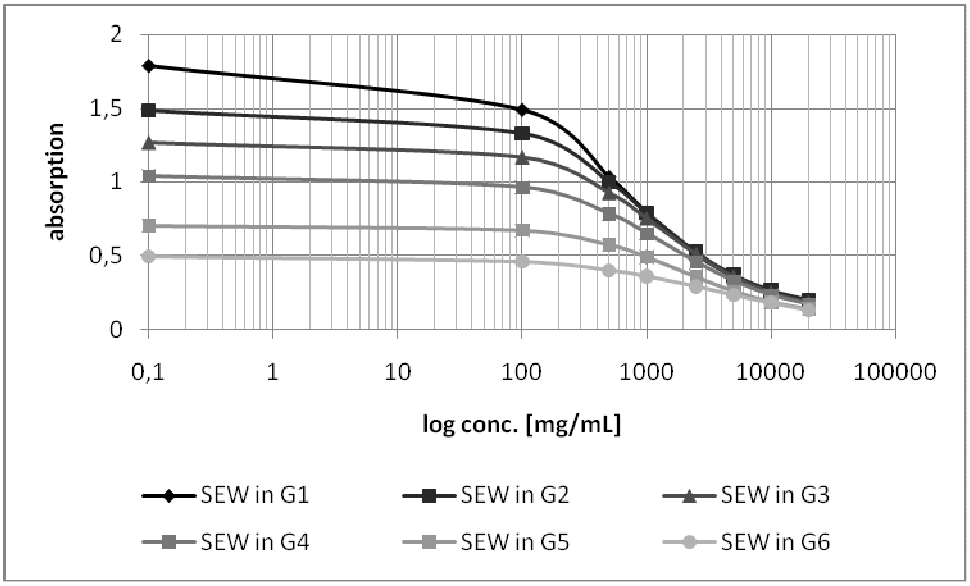


Figure 36. ELISA signal curves of SEW diluted in buffers with increasing urea concentration. The detection was performed with the corresponding antibody against SEW.

As the signal curves in figure 36 showed, the slope was lost with increasing urea concentrations, which inhibited the antigen-antibody binding reaction. However, to determine if the higher extraction yield caused by increased urea content in the extraction buffer could compensate the inhibiting effect, the pasta extracts of G1-G6 were diluted only with assay buffer and with the appropriate extraction buffer. For comparison the same volume of the extracts has been used for preparation of dilution series.

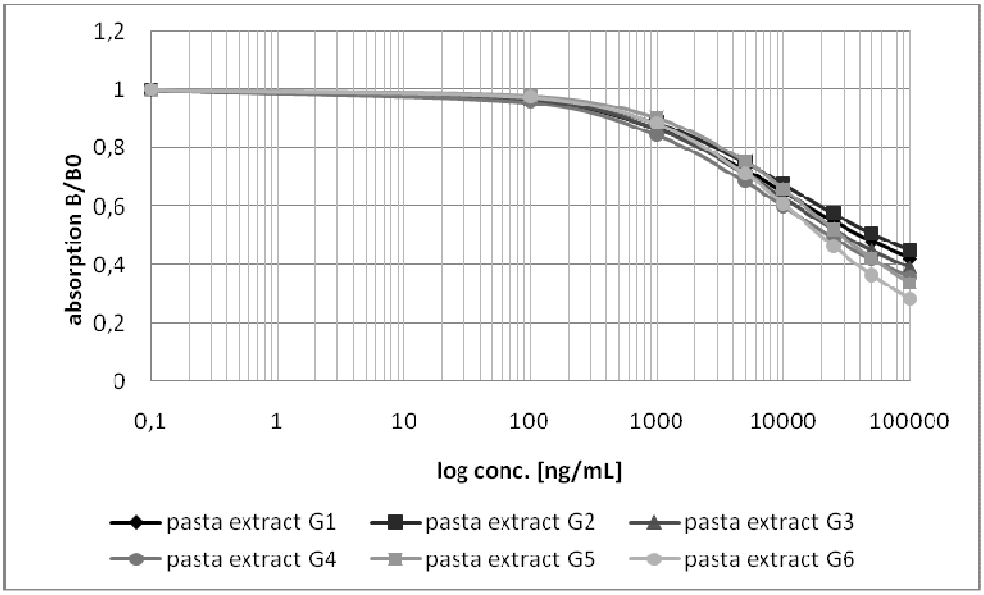


Figure 37. ELISA signal curves of pasta extracted with G1-G6 and diluted with ELISA assay buffer. The detection was performed with rabbit anti-SEW antibody.

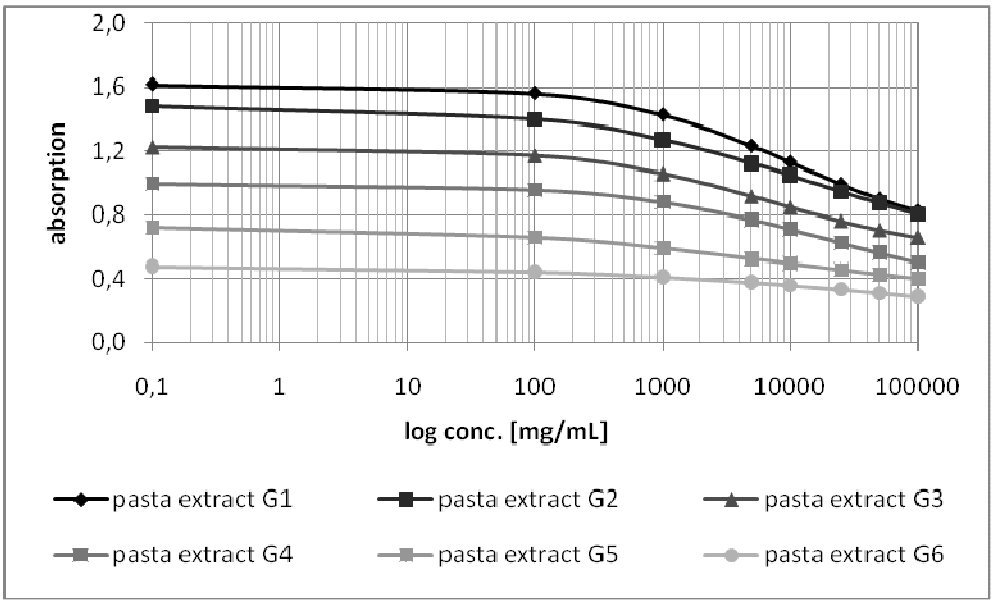


Figure 38. ELISA signal curves of pasta extracted with G1-G6 and diluted with the corresponding extraction buffer. The detection was performed with rabbit anti-SEW antibody.

The pasta sample extracted with 40% urea (G6) showed the best signal curve, if the dilutions had been prepared with assay buffer (figure 37). These results confirmed the protein yields measured via BCA. However, after adaption of the ELISA curves (B/B0) the expected signal enhancement by urea included in the extraction buffer was not obvious.
The dilutions prepared with extraction buffer showed that the increased protein yield of the pasta extract with buffer G6 (40% urea) was totally diminished by the negative effect of urea (see figure 38). The signal curves of the pasta extracts with buffer G3 (15% urea) and G4 (20% urea) offered similar IC_{50} values to G1, but less Δabs.

7.3 Discussion

Urea seems to be an efficient denaturing agent for food stuff extraction, but has a negative influence on the antigen-antibody binding in ELISA. A prior dilution step of the sample extract with assay buffer would decrease the urea concentration before ELISA performance. However, the advantage of increased protein yield caused by urea usage for extraction would disappear with major dilution factors. The sufficient urea concentration to achieve enough protein yields without inhibiting antibody binding to such an extent depends on sample composition. Therefore, every sample has to be tested for its extraction capability with urea before the most efficient extraction buffer could be defined.

8. The effect of the denaturating detergent SDS (Sodium dodecyl sulfate) for food stuff analysis

8.1 State of Art

The disadvantages of highly processed food samples are the less effective extraction ability of egg white allergens and the denatured state of the proteins, if the antibodies were produced with native protein. To solve this problems, antibodies raised against denatured or heated proteins [10, 19] are used to analyse processed food samples and/or a denaturising agent (SDS) is applied for food stuff extraction [1, 16, 17]. The working group of Kato [16] used 4% SDS in PBS buffer as optimal extraction solution for pasta samples, but performed only immunoblots and no ELISA analysis. Reed and Park (2010) worked with a final optimised extraction protocol using 10% SDS [17] and Watanabe et al. (2005) achieved extraction of boiled samples with a Tris/HCl buffer containing 1% SDS [1].

The study of Reed concerned about the extraction of highly processed samples and the determination of egg white with indirect ELISA, coating the MTPs with sample extracts, which were serially diluted with PBS, and detecting with an antibody against heat-denatured OVA. The advantage of the method is that no contact between SDS included in the extraction buffer and the antibody takes place. Otherwise, an efficient coating step with extracts containing max. 10% SDS overnight at 4 °C is critical and the sufficient dilution factor has to be determined.

Watanabe Y. from the Morinaga Institute of Biological Science developed 2005 a sandwich ELISA for the determination of ovalbumin in presence of 1% SDS and 7% 2-ME. Therefore, the antigen-antibody reaction took place in the presence of those agents in order to perform ELISA for proteins extracted with solutions containing SDS and 2-ME. The corresponding antibody was produced with SDS and 2-ME treated ovalbumin and the results showed that the antibody can tolerate 1% SDS and 7% 2-ME and the signal decrease was only minimal.

8.2 Determination of the adverse effect of SDS for protein extraction

8.2.1 Experimental

Extraction buffers:

- H0: 0.1 M TBS, pH 7.5
- H1: H0 + 0.5% SDS
- H2: H0 + 1% SDS
- H3: H0 + 2% SDS
- H4: H0 + 3% SDS
- H5: H0 + 5% SDS

Extraction procedure:

Spray dried egg white and cooked pasta were analysed for their extractability with SDS. 50 mg of SEW were mixed with 1.5 mL buffer and 2 g of cooked pasta with 5 mL buffer. The extraction was performed for 15 min at 60 °C, which is usual for commercial ELISA kits. After centrifugation the extracts were filtrated if it was required.

8.2.2 Results

BCA protein determination:

Table 19. Changes in extraction yield with increasing SDS concentration

Extraction buffer	protein yield [mg/mL] SEW	pasta
H0	21.21	0.49
H1	15.26	0.72
H2	17.24	0.90
H3	11.07	0.65
H4	18.39	0.98
H5	12.11	0.76

Interestingly, the protein yield of extracts from a native sample like spray dried egg white decreased with the usage of SDS and also the complex pasta extracts showed no linear correlation between extraction efficiency and SDS concentration. Therefore, the extracts had to be determined via ELISA to find the optimal buffer contents.

8.3 Determination of the negative effect of SDS in ELISA

8.3.1 Experimental

ELISA performance:

The indirect competitive ELISA was performed as normal, but the extracts were diluted with assay buffer (0.05 M PBS + 0.1% Tween) and for comparison also with extraction buffer to determine the inhibiting effect of SDS. For coating the same antigens were used as for immunization and antibody production.

8.3.2 Results

Indirect competitive ELISA:

As already mentioned, the extracts were diluted with assay buffer for ELISA analysis to identify the most effective SDS concentration for egg white extraction. The best result in case of anti-SEW antibody detection was obtained with the extraction buffer H4 containing 3% SDS (see figure 39). However, all buffers with various SDS concentrations enhanced the signal curve and the results agreed with the BCA values.

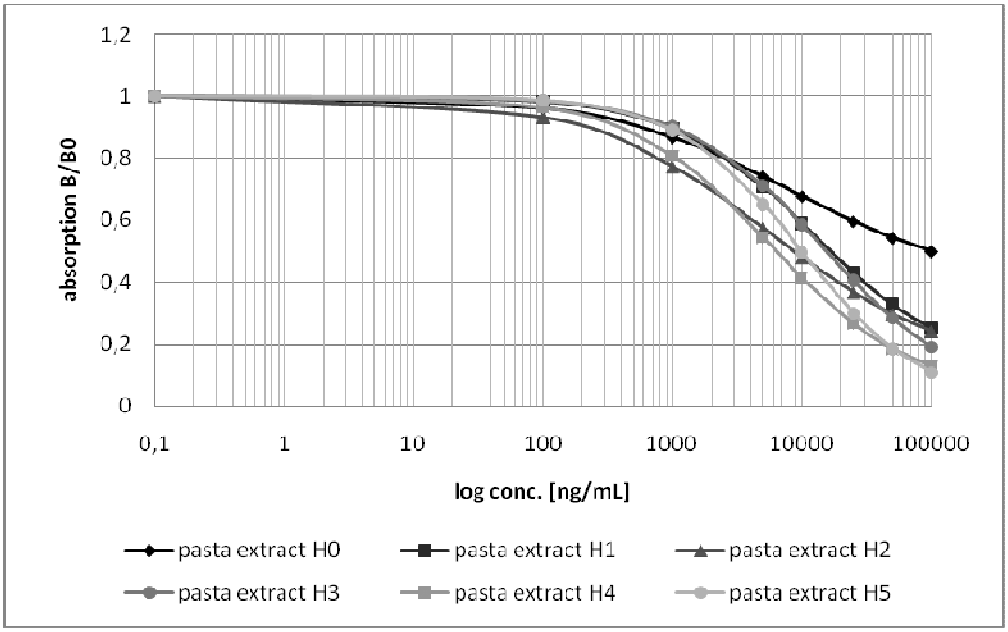


Figure 39. ELISA signal curves of pasta extracted with H0-H5 and diluted with ELISA assay buffer. The detection was performed with rabbit anti-SEW antibody.

To visualize the negative effect of SDS on antigen-antibody binding the same approach was repeated, but the pasta extracts were diluted with the corresponding extraction buffer instead of assay buffer (see figure 40). All antibodies were tested (data not shown) and the signal curves were dislocated and showed a flatter run with increasing SDS concentration. Even diluting the extract with a buffer containing 0.5% SDS decreased Δabs about one-third, using anti-SEW antibody for detection.

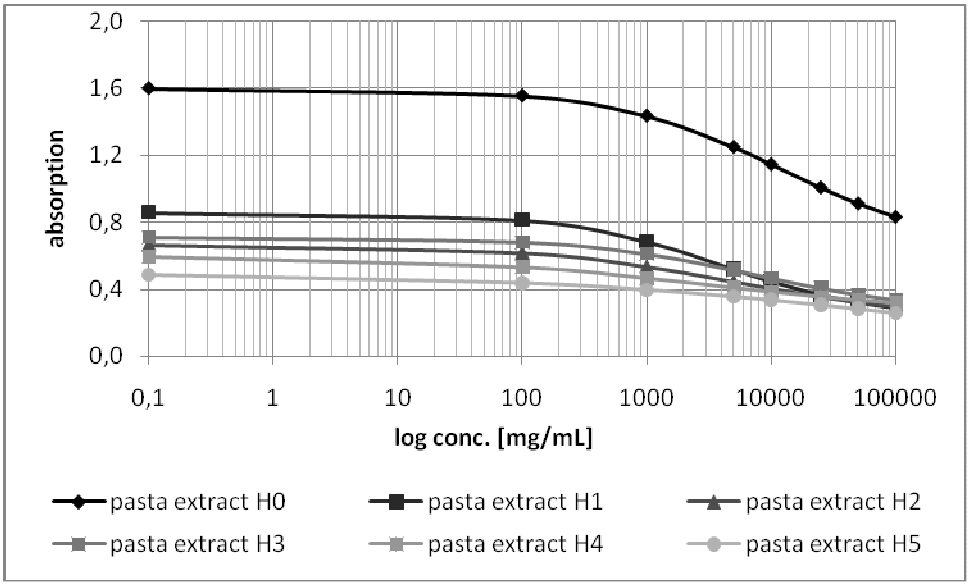


Figure 40. ELISA signal curves of pasta extracted with H0-H5 and diluted with the corresponding extraction buffer. The detection was performed with rabbit anti-SEW antibody.

8.4 Determination of the negative effect of SDS on coating efficiency

8.4.1 Experimental

Coating buffers:

- Sodium carbonate buffer, pH 9.6

  including 0%, 0.01%, 0.05%, 0.1%, 0.25% or 0.5% SDS

- PBS buffer, pH 7.5 (according to Reed)

  including 0%, 0.01%, 0.05%, 0.1%, 0.25% or 0.5% SDS

Coating procedure:

The MTPs were coated with SEW, which had been serially diluted in the various coating buffers. First the coating was performed with higher SDS concentrations (1-10%) and overnight at 4 °C, but the anionic detergent precipitated in the wells of the MTPs, which caused false signals in the following ELISA analysis. Therefore, SDS concentration was decreased and the coating step was performed at 15 °C overnight. Afterwards the SDS was completely removed by using the washing buffer containing 0.1% Tween 20.

ELISA procedure:

After the coating step the MTPs were blocked with 1% Ficoll for 2 h at 37 °C as usual. Because the sample extracts instead were used for coating instead of a standard, the detection was performed with 100 µL/well of rabbit anti-SEW antibody diluted 1:5000 in assay buffer for 1 h. No competitive reaction between the sample and the coated standard took place. Finally, the primary antibody was detected with anti-rabbit HRP antibody like in indirect competitive ELISA.

8.4.2 *Results*

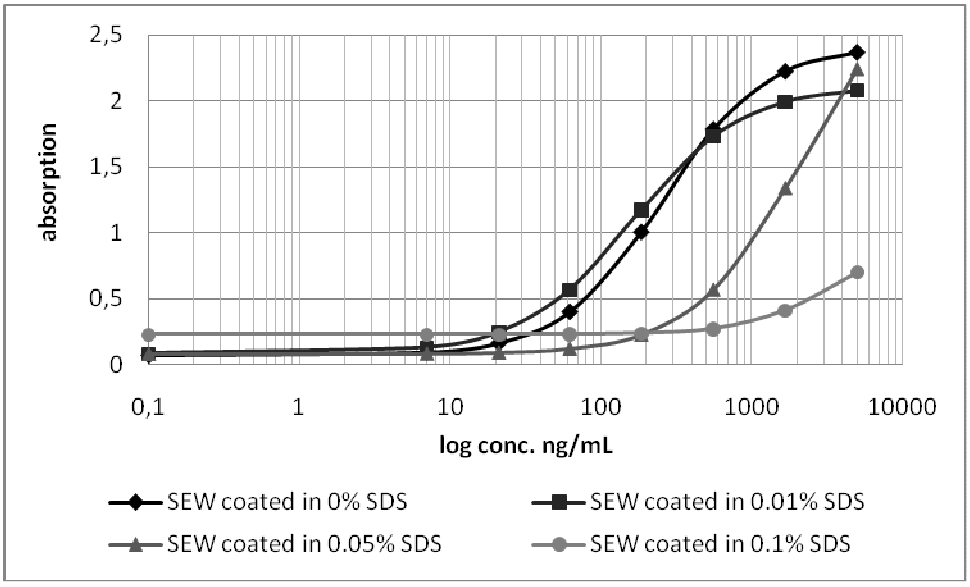


Figure 41. ELISA signal curves of coated SEW diluted with buffers containing increasing SDS concentration

The signal curves showed that SDS had an extensive negative effect on coating. Regardless of which concentration, the detergent SDS in PBS buffer or sodium carbonate buffer (data not shown) influenced the coating efficiency and caused signal reduction even in a low concentration like 0.01% (see figure 41).

8.5 Discussion

It can be concluded that the SDS concentration necessary for maximal protein extraction varies according to the composition of food sample, because the hydrophobic protein content (including denatured or altered proteins) varies. As mentioned in chapter 5.5. all food stuff samples offered higher protein yield using 1% or 2% SDS instead of 5% SDS for extraction. Furthermore, the standard SEW showed the best result within protein extraction without using SDS anyway. Otherwise the protein coagulum of a heat-processed food product may be considered as a random three-dimensional network of intertwined polypeptide chains held together by hydrogen and hydrophobic bonds. Protein can be adequately extracted from this mass only by using reagents capable of abolishing these interactions, such as a detergent like SDS. For efficient protein extraction of food stuff and also for native samples the SDS concentration should not exceed 4%. Finally, the limiting factor for using SDS within protein extraction in this work is its disruptive capacity for antigen-antibody binding in ELISA analysis.

Watanabe et al. (2005) used only 1% SDS for extraction and argued that the inhibiting effect of SDS on antibody-antigen binding could be abolished by using an antibody against SDS-treated ovalbumin. Interestingly, two studies were performed 2007 [18] and 2010 [19] to compare different commercial ELISA kits for egg white proteins in heat-treated samples. Both tested the Egg Protein ELISA Kit manufactured by Morinaga Institute of Biological Science, Inc. and both knew that the kit implies an extraction buffer containing 1% SDS and 7% 2-ME and works with an antibody against SDS/2-ME treated ovalbumin. The extracted samples were diluted 1:20 with Diluent 1 and further diluted with Diluent 2 according to the manufacturer‘s instructions independently of the extraction yield. Furthermore, Diluent 1 and Diluent 2 are expressions from the Morinaga kit protocol; they are different dilutions of the sample buffer (content not described) assuming the described Kit is the sandwich ELISA, which was developed from Watanabe 2005. In case of accuracy, that the presence of 1% SDS and 7% 2-ME do not inhibit reactions involved in antigen detection using the anti-SDS-OVA antibody, for which reason is a dilution step necessary? Why not using the concentrated extract?
Moreover, the dilution of the extracts has to be performed with a diluent and not with the extraction buffer. The risk to be under the LOD (limit of detection) level after a 1:20 dilution step increases with food samples containing only trace amounts of egg white resulting from contamination. The efficient performance of the Morinaga Kit in comparison to other commercial kits might be caused by longer extraction time (overnight) instead of the usual 15-20 min. Of course, the antibody against SDS-treated ovalbumin will detect denatured egg white better than an antibody against the native protein, but does its insensitivity against SDS also increases? Let´s assume, that the injection for immunization of rabbits includes 1% SDS and the SDS exposed antigen, after injection the components would be distributed by the circulatory system before antibody production and the free SDS molecules would bind to other proteins. Therefore, the antibody would be produced against the SDS-denatured antigen and doesn´t have to overcome big amounts of SDS. Nevertheless, in ELISA the antibody gets in contact with 1% SDS included in the extraction buffer and the immunoglobulin will be affected by the detergent.

To avoid this problem Reed et al. (2010) performed an ELISA without competitive reaction between the standard and the sample analyte by coating the serially diluted sample extract. Within this procedure the antibody never converged directly with the extracts and the included 10% SDS. However, a coating approach with various SDS concentrations showed that even 0.01% SDS influenced the coating efficiency and thus, the sample extracts have to be diluted 1000-fold to ensure a signal curve in ELISA analysis. Moreover this immuno-assay could not be used for commercial application, because the coating step, which has to be performed by the customer, takes place overnight.

8.6 Rescue of SDS inhibited antibody-antigen binding reaction by other detergents

8.6.1 Experimental

Antibody dilution buffers (DB):

- DB1: 0.05 M PBS + 0.5% Brij 35
- DB2: 0.05 M PBS + 1% Brij 35
- DB3: 0.05 M PBS + 2% Brij 35
- DB4: 0.05 M PBS + 3% Brij 35
- DB5: 0.05 M PBS + 0.5% CHAPS
- DB6: 0.05 M PBS + 1% CHAPS
- DB7: 0.05 M PBS + 2% CHAPS
- DB8: 0.05 M PBS + 3% CHAPS

ELISA performance:

The primary antibody was diluted with assay buffer and additionally with 0.05 M PBS + detergents in various concentrations (DB1-DB8) to capture SDS of the extraction buffer and to rescue the signal. Because of the anionic character of SDS, non-ionic detergents (Triton X-100, Tween 20, Brij 35), a zwitterionic detergent (CHAPS) and a cationic detergent (CTAB) were tested to compensate the negative effect of SDS. All experiments were performed with the antibodies against SEW, cEW, native OVM and heated OVM.

Sample order on MTPs

- Sample 1: H2/H4 pasta extract diluted in assay buffer
  Primary antibody diluted in assay buffer
- Sample 2: H2/H4 pasta extract diluted in extraction buffer
  Primary antibody diluted in assay buffer
- Sample 3: H2/H4 pasta extract diluted in extraction buffer
  Primary antibody diluted in DB1
- Sample 4: H2/H4 pasta extract diluted in extraction buffer
  Primary antibody diluted in DB2
- Sample 5: H2/H4 pasta extract diluted in extraction buffer
  Primary antibody diluted in DB3

- Sample 6: H2/H4 pasta extract diluted in extraction buffer

  Primary antibody diluted in DB4
- Sample 7: H2/H4 pasta extract diluted in extraction buffer

  Primary antibody diluted in DB5
- Sample 8: H2/H4 pasta extract diluted in extraction buffer

  Primary antibody diluted in DB6
- Sample 9: H2/H4 pasta extract diluted in extraction buffer

  Primary antibody diluted in DB7
- Sample 10: H2/H4 pasta extract diluted in extraction buffer

  Primary antibody diluted in DB8

8.6.2 Results

Pasta extract H2 signal rescue with 0.5%, 1%, 2% and 3% Brij 35

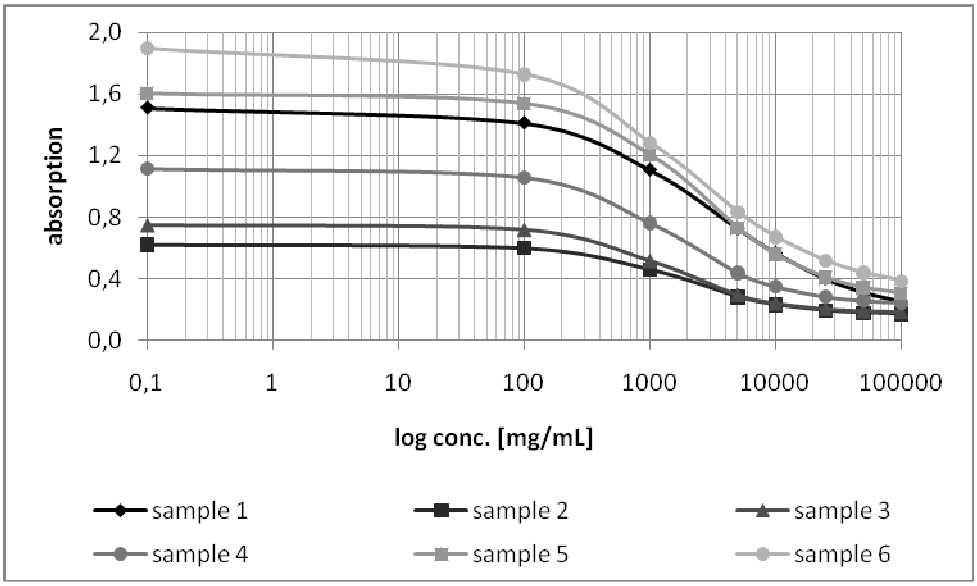


Figure 42. ELISA signal curves of pasta extracted with 1% SDS and rescued with non-ionic detergent Brij 35. The MTP was coated with 500 ng/mL SEW and the detection was performed with rabbit anti-SEW antibody.

The 4-parameter curves showed increased signal if the SDS molecules have been captured with the non-ionic detergent Brij 35 (figure 42). The best simulation of the signal curve produced with the assay buffer (sample 1) was generated with 2% Brij 35 in the antibody dilution buffer (sample 5).

Pasta extract H2 signal rescue with 0.5%, 1%, 2% and 3% CHAPS

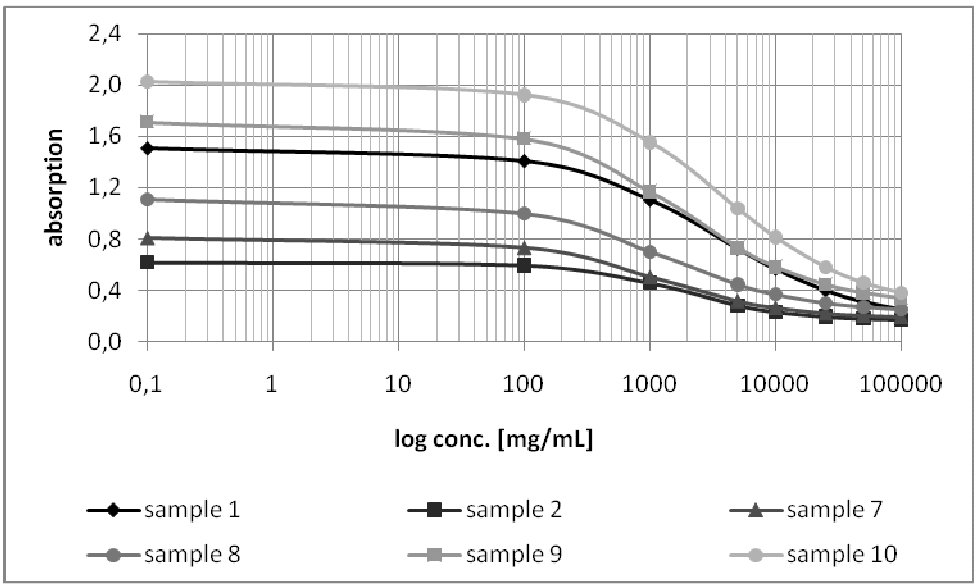


Figure 43. ELISA signal curves of pasta extracted with 1% SDS and rescued with zwitterionic detergent CHAPS. The MTP was coated with 500 ng/mL SEW and the detection was performed with rabbit anti-SEW antibody

Again the closest copy of the signal curve from the sample diluted in assay buffer (sample 1) was developed with 2% of the zwitterionic detergent CHAPS (sample 9). Furthermore, using 3% CHAPS to defend antibody-antigen binding against 1% SDS, increased the signal even more than in sample 1 (see figure 43).

Pasta extract H4 signal rescue with 0.5%, 1%, 2% and 3% Brij 35

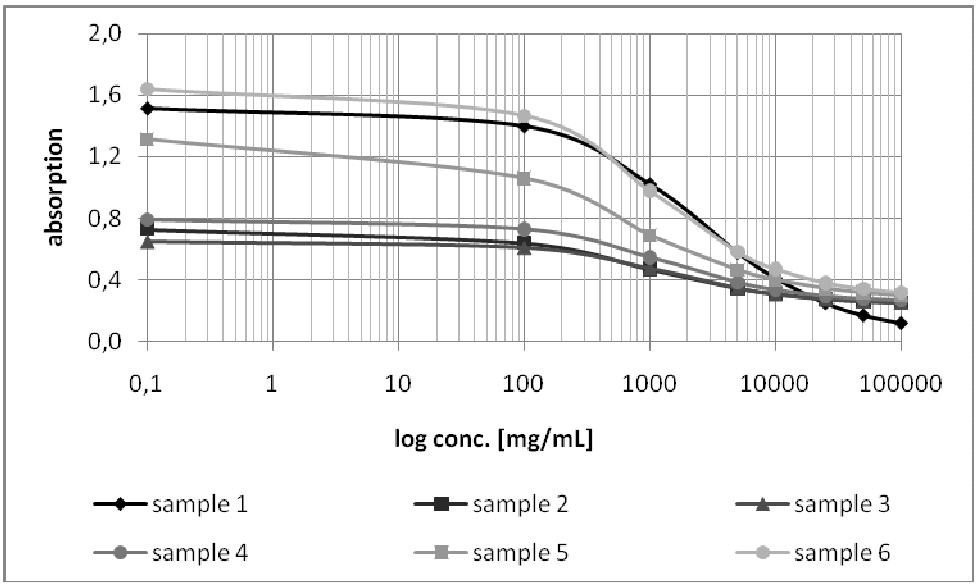


Figure 44. ELISA signal curves of pasta extracted with 3% SDS and rescued with nonionic detergent Brij 35. The MTP was coated with 500 ng/mL SEW and the detection was performed with rabbit anti-SEW antibody

Pasta extract H4 signal rescue with 0.5%, 1%, 2% and 3% CHAPS

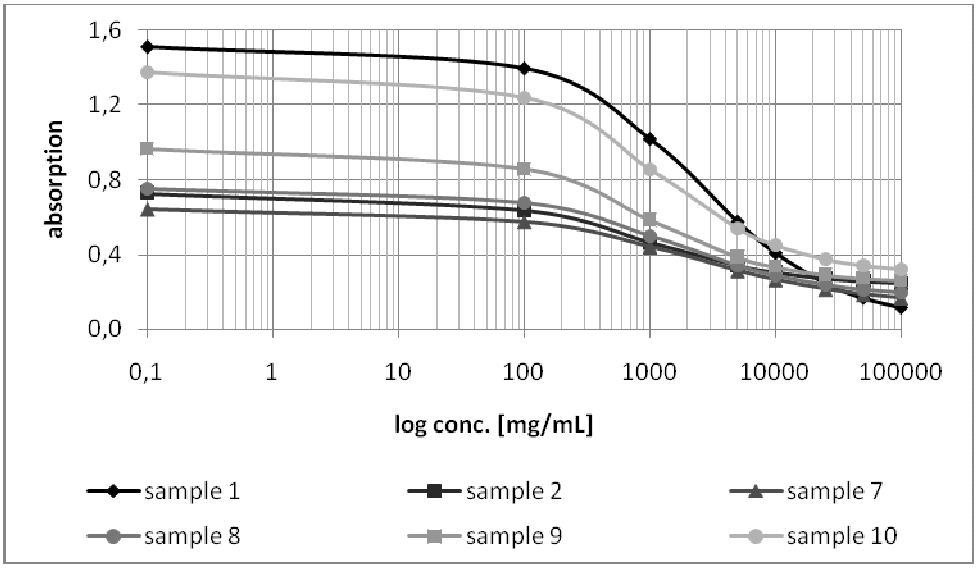


Figure 45. ELISA signal curves of pasta extracted with 3% SDS and rescued with zwitterionic detergent CHAPS. The MTP was coated with 500 ng/mL SEW and the detection was performed with rabbit anti-SEW antibody

An antibody dilution buffer containing 3% Brij 35 or 3% CHAPS was not sufficient to compensate 3% SDS in the sample buffer, therefore the slope of the signal curve of sample 1 could not be achieved (see figure 44 and 45).

8.6.3 Discussion

The results showed that the negative effect of 1%-2% SDS in the extraction buffer could be prevented by using at least the same concentration of the non-ionic detergent Brij 35 as antibody dilution buffer instead of diluting the sample extract before ELISA analysis. Other detergents, like CHAPS, Tween 20 or Triton X-100 offered also a positive effect on antibody-antigen binding in the presence of SDS, but less effective than Brij 35 (data not shown). Additionally, the cationic detergent CTAB alone and in combination with a nonionic detergent was tested for signal rescue efficiency. Surprisingly the cationic detergent was not capable to prevent the negative effect of SDS and caused lower signal than without any detergent in the antibody dilution buffer.

As control the extract diluted in assay buffer (sample 1) was also tested with the antibody diluted in 2% Brij 35 and 2% CHAPS to determine influences of those detergents on antibody-antigen binding (data not shown). The non-ionic detergent Brij 35 did not effect the signal curve, but CHAPS increased the signal compared to the usual assay buffer including 0.1% Tween 20 for antibody dilution. If the enhancement of the signal in ELISA was caused by unspecific interaction or increased accessibility of antibody recognition motive on the antigen is not clear. Finally, Brij 35 is more efficient in capturing SDS during the antigen-antibody binding reaction, seems to achieve no direct effect on antigen-antibody binding and is much cheaper than CHAPS.

9. The effect of the reducing agent DTT (Dithiothreitol) for food stuff analysis

The denaturation and aggregation of egg white proteins by heating has been investigated for isolated proteins, egg white, and whole egg. Many data have shown that isolated ovomucoid is not aggregated and precipitated by heating. Thus, heat-induced changes in antigenic and allergenic activities of OVM in egg white are well characterized, but little is known concerning changes in such immunological properties of OVM heated in the presence of other food proteins [16].

9.1 Determination of the adverse effect of DTT for egg white protein extraction

In the study of Kato et al., 2001, the heat-induced insolubilization of OVM was investigated in the egg white mixed with wheat flour as a model of pasta. By heating the model pasta OVM was effectively insolubilized, and almost no antigenic activity of OVM was detected for the used rabbit IgG antibodies probably due to aggregation through intermolecular disulfide bonds with wheat proteins. The working group determined the antigenicity of OVM in raw and cooked pasta extracted with 4% SDS and 10% 2-mercaptoethanol (2-ME). They demonstrate higher OVM yield particularly from the cooked pasta in immunoblots detected with rabbit anti-OVM serum, using 2-ME for extraction. The samples analyzed in competitive ELISA were only extracted with PBS and showed, that extracted OVM decreases with the processing level of the pasta. In the following experiment also self-made pasta was produced and analyzed by immunoblotting and in contrast to Kato by competitive ELISA, too. However, dithiothreitol (DTT) instead of 2-ME was used as reducing agent in different concentrations, because its not harmful and has no negative influence on antibody-antigen binding in ELISA like 10% of any alcohol. Furthermore, it can be concluded that the SDS concentration necessary for maximal protein extraction varies according to the food, because the hydrophobic protein content (including denatured or altered proteins) varies. The limiting factor for using SDS within protein extraction is its disruptive capacity for antigen-antibody binding in ELISA analysis. Therefore, the extractions were performed with only 1% SDS in PBS buffer.

9.1.1 Experimental

Pasta preparation:

First of all, the single ingredients were extracted to achieve a positive control (egg white) and negative controls (egg yolk and wheat flour) and to avoid any cross-reactivity within the used protein amounts. Afterwards the pasta ingredients (100 g wheat flour, 10.7 g crude egg yolk, 30.7 g egg white and 15 g olive oil) were mixed and kneaded for 10 min on a board to ensure a homogeneous sample. After the first extraction approach the raw mixture was benched for 4 h at RT, resulting in dough formation. The second extraction was performed and the dough was extended, cut into diamond forms and dried in the vacuum incubator at 50 °C for 20 h. Finally the pasta was cooked for 20 min in 500 mL water and before and after cooking samples were collected, extracted and their protein yield was determined with BCA.

Extraction buffers:

- K1: 0.1 M PBS, 1% SDS
- K2: 0.1 M PBS, 1% SDS, 1 mM DTT
- K3: 0.1 M PBS, 1% SDS, 2 mM DTT
- K4: 0.1 M PBS, 1% SDS, 5 mM DTT

Extraction procedure:

The samples were dissolved as good as possible in extraction buffer, vortexed and extracted for 15 min at 60 °C in the water bath shaking the samples every 5 min. Afterwards the extracts were centrifuged with 9500 rpm for 10 min and filtered if necessary.

9.1.2 Results

BCA protein determination:

Within this extraction buffer blanks were sufficient for protein quantification, because the reducing agent DTT influences the colorimetric reaction of BCA and falsifies the results. Therefore the buffer blanks were subtracted from the measured absorbencies. Surprisingly, only the highly processed cooked pasta showed better extraction results with increasing DTT concentration (see table 20). The protein yield of the crude ingredients egg white and yolk was diminished by the usage of DTT, also the extraction efficiency of the dough and the dried pasta samples decreased with increasing DTT concentration.

Table 20. Extraction yield of self-made pasta samples in various processing steps

Sample	Extraction buffer	Sample buffer ratio	protein [mg/ml]
crude egg white	0.1 M TBS, 1% SDS	0.1 g in 5 mL	2.95
	+ 1 mM DTT	0.1 g in 5 mL	2.02
	+ 2 mM DTT	0.1 g in 5 mL	1.89
	+ 5 mM DTT	0.1 g in 5 mL	1.66
crude egg yolk	0.1 M TBS, 1% SDS	0.1 g in 5 mL	3.71
	+ 1 mM DTT	0.1 g in 5 mL	2.87
	+ 2 mM DTT	0.1 g in 5 mL	2.53
	+ 5 mM DTT	0.1 g in 5 mL	1.75
wheat flour	0.1 M TBS, 1% SDS	0.1 g in 5 mL	1.77
	+ 1 mM DTT	0.1 g in 5 mL	3.23
	+ 2 mM DTT	0.1 g in 5 mL	2.58
	+ 5 mM DTT	0.1 g in 5 mL	2.68
kneaded dough	0.1 M TBS, 1% SDS	1 g in 5 mL	5.25
	+ 1 mM DTT	1 g in 5 mL	5.28
	+ 2 mM DTT	1 g in 5 mL	4.50
	+ 5 mM DTT	1 g in 5 mL	4.82
benched dough	0.1 M TBS, 1% SDS	1 g in 5 mL	3.20
	+ 1 mM DTT	1 g in 5 mL	2.16
	+ 2 mM DTT	1 g in 5 mL	2.86
	+ 5 mM DTT	1 g in 5 mL	1.10
dried pasta	0.1 M TBS, 1% SDS	1 g in 5 mL	10.03
	+ 1 mM DTT	1 g in 5 mL	9.77
	+ 2 mM DTT	1 g in 5 mL	8.99
	+ 5 mM DTT	1 g in 5 mL	8.98
cooked pasta	0.1 M TBS, 1% SDS	2 g in 5 mL	0.73
	+ 1 mM DTT	2 g in 5 mL	0.85
	+ 2 mM DTT	2 g in 5 mL	1.50
	+ 5 mM DTT	2 g in 5 mL	1.59
cooking water	0.1 M TBS, 1% SDS	1 mL in 5 mL	0.33
	+ 1 mM DTT	1 mL in 5 mL	0.32
	+ 2 mM DTT	1 mL in 5 mL	0.27
	+ 5 mM DTT	1 mL in 5 mL	n. d.*

* not detectable

SDS-PAGE and Western Blot:

To compare the composition of the extracts with the help of their band pattern, 7 µg protein was loaded on a 12 % Bis-Tris gel. The dough and the dried pasta samples showed the same band intensity; only the pattern of the samples extracted without or less DTT (lane 3, 7 and 8) was displaced below the others. This may be due to the conformational change after the loose of disulfide bridges. However, with increasing DTT concentration the protein content of the cooked pasta extracts also accelerated (see figure 46).

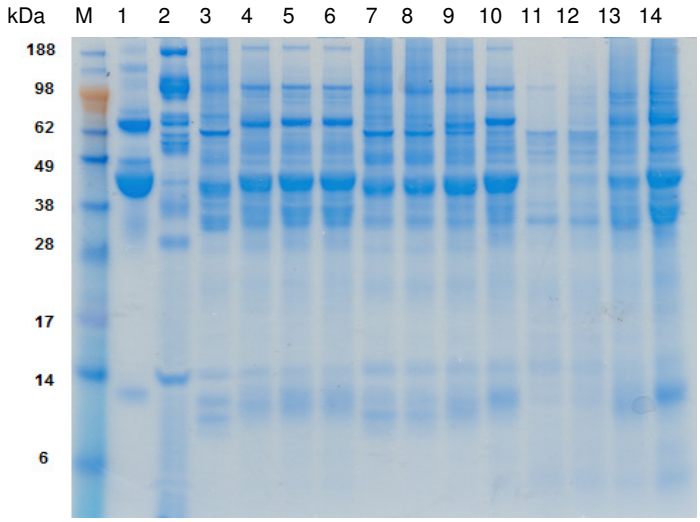


Figure 46. SDS-PAGE of self-made pasta extracts

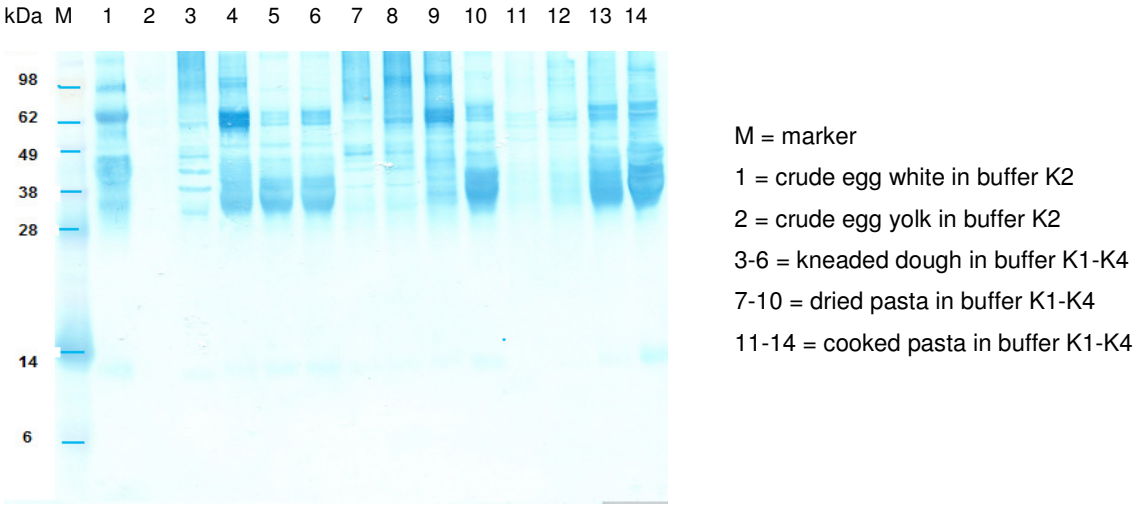


Figure 47. Western Blot of self-made pasta extracts detected with anti-native OVM antibody

The pasta also contained egg yolk and wheat flour, which consists of albumins, globulins, gliadin, and glutenin. Therefore, the protein pattern showed mainly other proteins than egg white and for quantification of extracted ovomucoid an immunoblot with rabbit antibodies against native OVM was performed (figure 47). The Western Blot showed that 1 mM DTT was effective for OVM and/or OVA extraction from the dough samples (lane 4, around 38 kDa), in contrast to the higher processed dried pasta samples, where only 5 mM DTT (lane 10) caused the same OVM/OVA yield. The cooked pasta extracts revealed the absolute benefit of DTT for food stuff extraction (lane 13 and 14).

Indirect competitive ELISA:

For final analysis of extraction efficiency of OVM with various DTT concentrations the pasta samples were also determined by ELISA using all four antibodies against egg white proteins. To achieve a relative comparison between the extracts of one approach the same volume of extracted protein was used to prepare the dilution series instead of a defined concentration. Therefore, the IC_{50} values were not absolute and are only helpful for comparison.

Table 21. ELISA results of pasta samples analysed with rabbit anti-cEW antibody

sample	extraction buffer	absmax	absmin	Δabs	IC50
kneaded dough	TBS + 1% SDS	1.497	0.089	1.408	67
	+ 1 mM DTT	1.483	0.120	1.363	244
	+ 2 mM DTT	1.489	0.133	1.356	559
	+ 5 mM DTT	1.807	0.208	1.599	272
benched dough	TBS + 1% SDS	1.494	0.092	1.402	130
	+ 1 mM DTT	1.505	0.159	1.346	419
	+ 2 mM DTT	1.354	0.158	1.196	491
	+ 5 mM DTT	1.314	0.300	1.014	791
dried pasta	TBS + 1% SDS	1.847	0.091	1.756	52
	+ 1 mM DTT	1.895	0.081	1.814	63
	+ 2 mM DTT	1.864	0.085	1.779	77
	+ 5 mM DTT	1.825	0.085	1.740	114
cooked pasta	TBS + 1% SDS	1.814	0.600	1.214	4854
	+ 1 mM DTT	1.764	0.404	1.360	3176
	+ 2 mM DTT	1.802	0.135	1.667	535
	+ 5 mM DTT	1.797	0.122	1.675	226
cooking water	TBS + 1% SDS	1.761	0.474	1.287	3120
	+ 1 mM DTT	1.713	0.424	1.289	4114
	+ 2 mM DTT	1.718	0.342	1.376	1470
	+ 5 mM DTT	1.656	0.424	1.232	915

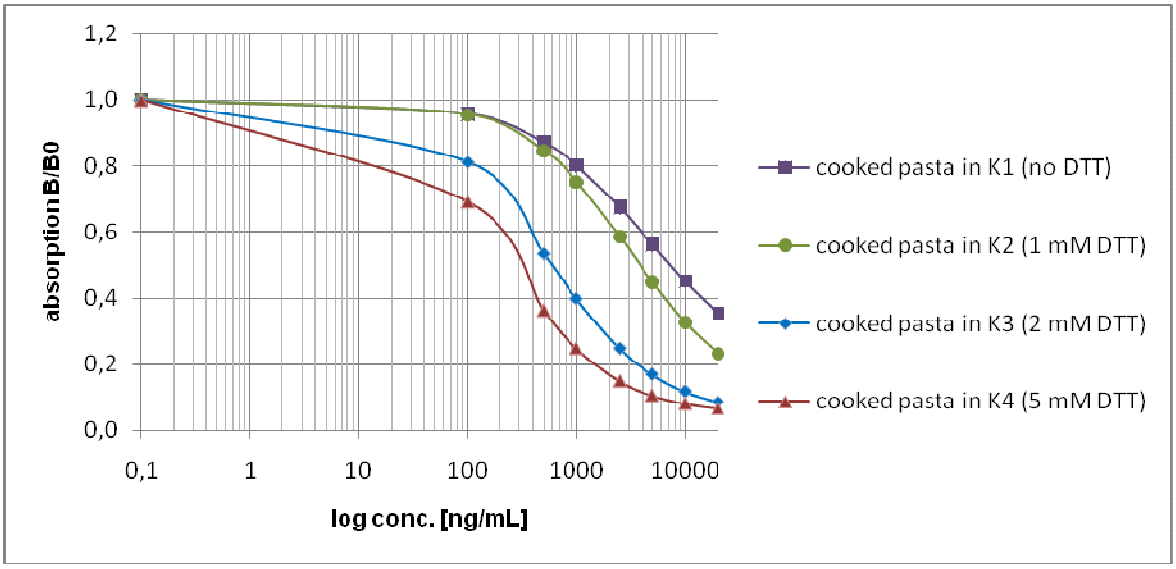


Figure 48. ELISA signal curves of cooked pasta extracts analysed with anti-cEW antibody

Table 22. ELISA results of pasta samples analysed with rabbit anti-SEW antibody

sample	extraction buffer	absmax	absmin	Δabs	IC50
kneaded dough	TBS + 1% SDS	2.026	0.098	1.928	122
	+ 1 mM DTT	2.061	0.116	1.945	269
	+ 2 mM DTT	2.064	0.232	1.832	464
	+ 5 mM DTT	2.049	0.332	1.717	512
benched dough	TBS + 1% SDS	2.047	0.097	1.950	190
	+ 1 mM DTT	2.024	0.160	1.864	445
	+ 2 mM DTT	1.750	0.187	1.563	506
	+ 5 mM DTT	1.713	0.420	1.293	741
dried pasta	TBS + 1% SDS	1.807	0.072	1.735	74
	+ 1 mM DTT	1.784	0.068	1.716	71
	+ 2 mM DTT	1.776	0.072	1.704	86
	+ 5 mM DTT	1.730	0.064	1.666	117
cooked pasta	TBS + 1% SDS	1.714	0.512	1.202	3847
	+ 1 mM DTT	1.716	0.323	1.393	2002
	+ 2 mM DTT	1.596	0.112	1.484	397
	+ 5 mM DTT	1.627	0.093	1.534	214
cooking water	TBS + 1% SDS	1.603	0.439	1.164	3571
	+ 1 mM DTT	1.555	0.391	1.164	3594
	+ 2 mM DTT	1.547	0.283	1.264	1318
	+ 5 mM DTT	1.526	0.346	1.180	1091

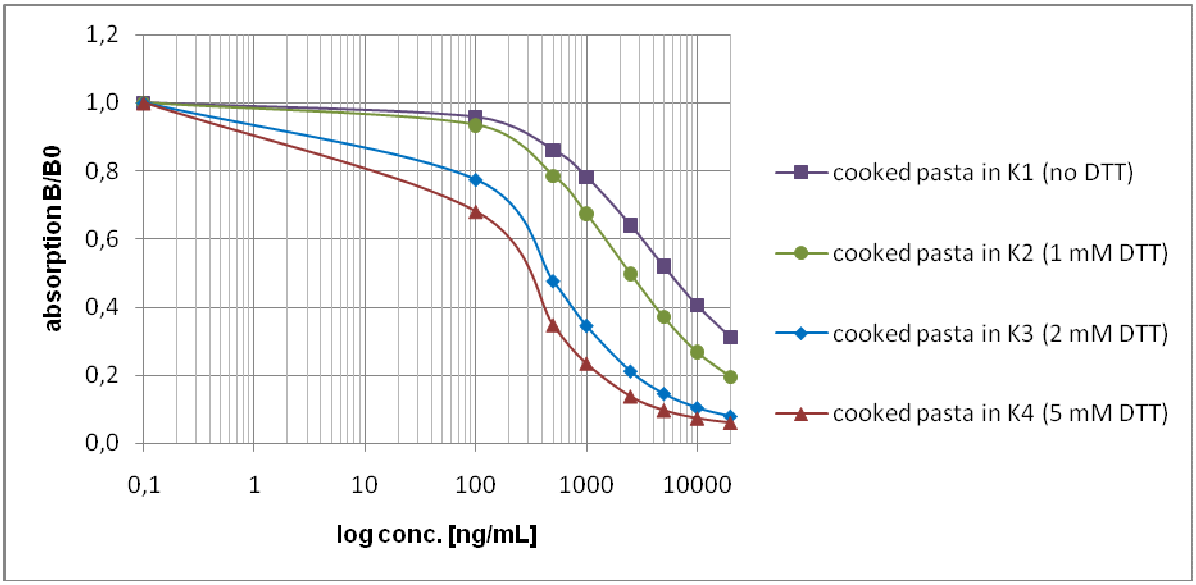


Figure 49. ELISA signal curves of cooked pasta extracts analysed with anti-SEW antibody

Table 23. ELISA results of pasta samples analysed with rabbit anti-native OVM antibody

sample	extraction buffer	absmax	absmin	Δabs	IC50
kneaded dough	TBS + 1% SDS	2.340	0.130	2.210	224
	+ 1 mM DTT	2.217	0.258	1.959	668
	+ 2 mM DTT	2.226	1.091	1.135	661
	+ 5 mM DTT	2.288	1.425	0.863	779
benched dough	TBS + 1% SDS	2.329	0.131	2.198	324
	+ 1 mM DTT	2.326	0.414	1.912	1863
	+ 2 mM DTT	1.894	0.868	1.026	1166
	+ 5 mM DTT	1.873	1.480	0.393	780
dried pasta	TBS + 1% SDS	1.618	0.077	1.541	195
	+ 1 mM DTT	1.537	0.078	1.459	216
	+ 2 mM DTT	1.527	0.084	1.443	336
	+ 5 mM DTT	1.475	0.073	1.402	379
cooked pasta	TBS + 1% SDS	1.530	0.944	0.586	30333
	+ 1 mM DTT	1.452	0.769	0.683	28252
	+ 2 mM DTT	1.887	0.32	1.567	2209
	+ 5 mM DTT	1.850	0.295	1.555	736
cooking water	TBS + 1% SDS	1.844	0.963	0.881	15207
	+ 1 mM DTT	1.797	1.268	0.529	7363
	+ 2 mM DTT	1.703	1.218	0.485	n.d.
	+ 5 mM DTT	1.748	1.390	0.358	n.d.

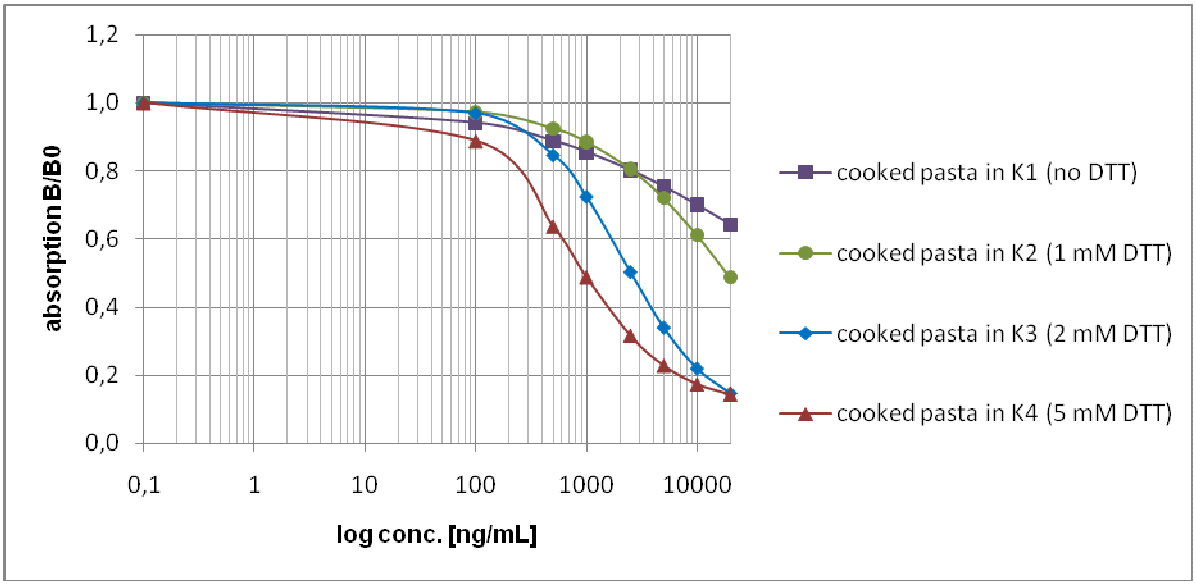


Figure 50. ELISA signal curves of cooked pasta extracts analysed with anti-native OVM antibody

Table 24. ELISA results of pasta samples analysed with rabbit anti-heated OVM antibody

sample	extraction buffer	absmax	absmin	Δabs	IC50
kneaded dough	TBS + 1% SDS	1.806	0.131	1.675	368
	+ 1 mM DTT	1.784	0.350	1.434	932
	+ 2 mM DTT	1.763	1.003	0.760	561
	+ 5 mM DTT	1.747	1.285	0.462	438
benched dough	TBS + 1% SDS	1.793	0.175	1.618	457
	+ 1 mM DTT	1.596	0.628	0.968	1487
	+ 2 mM DTT	1.679	0.879	0.800	1019
	+ 5 mM DTT	1.685	1.242	0.443	326
dried pasta	TBS + 1% SDS	1.645	0.088	1.557	217
	+ 1 mM DTT	1.630	0.093	1.537	300
	+ 2 mM DTT	1.635	0.105	1.530	423
	+ 5 mM DTT	1.575	0.134	1.441	443
cooked pasta	TBS + 1% SDS	1.573	1.133	0.440	39292
	+ 1 mM DTT	1.562	0.942	0.620	16580
	+ 2 mM DTT	1.501	0.309	1.192	2159
	+ 5 mM DTT	1.480	0.271	1.209	775
cooking water	TBS + 1% SDS	1.461	0.871	0.590	29658
	+ 1 mM DTT	1.418	1.094	0.324	n.d.
	+ 2 mM DTT	1.438	1.038	0.400	n.d.
	+ 5 mM DTT	1.450	1.203	0.247	n.d.

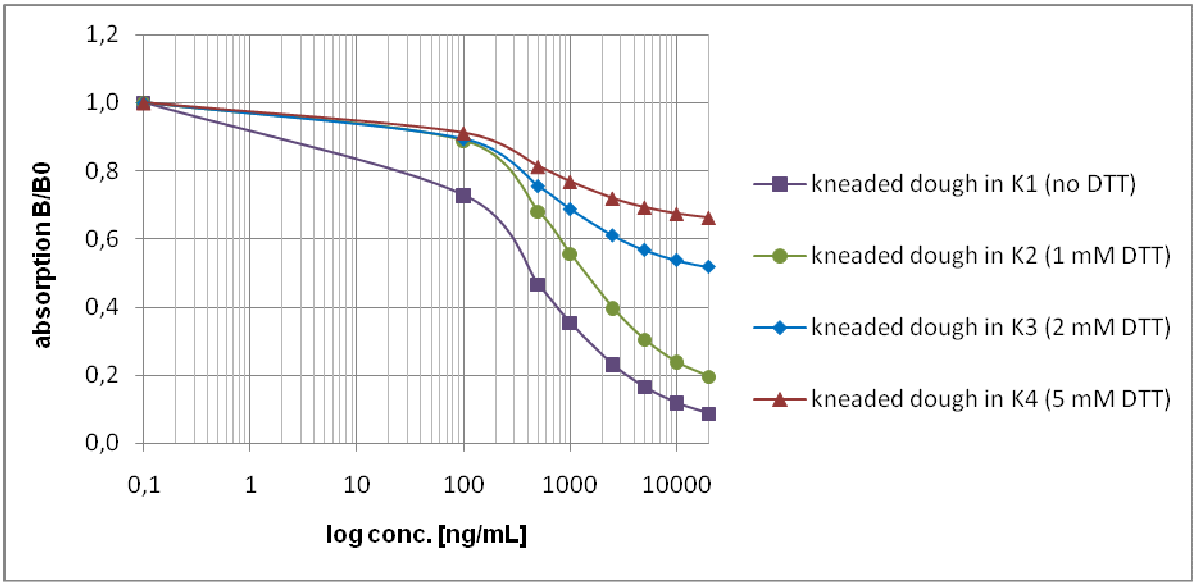


Figure 51. ELISA signal curves of dough extracts analysed with anti-heated OVM antibody

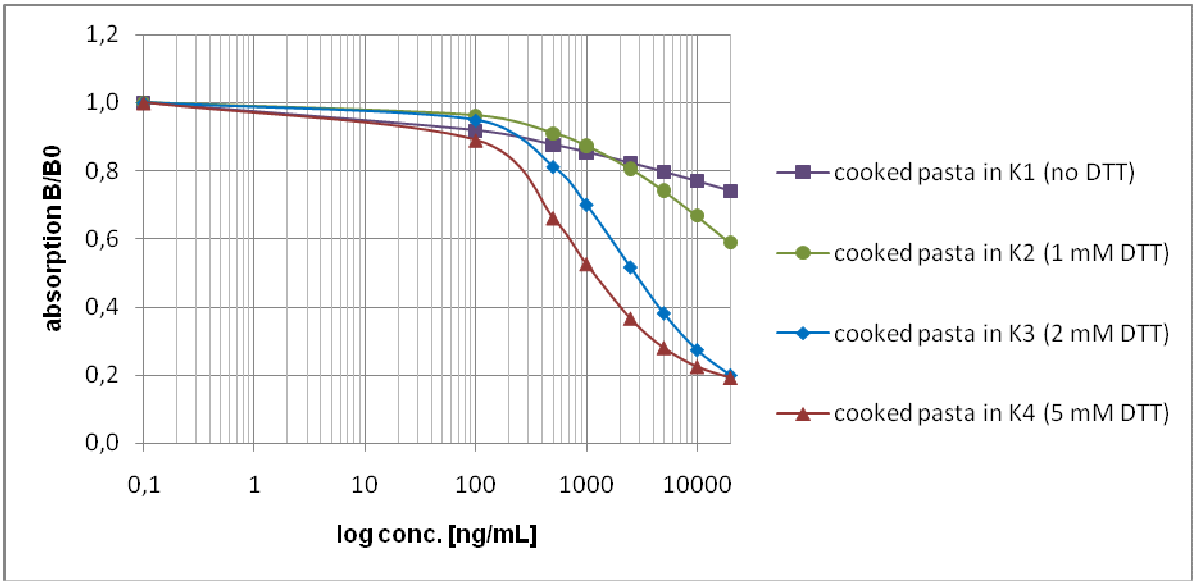


Figure 52. ELISA signal curves of cooked pasta extracts analysed with anti-heated OVM antibody

9.1.3 Discussion

The results showed that the use of DTT as reducing agent for extraction of proteins, in this case OVM and OVA, from highly processed food stuff can lead to higher protein yield. However, a negative effect for the extraction of native proteins from raw or only light processed samples (dried pasta) was observed with increasing DTT concentration (see table 20). Obviously the antibodies recognize primarily native egg white proteins, which were not reduced via DTT and kept their conformation, as expected for antibodies of rabbits immunized with native antigens (table 21-24). Nevertheless, DTT helps to solubilise egg white proteins, which were coupled to other food ingredients during processing, and to avoid aggregation of the allergens during extraction. Finally DTT alters the protein conformation by reducing disulfide bridges and decreases the affinity to the antibody, but without DTT nearly no egg white protein could be extracted from highly processed samples.

Great differences in extraction efficiency were obtained by using 1 mM or 2 mM DTT (see figures 48-52). Therefore, an extraction with 1 mM DTT would not be sufficient enough for highly denatured proteins, otherwise 2 mM DTT would affect native samples. However, the protein yield of extracts from processed samples is lower than from raw food stuff and extracting as much denatured protein as possible is important for detection. The negative effect of DTT for extraction of native proteins has to be compensated by less dilution of the sample extract for ELISA determination.

9.2 Determination of the negative effect of reducing agents on antibodies

As mentioned in chapter 8.3. the working group of Watanabe (2005) produced an antibody against SDS and 2-ME treated ovalbumin and argued, that this antibody can handle 1% SDS and 7% 2-ME included in the extraction buffer performing a sandwich ELISA. However, as shown in Fig.2 the light and heavy chains of IgG are kept together by the formation of disulfide bridges. Therefore the determination of DTT/2-ME concentration used for extraction could not be neglected, if the undiluted extract should be analysed in an immuno-based reaction.

9.2.1 Experimental

SDS-PAGE:

To determine the DTT concentration which causes antibody disassembly, 5 µg ProtA purified anti-SEW antibody alone and in 1 mM, 2 mM, 3 mM, 5 mM and 10 mM DTT were loaded on a gel and separated by denaturating electrophoresis.

Indirect competitive ELISA:

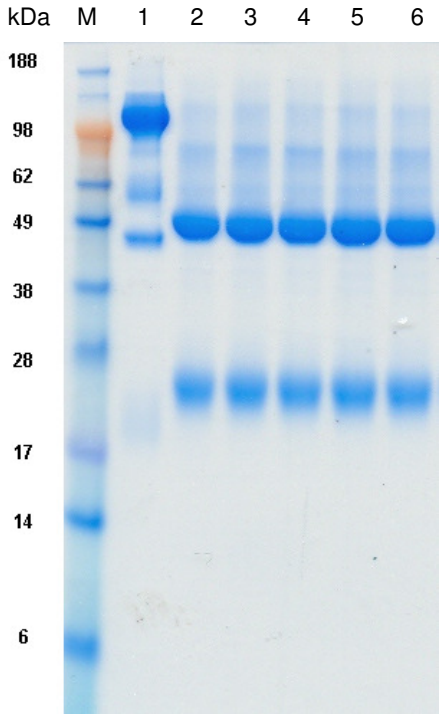
To approve the arguments of Watanabe, standard dilution series of ovomucoid, which offers 9 disulfide bridges, were produced with buffers containing increasing concentrations of 2-ME and and analysed with rabbit anti-SEW antibodies for signal comparison.

Dilution buffers:

- L0: 0.1 M PBS
- L1: L0 + 1 mM 2-ME
- L2: L0 + 2 mM 2-ME
- L3: L0 + 5 mM 2-ME
- L4: L0 + 2.5% 2-ME (~320 mM, according to Reed)
- L5: L0 + 7% 2-ME (~900 mM, according to Watanabe)

9.2.2 Results

SDS-PAGE:



M = marker
1 = anti-SEW ab without DTT
2 = anti-SEW ab in 1 mM DTT
3 = anti-SEW ab in 2 mM DTT
4 = anti-SEW ab in 3 mM DTT
5 = anti-SEW ab in 5 mM DTT
6 = anti-SEW ab in 10 mM DTT

Figure 53. SDS-PAGE of rabbit IgG treated with the reducing agent DTT

The SDS-PAGE band pattern in figure 53 showed that already a DTT concentration of 1 mmol/L causes decay of the antibody into the heavy chains (55 kDa) and light chains (24 kDa). It should be noticed that the antibody amounts used in ELISA are much lower than the loaded 5 µg in 10 µL.

Indirect competitive ELISA:

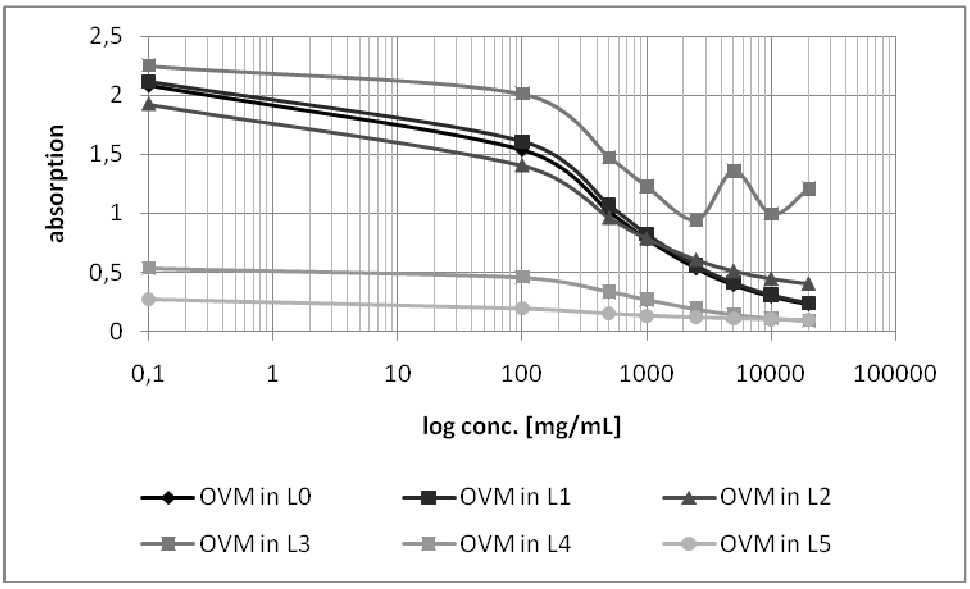


Figure 54. ELISA signal curves of OVM diluted in buffers with increasing 2-ME concentrations

The dilutions prepared without 2-ME, with 1 mM and with 2 mM 2-ME showed similar signal curves, whereas 2.5% or 7% 2-ME deleted the signal by degrading the antibody (see figure 54). The signal curve of the dilutions with 5 mM 2-ME offered a run without any correlation between OVM concentration and signal intensity and also exhibited increased standard deviation (data not shown). This might be due to the exact level of reducing agent, which causes disassembly of the antibody.

9.2.3 Discussion

The results showed that the reducing agents dithiothreitol and ß-mercaptoethanol could not be used in excess without any consequences because of their ability to degrade antibodies.
The problem of determining the optimal concentration is the unknown number of disulfide bridges in a sample. It could be assumed that concentrations up to 2 mM of a reducing agent are compatible for the antibody's function in the analysis of complex food stuff. However, Watanabe performed his analyses in another ELISA format (Sandwich ELISA), but the coated antibody is also exposed to the undiluted extract and will suffer from the 7% ß-mercaptoethanol.

10. BLOCKING DETERMINATION

10.1 General requirements

Solid phase immunoassays, such as ELISA, involve the immobilization of biomolecules, primarily proteins, to the surface via passive or covalent interactions. Non-specific binding of other proteins or biomolecules to unoccupied spaces on the surface during subsequent steps of the assay can be detrimental to the specificity and sensitivity of the assay results. Non-specific binding to the surface can be minimized by saturating these unoccupied binding sites with a blocking reagent – a collective term for various substances that are used to reduce non-specific binding without taking an active part in specific assay reactions.

Detection reagents cross-reacting with blocking reagents are one of the main causes of high background and low signal-to-noise ratio in ELISA systems. The ideal blocking substance will bind to all potential sites of non-specific interaction, eliminating background without altering or obscuring the epitope for antibody binding.

For true optimization of the blocking step for a particular immunoassay, empirical testing is essential. Many factors can influence nonspecific binding, including various protein-protein interactions unique to a given set of immunoassay reagents. The most important parameter when selecting a blocker is the signal-to-noise ratio, which is measured as the signal obtained with a sample containing the target analyte as compared to that obtained with a sample without the target analyte. No single blocking agent is ideal for every occasion because each antibody-antigen pair has unique characteristics [20].

10.2 Blocking efficiency

Detergents, proteins, synthetic blockers and carbohydrates were checked for their ability to reduce non-specific binding onto polystyrene microtiter plates used for ELISA. In food allergen analysis protein blockers like bovine serum albumin (BSA) or casein may interfere with the analyte and therefore alternative blockers like polyvinylalcohol (PVA), dextrans or Ficoll were tested for their blocking efficiency in ELISA. Finally, Ficoll was used in this working group as blocking reagent in the competitive indirect ELISA studies [21]. However, the usage of buffers containing detergents or denaturating agents to extract food allergens requires again a check-up of blocking efficiency to exclude negative effects of the buffer components. Also the presence of the non-ionic detergent Tween 20 in the washing step after blocking has to be determined. In general, Tween 20 can block areas on the surface that may be physically stripped of specifically bound biomolecules during the wash step and helps dislodge loosely bound blocking agent. Otherwise, detergent wash may break specific couplings in systems having weak immunologic affinities [22].

10.3 Determination of blocking efficiency

To ensure efficient blocking, the final extraction buffers (see chapter 11) were tested for their effects on the used blocking substance Ficoll (data not shown). Surprisingly, extraction buffers containing 20% urea and/or detergents had no influence on blocking, but the usual 0.1 M TBS buffer and buffer 8 (0.1 M TBS + 20% urea) caused a signal curve. However, both buffers had in common to include no detergent. Therefore, the effect of anionic, cationic and non-ionic detergent was analysed on the blocking efficiency of various blocking agents (Ficoll, Dextran, BSA, fish gelatine and PVA).

10.3.1 Experimental

ELISA procedure:

The ELISA performance was nearly the same as for all indirect competitive ELISAs except the lack of a coating step. Therefore, no coated standard existed to bind the specific antibody. After blocking for 2 h, the MTPs were washed with washing buffer without and with 0.1% Tween to look for any difference. To visualize the worst case the SEW samples, which were diluted with special buffers, were incubated without the primary antibody for 1 h. After a washing step the MTPs were incubated with rabbit anti-SEW antibody and if the blocking step was still comprehensive after the treatment with buffer components, the specific antibody had no chance to bind any antigen and would be washed away. Otherwise, if the extraction buffers had removed the blocking substance to a certain extent, the SEW samples would be able to bind to the plate and could be detected by the antibody. The result would be a signal curve, which escalates with increasing SEW sample concentration.

Dilution buffers:

- N1: 0.1 M Tris-HCl, pH 7.5
- N2: 0.1 M TBS
- N3: 0.1 M TBS + 0.1% Tween
- N4: 0.1 M TBS + 0.5% SDS
- N5: 0.1 M TBS + 0.5% CTAB

Standard:

Spray dried egg white extracted with TBS was used to produce the dilution series with the dilution buffers. The maximal concentration of SEW was 20000 ng/mL.

10.3.2 *Results*

Table 25. Absorption results of SEW in various buffers to determine blocking efficiency. No antigen was used for coating, thus the difference between the maximum and minimum of absorption corresponds to background signals.

blocking agent in carbonate buffer pH 9.6	sample dilution buffer	washed without Tween Δabs	washed with Tween Δabs
1 % Ficoll	N1	1,615	0,467
	N2	1,622	0,235
	N3	0,166	0,043
	N4	0,282	0,059
	N5	0,276	0,055
1 % Dextran	N1	1,300	0,554
	N2	1,254	0,397
	N3	0,101	0,064
	N4	0,043	0,028
	N5	0,051	0,029
1 % BSA	N1	1,593	0,648
	N2	1,709	0,496
	N3	0,373	0,180
	N4	0,070	0,085
	N5	0,190	0,129
1 % fish gelatine	N1	1,696	1,036
	N2	1,686	0,610
	N3	0,406	0,334
	N4	0,002	0,060
	N5	0,164	0,115
1 % Polyvinylalcohol	N1	2,510	0,597
	N2	2,528	0,580
	N3	0,040	0,082
	N4	0,208	0,115
	N5	0,373	0,506

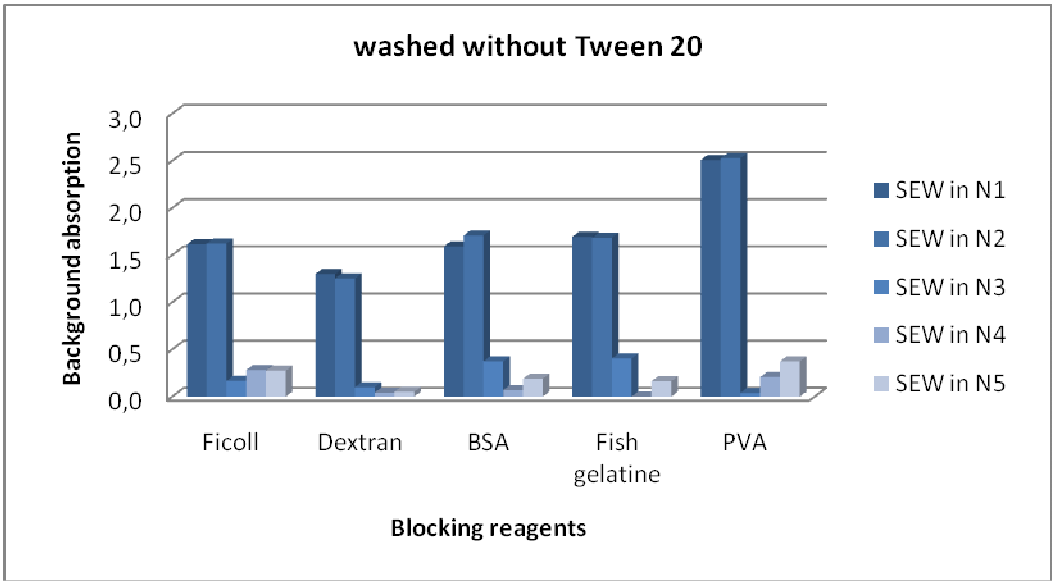


Figure 55. Background signals of the buffers N1-N5 caused by reduction of blocking efficiency after washing without Tween

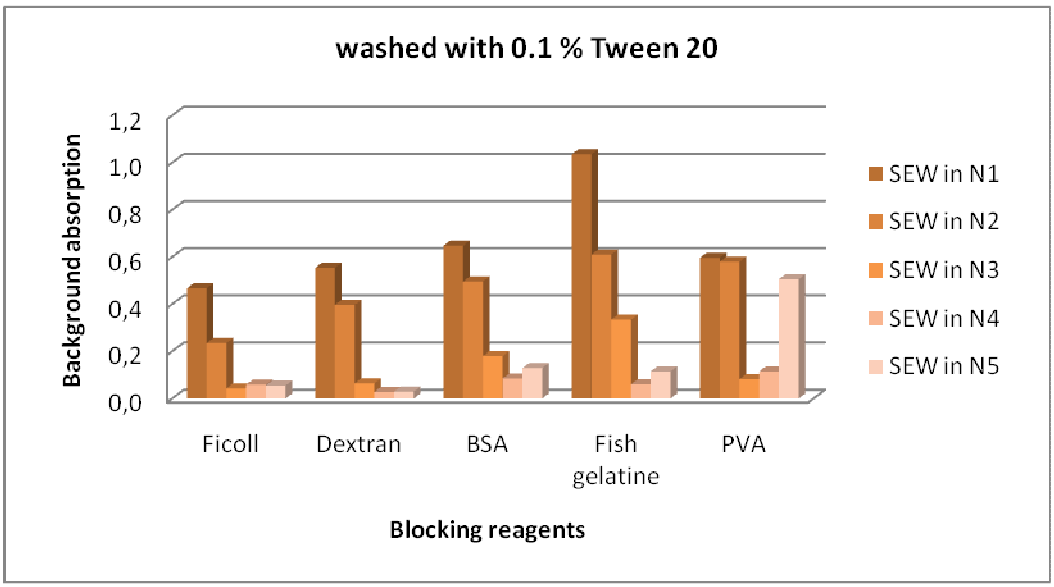


Figure 56. Background signals of the buffers N1-N5 caused by reduction of blocking efficiency after washing with Tween

10.3.3 *Discussion*

The efficiency of the washing step after the blocking process was influenced by the use of Tween 20. All background signal values decreased with the applied 0.1% Tween in the washing buffer independent of the dilution buffer and the blocking agent (see table 25). As already described from Huber et al., 2009 [21], the carbohydrates Ficoll and Dextran showed less background signal than the others. Furthermore, the positive effect of detergents was also evident for each blocking reagent, whereas the dilution buffers Tris-HCl and TBS caused the strongest background (figures 55 and 56). Therefore, 0.1% Tween 20 was added to the extraction buffers P2 (0.1 M TBS) and P7 (0.1 M TBS + 20% urea) in the final extraction approach to inhibit such background signals (see chapter 11).

Finally, it has to be noticed that the common PBS extracts have a negative effect on the blocking efficiency in ELISA if they are undiluted. Moreover, the signal curve would be falsified if the dilution series of a PBS extract are produced with a dilution buffer containing Tween, because each dilution would offer another final concentration of Tween 20.

11. FINAL EXTRACTION APPROACH

11.1 Background

The protein coagulum of a heat-processed food product may be considered as a random three-dimensional network of intertwined polypeptide chains held together by hydrogen and hydrophobic bonds. Protein can be adequately extracted from this mass only by using reagents capable of abolishing these interactions, such as detergents and concentrated solutions of urea [23].
Detergent molecules are characterized by the presence of segregated hydrophobic and hydrophilic moieties, a property that allows them to self-associate into micellar structures above a certain concentration termed the critical micellar concentration (CMC) [24].

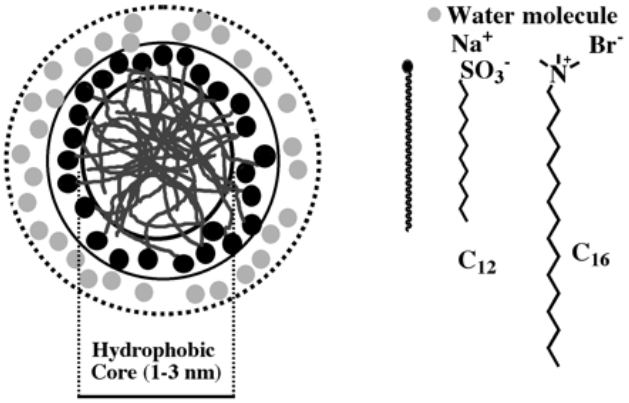


Figure 57. Schematic representation of a micelle. The black circle represents the ionic portion of the head group (e.g.,SO_3), whereas the long tail depicts the hydrophobic alkyl chain. SDS (C_{12}) and cetyltrimethylammonium bromide/CTAB (C_{16}) are typical anionic and cationic micelle-forming surfactants, respectively (Turro N J PNAS 2002;99:4805-4809).

It is generally accepted that with ionic detergents micelle formation is ascribed to a balance between hydrocarbon-chain attractions and electrostatic repulsions, whereas with nonionic detergents the hydrocarbon-chain attractions are opposed by the hydration and space requirement of the tail chains. Consequently, it is expected that this balance between cohesive and opposing electrostatic or hydration forces, respectively, varies in mixed ionic-nonionic micelles as a function of the mixing ratio.

11.2 Trial setup

For the last approach 20 different extraction buffers were tested and compared for their protein yield and for the IgG detection capability of the extracted egg white proteins in indirect competitive ELISA.
The list of determined buffers included usual PBS and TBS buffer, two commercial buffers for egg protein extraction (R-Biopharm and Romer), buffers containing an anionic, cationic or nonionic detergent alone or in combination with each other and buffers with urea combined with various detergents and fish gelatine (supplement used by Tepnel).

The anioinic detergent SDS is a common denaturating agent used for efficient protein extraction, but as already described in chapter 8. SDS influences the antigen-antibody binding reaction in a negative way. Therefore, SDS was emploid at concentrations above the CMC (10 mM ~ 0.3%) and below CMC in combination with the nonionic detergent Brij 35, used in concentrations above CMC (0.1 mM ~ 0.01%). It can be assumed, that below the CMC the surface-active anions of SDS are absorbed by the nonionic micelles, which causes increased suppression of the ionic head group of SDS by the incorporation of the nonionic head groups into the micellar interface. In this case the inhibiting effect of SDS may be alleviated in the immunological detection of a protein. The same idea was implemented for the cationic detergent CTAB, which is a declared detergent for the extraction of DNA and membrane proteins exhibiting a lipid anchor. The usage of CTAB in the approach for the extraction of egg white proteins is based on the fact that the main egg white allergens ovomucoid and ovalbumin offer isoelectric points at 4.1 and 4.5., which implicates acidic properties in neutral environment (extraction buffer with pH 7.5). An acid releases protons and thus possesses in total a negative charge. Finally it could be expected that the deprotonated forms of OVM and OVA show higher affinity for a cationic detergent. To confirm this theory, the extraction buffers including CTAB were also prepared with a pH of 5.5 instead of 7.5., expecting a decrease of total protein content in the extracts, but same amount of OVM and OVA.

Furthermore, detergents were used to alter the nature of the coated antigens, which influenced the competitive reaction between the coated protein and the target analyte in the sample extracts. The antibodies were produced with native antigens (except heated OVM) and the same antigens should be used for the coating step to analyse even high processed samples. Of course, the antibodies also detect denatured proteins and thus, the chance to recognize a protein of a processed sample increases in front of a coated denatured antigen instead of a native one. Another concept would be the same treatment (with a detergent) of the sample and the coating antigen to ensure the same conformation of the target protein. To confirm this theory the extracts of buffers including SDS should show optimal signals in ELISA with SDS-treated antigen used for coating.
However, to avoid such problems, the antigens will be treated with the corresponding detergent used for extraction (SDS, CTAB) before immunization takes place. The concentration of the detergent has to be determined to achieve complete 3-dimensional change in protein structure without harming the animal.

11.3 Experimental

Extraction procedure:

50 mg of SEW were dissolved in 2 mL buffer and 2 g of cooked egg white were extracted with 5 mL buffer for 15 min at 60 °C, vortexing every 5 min. Afterwards the extracts were centrifuged at 9500 rpm, for 15 min at RT. The analyses of the samples had to be performed 1-2 days after extraction, because storage at 4 °C or -20 °C caused irreversible protein precipitation in some extracts, especially if the extraction buffer included CTAB.

Extraction buffers:

- P1: 0.2 M PBS + 0.1% Tween, pH 7.5
- P2: 0.1 M TBS + 0.1% Tween, pH 7.5
- P3: 0.1 M TBS + 1% SDS/1 mM DTT
- P4: 0.1 M TBS + 2% SDS/1 mM DTT
- P5: 0.1 M TBS + 0.5% CTAB/1 mM DTT
- P6: 0.1 M TBS + 1% CTAB/1 mM DTT
- P7: 0.1 M TBS + 20% urea/1 mM DTT + 0.1% Tween
- P8: 0.1 M TBS + 20% urea/1 mM DTT + 2% fish gelatine
- P9: 0.1 M TBS + 20% urea/1 mM DTT + 1% Triton X-100
- P10: 0.1 M TBS + 20% urea/1 mM DTT + 0.5% SDS
- P11: 0.1 M TBS + 20% urea/1 mM DTT + 0.5% CTAB
- P12: 0.1 M TBS + 1% Brij 35/1 mM DTT
- P13: 0.1 M TBS + 1% Brij 35/1 mM DTT + 0.29% SDS
- P14: 0.1 M TBS + 1% Brij 35/1 mM DTT + 0.18% CTAB
- P15: 0.1 M TBS + 1% CTAB/1 mM DTT, pH 5.5
- P16: 0.1 M TBS + 1% Brij 35/1 mM DTT + 0.18% CTAB, pH 5.5
- P17: 0.1 M TBS + 10% urea/1 mM DTT + 0.5% SDS
- P18: 0.075 M Pot. acetat, 0.3 M NaCl, 0.01 M EDTA + 0.25% Triton X-100, pH 7.4
- P19: Romer AgraQuant for egg white
- P20: R-Biopharm RidaScreen for egg

ELISA performance:

For signal comparison the same volume of the extracts was used to start the dilution series, 1:770 (min. 20000 ng/mL) for the SEW extracts and 1:100 for the cooked egg white extracts (min. 50000 ng/mL). Therefore, the concentration applied as x-axis corresponded to the diluted extract concentration instead to the absolute protein content. The values for Δabs and IC_{50} were also not absolute and were only used to compare the extraction efficiency of the various buffers.

11.4 Results

BCA protein determination:

Table 26. Protein yield of samples extracted with the buffers P1-P20.

(n.d. …not determined, because the protein additive fish gelatine falsified the BCA result)

Extraction buffer	protein yield [mg/mL]		
	SEW	cooked EW	dried noodles
P1	17.31	3.79	3.11
P2	18.67	4.44	4.42
P3	15.55	4.82	8.06
P4	18.74	4.79	9.16
P5	6.53	3.83	3.58
P6	8.92	4.36	5.60
P7	10.74	3.87	4.61
P8	8.66	n.d.	5.77
P9	16.65	5.22	7.07
P10	19.41	4.64	7.90
P11	5.48	3.79	18.22
P12	21.59	3.97	4.69
P13	23.87	3.50	7.13
P14	17.63	3.33	5.88
P15	9.98	3.12	5.89
P16	19.86	3.38	5.08
P17	18.89	3.56	11.79
P18	19.66	3.46	5.89
P19	20.21	2.59	6.67
P20	22.86	3.93	5.62

For the extraction of spray dried egg white powder as native sample the commercial buffers (P19, P20) and the buffers including 1% Brij 35 alone and with 0.29% SDS (P12, P13) offered the best results. Worst extraction yield was obtained with buffers containing the cationic detergent CTAB alone (P5, P6), but the protein content increased when CTAB was present in addition with the nonionic detergent Brij 35 (P14). Moreover, the protein content could be accelerated by lowering the pH of the CTAB-buffers (P15, P16). The cooked egg white extracts of the buffers containing urea + Triton X-100 (P9) and SDS alone (P3, P4) showed high protein yield. The buffer purchased from Romer (P19) and the buffers including Brij 35 and a second detergent (P12-P14) exhibited decreased extraction capability. The dried noodles, representing a food sample with complex matrix, favoured another extraction buffer than cooked egg white. Best results were achieved with the buffer containing urea and CTAB (P11), whereas the other extraction buffers including urea (P7-P10) or CTAB (P5, P6) showed decreased extraction cabability. The usual buffers PBS and TBS were not suitable for the extraction of the noodles (see table 26).

SDS-PAGE:

M = marker	7 = noodles in P7	14 = noodles in P15
1 = noodles in P1	8 = noodles in P9	15 = noodles in P16
2 = noodles in P2	9 = noodles in P10	16 = noodles in P17
3 = noodles in P3	10 = noodles in P11	17 = noodles in P18
4 = noodles in P4	11 = noodles in P12	18 = noodles in P19
5 = noodles in P5	12 = noodles in P13	19 = noodles in P20
6 = noodles in P6	13 = noodles in P14	20 = noodles in P8

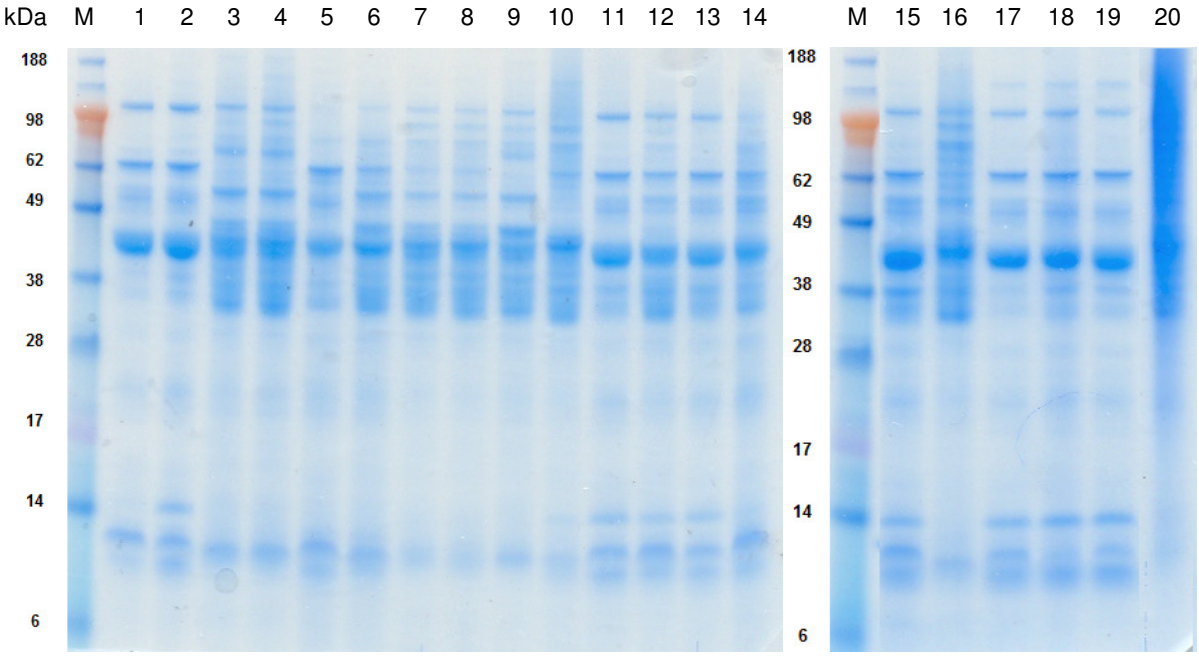


Figure 58. SDS-PAGE of dried noodle samples extracted with the final buffers P1-P20

The buffers containing denaturating agents like SDS (P3, P4) or urea (P7-P11) caused a more diffuse band pattern on SDS-PAGE (see figure 58) and offered also more proteins bands (lanes 3, 4, 7-10). The detergents Brij 35 and CTAB do not possess such denaturing character, which could be visualized in the SDS gel (lanes 5, 6, 11-15). Furthermore, the commercial extraction buffers showed the same band pattern like the usual PBS and TBS buffer, suggesting that no denaturing agent is included. The protein composition of the buffer P8 extract could not be determined, because of the high protein content caused by the added fish gelatine.

Indirect competitive ELISA:

Table 27. ELISA results of SEW samples extracted with the buffers P1-P20 and analysed with rabbit anti-SEW antibody. The coating was performed with 500 ng/mL SEW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB, additionally with OVA and OVM (250 ng/mL each) extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in the following graph.

sample	coating	extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
SEW1	SEW in PBS	P1	1.630	0.231	1.399	999
SEW2		P2	1.125	0.334	0.791	3311
SEW3		P3	0.561	0.111	0.450	321
SEW5		P5	1.317	0.211	1.107	668
SEW6		P6	1.232	0.221	1.012	574
SEW7		P7	0.952	0.550	0.402	89407
SEW8		P8	1.067	0.217	0.850	2632
SEW9		P9	1.285	0.182	1.104	988
SEW10		P10	0.237	0.073	0.164	2629
SEW11		P11	0.746	0.244	0.503	3219
SEW12		P12	1.525	0.219	1.306	412
SEW13		P13	1.844	0.128	1.716	302
SEW14		P14	1.314	0.122	1.192	511
SEW15		P15	1.523	0.215	1.308	672
SEW16		P16	1.301	0.106	1.195	406
SEW17		P17	0.423	0.074	0.349	82
SEW18		P18	1.769	0.284	1.485	563
SEW19		P19	1.453	0.200	1.254	380
SEW20		P20	1.253	0.132	1.121	487
SEW1	SEW in SDS	P1	1.567	0.217	1.350	980
SEW2		P2	1.748	0.481	1.267	8625
SEW3		P3	0.633	0.094	0.539	346
SEW5		P5	1.865	0.282	1.583	853
SEW6		P6	1.722	0.288	1.434	585
SEW7		P7	0.995	0.515	0.481	9540
SEW8		P8	0.998	0.227	0.772	1777
SEW9		P9	1.378	0.166	1.213	1168
SEW10		P10	0.303	0.065	0.238	800
SEW11		P11	0.841	0.200	0.642	2060
SEW12		P12	1.360	0.187	1.174	488
SEW13		P13	1.831	0.124	1.708	320
SEW14		P14	1.367	0.121	1.246	585
SEW15		P15	1.500	0.225	1.276	861
SEW16		P16	1.270	0.112	1.158	475
SEW17		P17	0.434	0.085	0.349	52
SEW18		P18	1.669	0.350	1.319	793
SEW19		P19	1.442	0.194	1.248	469
SEW20		P20	1.406	0.162	1.244	345

Table 27 continued. ELISA results of SEW samples extracted with the buffers P1-P20 and analysed with rabbit anti-SEW antibody. The coating was performed with 500 ng/mL SEW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB, additionally with OVA and OVM (250 ng/mL each) extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in the following graph.

sample	coating	extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
SEW1	SEW in CTAB	P1	1.422	0.118	1.304	568
SEW2		P2	1.635	0.451	1.184	3828
SEW3		P3	0.558	0.152	0.406	643
SEW5		P5	1.492	0.219	1.274	732
SEW6		P6	1.459	0.171	1.288	443
SEW7		P7	1.104	0.796	0.308	2748
SEW8		P8	1.134	0.226	0.909	1494
SEW9		P9	1.091	0.180	0.912	1010
SEW10		P10	0.254	0.135	0.119	690
SEW11		P11	0.991	0.247	0.744	1967
SEW12		P12	1.268	0.121	1.147	312
SEW13		P13	1.441	0.122	1.320	325
SEW14		P14	1.292	0.099	1.193	410
SEW15		P15	1.520	0.135	1.385	653
SEW16		P16	1.186	0.079	1.107	366
SEW17		P17	0.386	0.107	0.279	89
SEW18		P18	1.648	0.146	1.502	320
SEW19		P19	1.560	0.128	1.432	367
SEW20		P20	1.377	0.111	1.266	279
SEW1	Ova/Ovm in PBS	P1	1.398	0.180	1.218	1446
SEW2		P2	1.419	0.371	1.048	8271
SEW3		P3	1.041	0.462	0.579	1046
SEW5		P5	1.460	0.437	1.023	1620
SEW6		P6	1.503	0.345	1.158	767
SEW7		P7	1.413	0.642	0.772	25698
SEW8		P8	1.371	0.415	0.956	3777
SEW9		P9	1.386	0.386	1.000	2325
SEW10		P10	0.502	0.201	0.301	2333
SEW11		P11	1.338	0.527	0.811	3900
SEW12		P12	1.632	0.248	1.384	882
SEW13		P13	1.376	0.239	1.137	883
SEW14		P14	1.283	0.179	1.104	1231
SEW15		P15	1.447	0.234	1.213	1835
SEW16		P16	1.514	0.129	1.385	547
SEW17		P17	0.517	0.153	0.365	173
SEW18		P18	1.576	0.192	1.385	829
SEW19		P19	1.335	0.157	1.179	768
SEW20		P20	1.316	0.117	1.199	660

Table 27 continued. ELISA results of SEW samples extracted with the buffers P1-P20 and analysed with rabbit anti-SEW antibody. The coating was performed with 500 ng/mL SEW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB, additionally with OVA and OVM (250 ng/mL each) extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in the following graph.

sample	coating	extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
SEW1	Ova/Ovm in SDS	P1	1.383	0.204	1.179	1520
SEW2		P2	1.529	0.312	1.217	3365
SEW3		P3	1.220	0.494	0.727	909
SEW5		P5	1.317	0.407	0.910	1373
SEW6		P6	1.282	0.330	0.952	1010
SEW7		P7	1.551	0.658	0.893	11233
SEW8		P8	1.499	0.450	1.049	4285
SEW9		P9	1.517	0.397	1.121	2064
SEW10		P10	0.611	0.207	0.405	1242
SEW11		P11	1.304	0.556	0.749	4818
SEW12		P12	1.662	0.282	1.380	966
SEW13		P13	1.165	0.214	0.951	1227
SEW14		P14	1.083	0.165	0.918	1408
SEW15		P15	1.169	0.219	0.951	2104
SEW16		P16	1.019	0.124	0.895	1173
SEW17		P17	0.452	0.148	0.305	187
SEW18		P18	1.339	0.189	1.151	1046
SEW19		P19	1.541	0.177	1.364	1108
SEW20		P20	1.599	0.138	1.461	814
SEW1	Ova/Ovm in CTAB	P1	1.595	0.333	1.262	4001
SEW2		P2	1.644	0.438	1.206	8776
SEW3		P3	1.606	0.799	0.808	1224
SEW5		P5	1.500	0.611	0.889	2962
SEW6		P6	1.579	0.508	1.071	1463
SEW7		P7	1.758	0.746	1.012	13905
SEW8		P8	1.590	0.595	0.995	8133
SEW9		P9	1.705	0.522	1.183	2494
SEW10		P10	0.935	0.344	0.591	1237
SEW11		P11	1.459	0.690	0.769	14368
SEW12		P12	1.728	0.355	1.373	1794
SEW13		P13	1.377	0.276	1.102	1518
SEW14		P14	1.264	0.227	1.037	1840
SEW15		P15	1.315	0.305	1.010	3235
SEW16		P16	1.181	0.168	1.013	1762
SEW17		P17	0.582	0.193	0.389	511
SEW18		P18	1.432	0.247	1.186	1458
SEW19		P19	1.555	0.356	1.199	2587
SEW20		P20	1.685	0.217	1.468	1312

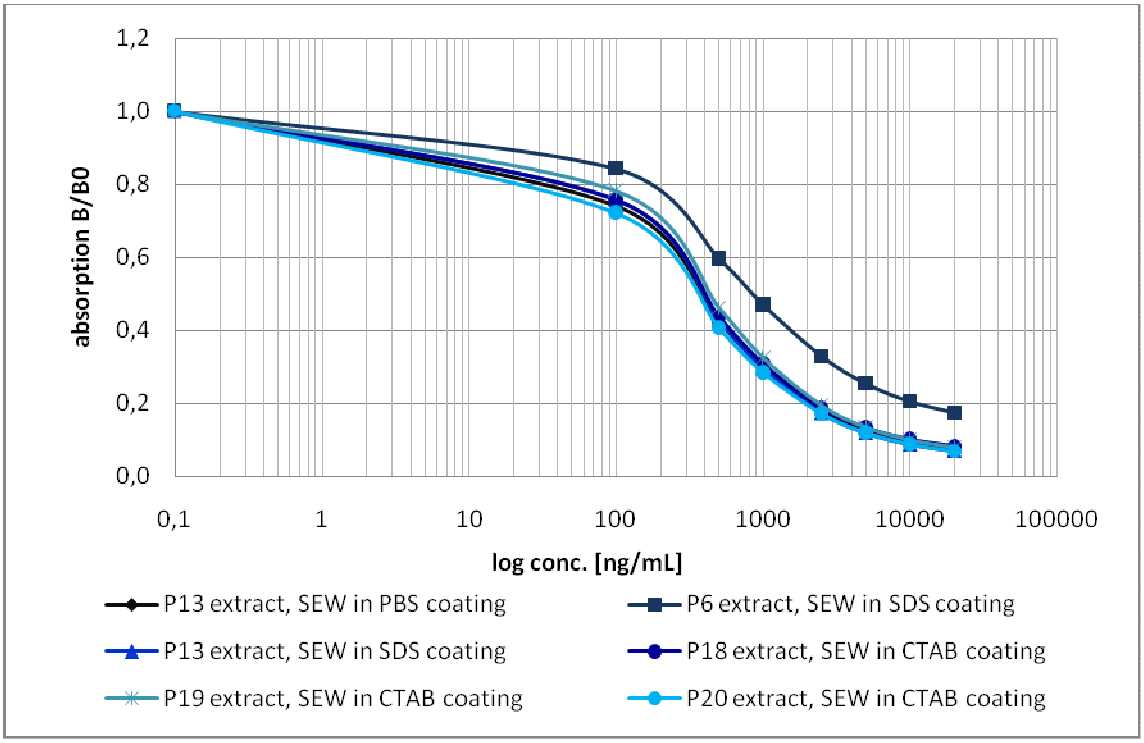


Figure 59. Best ELISA signal curves of SEW extracts analysed with rabbit anti-SEW antibody.

In general, the SEW extracts showed better results, if the coating had been performed with SEW and not with Ova/Ovm, which corresponded to the used immunogen for antibody production (see table 27). The ELISA signal values agreed well with BCA results except the P6 extract, which offered low protein yield in BCA determination (table 26), but caused a sufficient signal curve. Moreover, the extracts prepared with buffers including urea exhibited worst signals in ELISA despite their moderate protein amount according BCA quantification. However, the signals of the extracts from urea-buffers P7-P11 increased with coating of MTPs with SDS- and CTAB treated SEW, which approves the idea of a denatured coating protein in processed food stuff analysis. Surprisingly, the commercial buffers and P18 achieved high extraction yield and optimal ELISA results (see figure 59), if the coating step was performed with CTAB-treated SEW. Finally, it should be noticed that the best result was obtained with buffer P13 containing the detergents Brij 35 and SDS in the standardized ELISA version, in which the same protein is used for immunization and coating (SEW in PBS).

Table 28. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-SEW antibody. The coating was performed with 500 ng/mL SEW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB, additionally with OVA and OVM (250 ng/mL each) extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in the following graph.

sample	Coating	Extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
cookEW1	SEW in PBS	P1	1.332	0.562	0.770	1387
cookEW2		P2	1.280	0.494	0.787	1601
cookEW3		P3	0.702	0.120	0.582	171
cookEW5		P5	1.235	0.190	1.045	371
cookEW6		P6	1.241	0.161	1.081	155
cookEW7		P7	0.970	0.569	0.401	2742
cookEW8		P8	1.125	0.552	0.573	4786
cookEW9		P9	1.043	0.509	0.534	2588
cookEW10		P10	0.237	0.104	0.134	210
cookEW11		P11	0.615	0.169	0.446	473
cookEW12		P12	1.134	0.355	0.779	4198
cookEW13		P13	1.619	0.483	1.136	1476
cookEW14		P14	1.118	0.434	0.684	499
cookEW15		P15	1.413	0.251	1.163	379
cookEW16		P16	1.252	0.407	0.845	567
cookEW17		P17	0.355	0.114	0.241	2119
cookEW18		P18	2.230	0.988	1.242	1237
cookEW19		P19	1.571	0.571	1.000	3364
cookEW20		P20	1.349	0.422	0.927	1568
cookEW1	SEW in SDS	P1	1.251	0.509	0.743	426
cookEW2		P2	1.475	0.587	0.888	904
cookEW3		P3	0.832	0.124	0.708	197
cookEW5		P5	1.234	0.180	1.054	308
cookEW6		P6	1.139	0.125	1.015	198
cookEW7		P7	1.374	0.810	0.564	3422
cookEW8		P8	1.510	0.724	0.786	4602
cookEW9		P9	1.429	0.728	0.701	2952
cookEW10		P10	0.294	0.092	0.202	183
cookEW11		P11	0.809	0.166	0.643	746
cookEW12		P12	0.950	0.397	0.553	7703
cookEW13		P13	1.749	0.507	1.242	1280
cookEW14		P14	1.184	0.379	0.805	436
cookEW15		P15	1.557	0.235	1.323	464
cookEW16		P16	1.364	0.365	0.999	297
cookEW17		P17	0.568	0.115	0.453	778
cookEW18		P18	1.478	0.624	0.854	1179
cookEW19		P19	1.687	0.614	1.074	1258
cookEW20		P20	1.492	0.451	1.041	1320

Table 28 continued. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-SEW antibody. The coating was performed with 500 ng/mL SEW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB, additionally with OVA and OVM (250 ng/mL each) extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in the following graph.

sample	Coating	Extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
cookEW1	SEW in CTAB	P1	1.364	0.176	1.189	88
cookEW2		P2	1.530	0.210	1.320	93
cookEW3		P3	0.935	0.106	0.829	110
cookEW5		P5	1.404	0.098	1.306	123
cookEW6		P6	1.354	0.087	1.267	106
cookEW7		P7	1.095	0.175	0.920	225
cookEW8		P8	1.177	0.191	0.987	293
cookEW9		P9	1.142	0.176	0.967	237
cookEW10		P10	0.446	0.131	0.315	432
cookEW11		P11	1.244	0.136	1.108	298
cookEW12		P12	1.380	0.163	1.217	175
cookEW13		P13	1.413	0.127	1.287	206
cookEW14		P14	1.721	0.155	1.567	147
cookEW15		P15	1.421	0.116	1.306	254
cookEW16		P16	1.299	0.103	1.196	238
cookEW17		P17	0.649	0.094	0.555	312
cookEW18		P18	1.612	0.219	1.393	188
cookEW19		P19	1.426	0.196	1.230	352
cookEW20		P20	1.389	0.137	1.252	103
cookEW1	Ova/Ovm in PBS	P1	1.591	0.308	1.283	325
cookEW2		P2	1.715	0.351	1.364	435
cookEW3		P3	1.258	0.113	1.146	229
cookEW5		P5	1.534	0.101	1.433	317
cookEW6		P6	1.469	0.108	1.361	289
cookEW7		P7	1.140	0.225	0.915	402
cookEW8		P8	1.191	0.204	0.988	492
cookEW9		P9	1.178	0.201	0.977	555
cookEW10		P10	0.757	0.128	0.630	673
cookEW11		P11	1.028	0.100	0.928	625
cookEW12		P12	1.368	0.183	1.185	388
cookEW13		P13	1.696	0.146	1.550	521
cookEW14		P14	1.633	0.115	1.518	358
cookEW15		P15	1.791	0.132	1.659	779
cookEW16		P16	1.654	0.132	1.522	612
cookEW17		P17	1.056	0.098	0.959	573
cookEW18		P18	1.785	0.232	1.553	475
cookEW19		P19	1.636	0.254	1.382	640
cookEW20		P20	1.523	0.156	1.367	452

Table 28 continued. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-SEW antibody. The coating was performed with 500 ng/mL SEW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB, additionally with OVA and OVM (250 ng/mL each) extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in the following graph.

sample	Coating	Extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
cookEW1	Ova/Ovm in SDS	P1	1.670	0.447	1.223	475
cookEW2		P2	1.802	0.534	1.268	609
cookEW3		P3	1.403	0.135	1.268	265
cookEW5		P5	1.530	0.138	1.392	422
cookEW6		P6	1.467	0.104	1.363	278
cookEW7		P7	1.213	0.413	0.801	519
cookEW8		P8	1.239	0.350	0.889	750
cookEW9		P9	1.178	0.365	0.814	821
cookEW10		P10	0.929	0.111	0.819	908
cookEW11		P11	1.201	0.134	1.067	847
cookEW12		P12	1.263	0.284	0.980	592
cookEW13		P13	1.852	0.304	1.549	653
cookEW14		P14	1.633	0.247	1.386	341
cookEW15		P15	1.854	0.206	1.648	797
cookEW16		P16	1.670	0.246	1.424	526
cookEW17		P17	1.214	0.130	1.084	750
cookEW18		P18	2.045	0.645	1.401	830
cookEW19		P19	1.576	0.416	1.161	852
cookEW20		P20	1.409	0.300	1.110	680
cookEW1	Ova/Ovm in CTAB	P1	1.977	0.547	1.430	618
cookEW2		P2	2.072	0.645	1.428	577
cookEW3		P3	1.503	0.108	1.395	295
cookEW5		P5	1.876	0.175	1.701	496
cookEW6		P6	1.807	0.131	1.676	445
cookEW7		P7	1.758	0.746	1.012	497
cookEW8		P8	1.590	0.595	0.995	797
cookEW9		P9	1.705	0.522	1.183	815
cookEW10		P10	0.935	0.344	0.591	788
cookEW11		P11	1.459	0.690	0.769	1547
cookEW12		P12	1.728	0.355	1.373	713
cookEW13		P13	1.945	0.364	1.581	911
cookEW14		P14	1.766	0.251	1.515	477
cookEW15		P15	1.980	0.228	1.752	1077
cookEW16		P16	1.821	0.269	1.552	657
cookEW17		P17	1.329	0.133	1.197	1002
cookEW18		P18	2.077	0.755	1.323	1039
cookEW19		P19	1.625	0.486	1.139	1175
cookEW20		P20	1.541	0.362	1.180	947

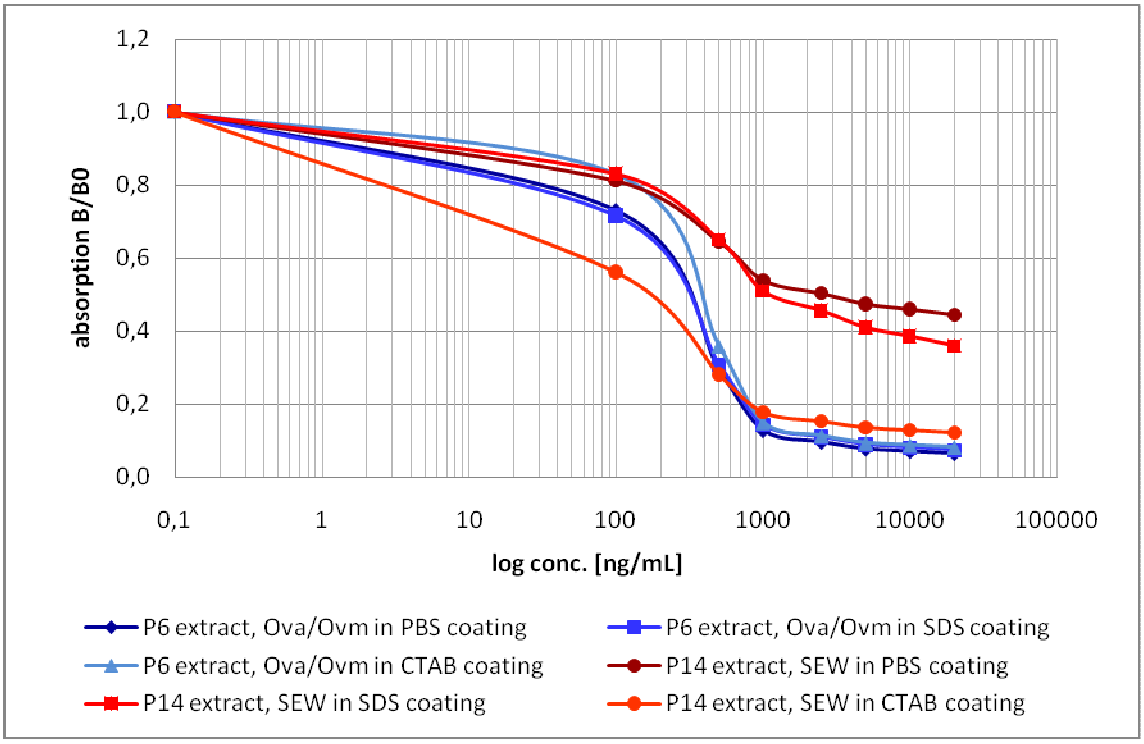


Figure 60. ELISA signal curves of cooked EW extracts analysed with rabbit anti-SEW antibody. The results showed the great influence of the detergent-treated antigen used for coating.

The best result was obtained with the cooked EW extract of buffer P14 including Brij35 and CTAB, but only if the coating step had been performed with CTAB-treated SEW (see figure 28). In comparison, the different coating approaches showed great influence in the ELISA signal curves (see figure 60). Unfortunately, the extract of buffer P6 including CTAB offered worst results in the CTAB-coating approach. Overall, the approach using Ova/Ovm instead of SEW for coating showed better results and the buffers containing the cationic detergent CTAB seemt to present the antigen to the anti-SEW antibody in the correct conformation for recognition and binding. Again the buffers with urea inhibited the signal in ELISA and the commercial buffers caused decreased protein yield according to BCA determination, which matched to the ELISA signals.

Table 29. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-native OVM antibody. The coating was performed with 500 ng/mL of native OVM extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB (only OVM in PBS coating showed). The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in following graph.

sample	coating	extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
cookEW1	nativ OVM in PBS	P1	2.425	0.128	2.297	804
cookEW2		P2	2.453	0.112	2.341	561
cookEW3		P3	0.788	0.353	0.435	2345
cookEW4		P4	0.593	0.342	0.251	1048
cookEW5		P5	2.386	0.139	2.247	932
cookEW6		P6	2.375	0.152	2.223	836
cookEW7		P7	1.686	0.148	1.538	2363
cookEW8		P8	1.740	0.112	1.628	952
cookEW9		P9	1.645	0.164	1.481	2201
cookEW10		P10	0.732	0.066	0.666	1059
cookEW11		P11	1.379	0.239	1.140	6987
cookEW12		P12	1.906	0.165	1.741	1036
cookEW13		P13	1.886	0.094	1.792	537
cookEW14		P14	1.744	0.085	1.659	452
cookEW15		P15	1.799	0.121	1.678	1014
cookEW16		P16	1.683	0.062	1.621	406
cookEW17		P17	0.796	0.077	0.692	687
cookEW18		P18	1.809	0.083	1.726	416
cookEW19		P19	1.620	0.093	1.527	552
cookEW20		P20	1.608	0.080	1.528	367

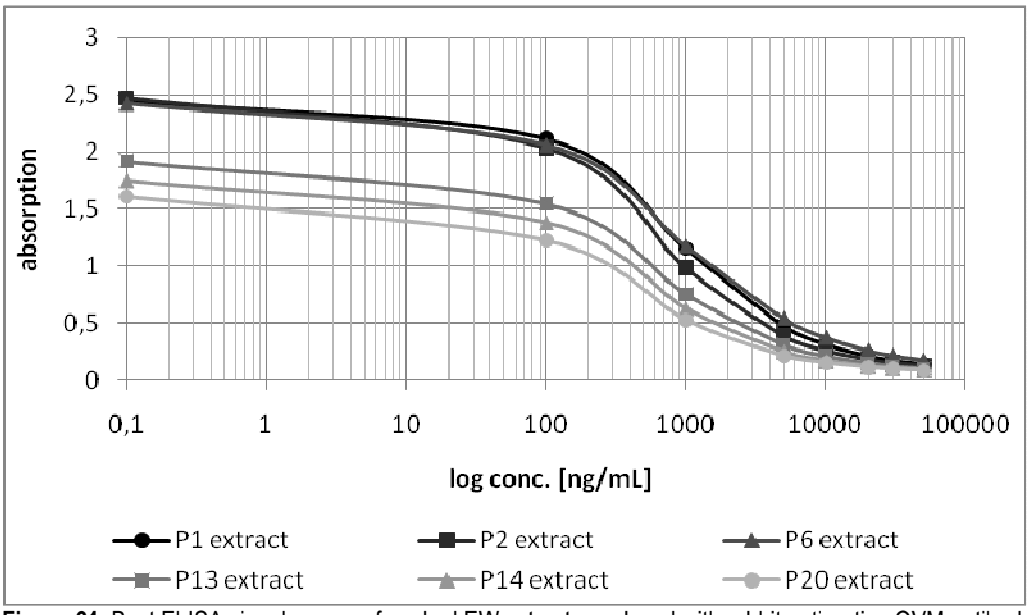


Figure 61. Best ELISA signal curves of cooked EW extracts analysed with rabbit anti-native OVM antibody.

The ELISA analysis of the cooked egg white extracts with the anti-native OVM antibody offered optimal signal curves with the usual buffers PBS and TBS without any supplements except 0.1% Tween 20 (see table 29). Therefore, the coating approaches with SDS-treated OVM impaired the ELISA results, because the affinity of the antibody for the antigen has been lost with its denaturation. However, CTAB might not effect the antigen-antibody binding in such a negative way like SDS and urea, which was approved by the extract of buffer P6 (see figure 61). Furthermore, the combination of less SDS with the nonionic detergent Brij 35 (P13 extract) diminished the negative influence of the anionic detergent.

Table 30. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-heated OVM antibody. The coating was performed with 500 ng/mL of heated OVM extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB (only OVM in PBS coating showed). The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in following graph.

sample	coating	extraction buffer	abs max.	abs min.	abs (max-min)	IC 50
cookEW1	heated OVM in PBS	P1	2.353	0.138	2.215	755
cookEW2		P2	2.387	0.115	2.272	522
cookEW3		P3	1.045	0.403	0.642	722
cookEW4		P4	0.769	0.363	0.406	841
cookEW5		P5	2.352	0.136	2.216	553
cookEW6		P6	2.345	0.155	2.190	682
cookEW7		P7	1.438	0.123	1.315	1580
cookEW8		P8	1.348	0.103	1.245	733
cookEW9		P9	1.325	0.140	1.185	1533
cookEW10		P10	0.750	0.079	0.671	579
cookEW11		P11	1.073	0.159	0.914	2951
cookEW12		P12	1.533	0.130	1.403	1017
cookEW13		P13	1.868	0.122	1.746	868
cookEW14		P14	1.821	0.106	1.715	575
cookEW15		P15	1.825	0.142	1.683	1088
cookEW16		P16	1.745	0.080	1.665	629
cookEW17		P17	1.196	0.110	1.086	582
cookEW18		P18	1.772	0.107	1.665	583
cookEW19		P19	1.519	0.078	1.441	370
cookEW20		P20	1.469	0.068	1.401	265

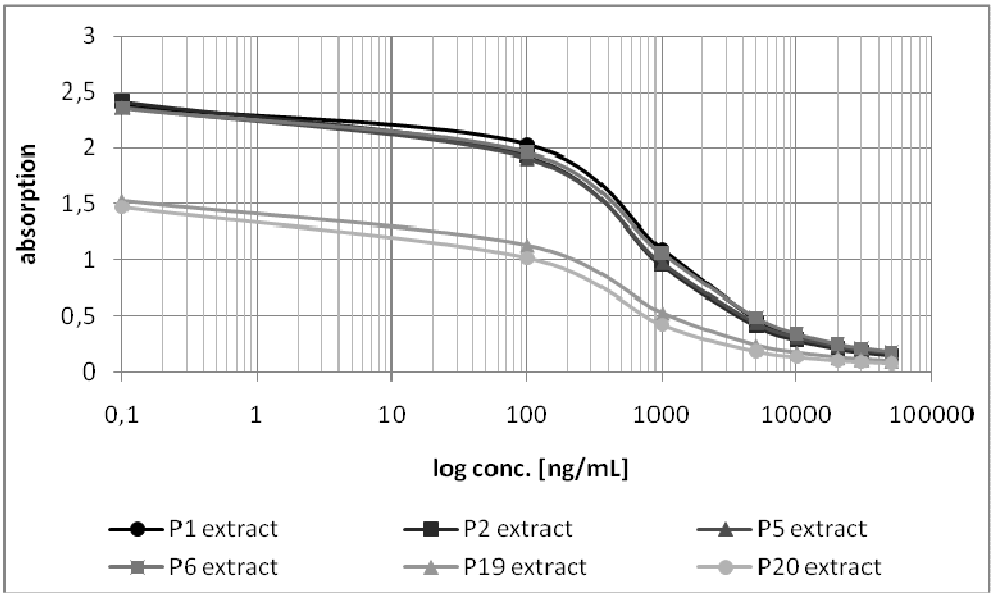


Figure 62. Best ELISA signal curves of cooked EW extracts analysed with rabbit anti-heated OVM antibody.

The antibody against heated OVM showed similar results like the antibody against native OVM and was not able to achieve higher signals in the analysis of a heat-processed sample.

Table 31. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-crude EW antibody. The coating was performed with 500 ng/mL of crude EW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in following graph.

sample	Coating	Extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
cookEW1	crude EW in PBS	P1	2.089	0.246	1.843	54
cookEW2		P2	1.752	0.343	1.409	71
cookEW3		P3	2.020	0.090	1.930	50
cookEW4		P4	1.692	0.081	1.611	43
cookEW5		P5	1.663	0.206	1.457	93
cookEW6		P6	1.976	0.217	1.759	91
cookEW7		P7	1.961	0.821	1.140	1122
cookEW8		P8	2.075	1.115	0.960	664
cookEW9		P9	1.829	0.773	1.056	3041
cookEW10		P10	1.030	0.090	0.940	342
cookEW11		P11	1.687	0.277	1.410	576
cookEW12		P12	2.081	1.393	0.688	1182
cookEW13		P13	1.465	0.156	1.309	59
cookEW14		P14	1.651	0.257	1.394	75
cookEW15		P15	1.530	0.239	1.291	262
cookEW16		P16	1.597	0.248	1.349	139
cookEW17		P17	0.445	0.065	0.380	101
cookEW18		P18	1.713	0.505	1.208	92
cookEW19		P19	1.937	0.475	1.462	83
cookEW20		P20	1.778	0.247	1.531	42
cookEW1	crude EW in SDS	P1	2.123	0.872	1.251	352
cookEW2		P2	2.136	1.059	1.077	376
cookEW3		P3	1.882	0.115	1.767	88
cookEW4		P4	1.538	0.113	1.425	103
cookEW5		P5	1.944	0.449	1.495	771
cookEW6		P6	2.005	0.345	1.660	438
cookEW7		P7	1.767	0.892	0.875	875
cookEW8		P8	1.879	1.043	0.836	812
cookEW9		P9	1.499	1.005	0.494	19057
cookEW10		P10	1.028	0.132	0.896	415
cookEW11		P11	2.341	0.434	1.907	811
cookEW12		P12	1.902	0.938	0.964	1383
cookEW13		P13	1.782	0.578	1.204	274
cookEW14		P14	1.751	0.466	1.285	383
cookEW15		P15	1.845	0.348	1.497	810
cookEW16		P16	1.733	0.493	1.240	400
cookEW17		P17	0.767	0.093	0.674	212
cookEW18		P18	2.004	0.962	1.042	1101
cookEW19		P19	1.728	0.716	1.012	389
cookEW20		P20	1.652	0.559	1.093	363

Table 31 continued. ELISA results of cooked egg white samples extracted with the buffers P1-P20 and analysed with rabbit anti-crude EW antibody. The coating was performed with 500 ng/mL of crude EW extracted with PBS, PBS + 1% SDS and PBS + 1% CTAB. The coating solutions were prepared with the usual ELISA coating buffer. The values marked in the table were also visualized in following graph.

sample	Coating	Extraction buffer	abs max.	abs min.	Δabs (max-min)	IC 50
cookEW1	crude EW in CTAB	P1	1.878	0.261	1.617	122
cookEW2		P2	1.940	0.384	1.556	300
cookEW3		P3	1.575	0.102	1.473	67
cookEW4		P4	1.267	0.107	1.160	67
cookEW5		P5	1.704	0.184	1.520	230
cookEW6		P6	1.753	0.162	1.591	205
cookEW7		P7	2.187	0.214	1.973	544
cookEW8		P8	2.441	0.286	2.155	1265
cookEW9		P9	1.273	0.321	0.952	7721
cookEW10		P10	0.903	0.074	0.829	207
cookEW11		P11	2.248	0.147	2.101	572
cookEW12		P12	2.543	0.198	2.345	505
cookEW13		P13	1.523	0.115	1.408	133
cookEW14		P14	2.013	0.100	1.913	253
cookEW15		P15	1.555	0.132	1.423	626
cookEW16		P16	1.902	0.077	1.825	392
cookEW17		P17	0.738	0.064	0.674	198
cookEW18		P18	1.670	0.184	1.486	252
cookEW19		P19	2.173	0.153	2.020	248
cookEW20		P20	2.130	0.123	2.007	119

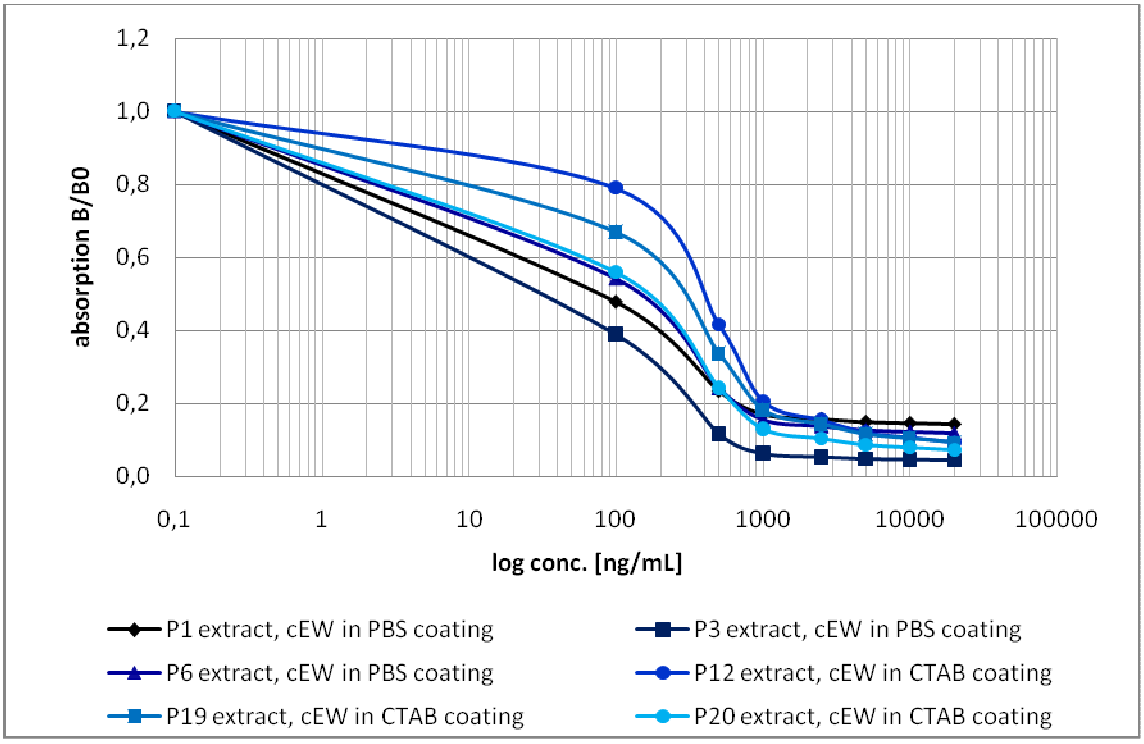


Figure 63. ELISA signal curves of cooked EW extracts analysed with rabbit anti-crude EW antibody.

The antibody against crude EW showed unexpected good results in detection of the heat-processed cooked egg white (table 31). Of course, the AS purified antibody was used for ELISA analysis and also less diluted (1:1000) than the others because of decreased IgG yield after purification. Additionally the antibody was able to handle the strong negative influence of 1% SDS without using SDS-treated cEW for coating. Furthermore, the commercial buffers, PBS and the buffer P12 including the non-ionic detergent Brij 35 offered sufficient signal curves but highly dependent on the coating procedure (worst with SDS-treated cEW).

11.5 Discussion

The last extraction approach revealed the complex interaction of extraction buffer, antibody and coated antigen and their influence on signal strength. However, the advantage of using the same detergent for extraction and for the coating step could not be demonstrated, but an enhancement of the signal was observed for some extraction buffers in combination with specific coating (commercial buffers P19 and P20 favoured CTAB-treated antigen for coating). The extraction of cooked egg white using buffers including CTAB with decreased pH (P15 and P16) achieved similar protein yield than the buffers with neutral pH (see table 26). The ELISA signal curves were dislocated in the upper absorbance range, but the IC_{50} values increased, thus no signal improvement was observed. The possible inhibiting effect of lower pH on antigen-antibody binding was compensated by neutralization of the extract before the ELISA was performed (data not shown), but the signals were similar. However, more analyses of complex food stuff would be necessary to determine the specificity of CTAB for the extraction of proteins exhibiting a pI below the pH of the sample buffer.

The same ELISA results obtained for the antibodies against native and heated OVM might be caused by incomplete denaturation of the immunogen. In literature, OVM is declared as very heat-stable and temperatures up to 190 °C may not alter its conformation because of stabilization by disulfide bonds. Therefore, the treatment with a reducing agent will be necessary to allow the complete loss of folding by heating the protein. Additionally, exposing the immunogen to SDS before antibody production starts may accelerate the detection capability of antigens extracted with buffers containing SDS.

12. CONCLUSION

The extraction of the analyte from a complex sample matrix is the first limiting factor in food stuff analyses. In most cases considerable loss in target extraction can not be compensated by a sensitive detection method or specific antibodies. Moreover, the extraction yield of a buffer and subsequent detection outcome depends on the character of the target analyte, the detection antibody and the immunogen used for IgG production. The problem of food stuff extraction increases with the processing level of the sample, which causes protein denaturation, aggregation and coagulum formation.

The anionic detergent SDS helps to handle these effects by its stringent denaturing nature. Of course, SDS only in low concentrations increases the extraction yield without influencing the antigen-antibody binding in subsequent analysis. In some cases the negative effect of SDS can be avoided by adding a nonionic detergent (≥ SDS content) to protect the antibody and to "neutralize" the SDS molecules. Urea is another denaturating agent and affects the immunological detection of antigens by antibodies, a fact which could not be prevented by detergents. However, treating the immunogen with SDS before IgG production may lead to an antibody, which recognizes the denatured antigen more effective than the native one, but the sensitivity against SDS is still present. Moreover, antibodies against denatured proteins may discriminate native antigens, thus a combination of two antibodies against a protein in different conformational states will be sufficient for food stuff analysis.
The main components and allergens of chicken´s egg white, ovalbumin and ovomucoid, have disulfide bonds and free –SH groups, which may cause aggregation with other food ingredients (e.g. wheat flour) during production. Therefore, the usage of a reducing agent is important for egg white protein extraction to remove the S-S bridges and to allow the complete contact of the protein with buffer components (SDS). It should be noticed, that also immunoglobulins possess disulfide bridges and reducing these bonds may alter the activity of the antibody and consequently the detection of antigens.

The antibodies against egg white proteins SEW, cEW, native and heated OVM showed nearly no cross-reactivity after ELISA analysis, but the results revealed that the cross-reactivity depends on the extraction buffer. Different buffers exhibit selective extraction capability for a specific antigen and different components may change the conformation of the antigen, which influences the recognition epitope of the corresponding antibody. Therefore, the same extraction buffer has to be used for extraction of potential cross-reacting substances and an existing cross-reactivity within one detection method (e.g. immunoblotting) may not involve the same results in another method (e.g. ELISA).

A signal enhancement in indirect competitive ELISA, analysing processed samples with antibodies against a native antigen, can be achieved by altering the conformation of the coated standard to some extent with detergents. However, direct correlation between the detergent used for extraction of the analyte and for treatment of the coating antigen to adapt both conformations of the target could not be demonstrated.

The extraction approaches showed that the optimal extraction buffer for the standard (e.g. SEW or cEW) differs from the most efficient buffer for complex food stuff extraction. For SEW extraction best results were obtained with buffers excluding denaturating agents like urea or SDS, but non-ionic detergents might increase protein yield and signals in ELISA using anti-SEW antibodies. In contrast, cooked egg white offered decreased extraction yield with buffers including the cationic detergent CTAB, but best results in ELISA using the same antibody as for SEW determination. Therefore, an extraction buffer containing CTAB only or in combination with a non-ionic detergent improves food stuff analysis with the rabbit anti-SEW antibody.
The antibodies against native- and heated OVM were not able to deal with detergents in the extraction buffer and offered only sufficient signal curves in ELISA with the usual PBS or TBS buffer. In comparison to the other antibodies produced by rabbits, the animals which had been immunized with OVM showed higher immune response and antibody production, but the antibodies could not detect processed antigens in such extent as expected for anti-heated OVM antibody.
Surprisingly, high sensitivity for antigens from highly processed samples (cooked egg white) was observed using the antibody against crude EW. Additionally, SDS exhibited less negative influence on antigen-antibody binding without using a non-ionic detergent for signal rescue. Moreover, buffers including no supplements like PBS or the commercial buffers showed sufficient signal curves in ELISA analysis.

Finally, more extractions of food samples will be sufficient to determine the optimal extraction buffer. The appropriate choice of a buffer depends on the detection method, the detection antibody, the immunogen used for antibody production and at least on the biochemical properties of the target analyte.
In some cases, only extraction approaches with processed samples may lead to the most efficient antibody production using the correct immunogen. Overall, each antibody and each antigen require optimisation of the extraction buffer and a general concept for protein extraction from food stuff does not exist because of the impact of various matrix components on the target analyte.

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14. APPENDIX

14.1 List of Tables

14.2 List of Figures

14.3 KURZZUSAMMENFASSUNG

Der Schwerpunkt der vorliegenden Diplomarbeit liegt in der Optimierung eines Puffers zur Extraktion von Hühnereiweißallergenen aus prozessierten Lebensmitteln. Zusätzlich wurde der Einfluss der Pufferkomponenten auf die Bindungsreaktion zwischen Antigen und Antikörper untersucht und ein Vergleich zwischen extrahiertem Gesamtprotein und Signalstärke im indirekten competitive ELISA erstellt. Dazu wurden vier verschiedene polyklonale Antikörper aus Hasen verwendet, welche mit Sprüheiweiß, rohem Hühnereiweiß, nativem Ovomucoid und erhitztem Ovomucoid immunisiert wurden.

In den ersten Ansätzen wurden Puffer aus verschiedenen Literaturquellen miteinander verglichen, welche allgemein zur Proteinextraktion verwendet worden waren. Die Zusammensetzung der Proteinextrakte wurde mittels SDS-PAGE und Western Blot bestimmt, und die Gesamtkonzentration an Protein wurde mit Hilfe eines BCA-Testkits gemessen. Danach wurde der notwendige Einsatz von denaturierenden Agenzien wie Urea oder SDS ersichtlich, welche den Proteingehalt der Extrakte um ein Vielfaches steigerten. Nach anschließender Messung der Extrakte im indirekt kompetitiven ELISA, wurde allerdings ein negativer Effekt der Agenzien festgestellt. Im Falle von Natriumdodecylsulfat konnte der inhibierende Einfluss des anionischen Detergens auf die Antigen-Antikörper Bindung durch die Zugabe von nonionische Detergentien (z.B. Brij 35) fast gänzlich aufgehoben werden. Allerdings eignete sich SDS nur als Pufferzusatz für die Proteinextraktion, wenn die Detektion im ELISA mit den Antikörpern gegen Sprüheiweiß und gegen rohes Hühnereiweiß erfolgte.
Weiters konnte festgestellt werden, dass Dithiothreitol als Reduktionsmittel die Extraktion von Ovalbumin und Ovomucoid aus denaturierten Lebensmitteln erleichtert, indem es Disulfidbrücken aufhebt und somit die Bildung von Proteinaggregaten verhindert.

Im letzten Extraktionsansatz wurde gekochtes Hühnereiweiß als Vertretung für prozessierte Lebensmittel mit 20 Puffern extrahiert und im ELISA auf vorhandene Allergene untersucht. Dabei erzielten Extrakte, welche mit Puffern hergestellt wurden, die das kationische Detergens CTAB enthielten, die besten Resultate, wenn der Antikörper gegen Sprüheiweiß verwendet wurde.
Die Signalkurve konnte zusätzlich durch Coating der Mikrotiterplatte mit Sprüheiweiß als Standard verbessert werden, welches ebenfalls mit einem CTAB-Puffer anstatt mit PBS extrahiert worden war.
Unerwartet zufriedenstellende Ergebnisse lieferte der Antikörper gegen rohes Hühnereiweiß, der trotz seiner Herstellung mittels eines nativen Immunogens in der Lage war, mit SDS-Puffern extrahierte und denaturierte Proteine zu erkennen. Die Antikörper gegen natives und erhitztes Ovomucoid zeigten nur mit TBS und PBS Puffer ohne jeglichen Zusatz von Detergentien passable Signalkurven. Abschließend konnte gezeigt werden, dass der verwendete Extraktionspuffer maßgeblichen Einfluss auf die darauffolgende Analyse der Allergene im ELISA hat und für jeden Antikörper neu optimiert werden muss.

14.4 ABSTRACT

The aim of the diploma thesis is the optimisation of an extraction buffer for egg white allergens from processed food stuff. Additionally, the influence of different buffer components on antigen-antibody binding was determined and a comparison between extracted total protein and signal strength in indirect competitive ELISA was performed. For that purpose four different polyclonal rabbit-antibodies were used, which were produced with spray dried egg white, crude egg white, native and heated ovomucoid.

In the first approaches various buffers from literature were compared, which had been used for general protein extraction. The composition of the protein extracts was determined via SDS-PAGE and Western Blot, and the total amount of protein was measured with a commercial BCA-test kit. Afterwards the essential usage of denaturating agents like urea or SDS became evident, which increased the protein yield of the extracts. After ELISA analysis of the extracts a negative effect was observed caused by those denaturing agents. In the case of sodium dodecylsulphate, the inhibiting influence of the anionic detergent on antigen-antibody binding was nearly compensated by adding non-ionic detergents like Brij 35. However, SDS was only suitable as buffer additive for protein extraction, if the detection in ELISA had been performed with the antibodies against spray dried egg white and crude egg white. Moreover it could be noticed, that the reducing agent dithiothreitol is able to facilitate the extraction of ovalbumin and ovomucoid from denatured food samples by removing disulfide bridges and therefore avoiding the formation of protein aggregates.

In the last extraction approach cooked egg white, which represents processed food stuff, was extracted with 20 different buffers and analysed in ELISA for accessible allergens. Thereby, extracts which were produced with buffers containing the cationic detergent CTAB, offered the best results if the antibody against spray dried egg white had been used.
Furthermore, the signal curve could be optimised by coating the microtiterplate with spray dried egg white as standard, extracted also with a CTAB-buffer instead of the usual PBS. Surprisingly the antibody against crude egg white showed good results in the detection of SDS-extracted and denatured proteins in spite of its production with a native immunogen. A sufficient signal with the antibodies against native and heated ovomucoid was only achieved, if the extraction had been performed with TBS or PBS buffer without any supplements. Finally it could be concluded, that the used extraction buffer influences the subsequent ELISA analysis of the allergens to a large extent and optimisation of the buffer is essential for every new type of antibody.

14.5 CURRICULUM VITAE

Personal Information

Name:	Kern Corinna
E-mail:	a0325071@univie.ac.at
Citizenship:	Austrian
Date of Birth:	24-05-1985

Education

University:	University Vienna since Oct. 2004 Molecular biology
	TU (Technical University)Vienna Oct. 2003 – June 2004 Technical chemistry
Secondary school:	BORG St. Pölten Sept.1999 – June 2003 main focus on sciences

Professional Qualifications

Language skills:	German (mother tongue) English (independent user) Spanish (advanced)
PC skills:	Microsoft Office (Word, Excel, PowerPoint) GelCompare

Compulsory internship

Biochemistry

Max Perutz Laboratories	Identification of CPK (Ca^{2+} dependent protein kinase)
Campus Vienna Biocenter	in plant cells (Arabidopsis thaliana, Tobacco)
Sept. & Oct. 2007	Cloning and production of plasmids
	Protein expression and purification
	Promoter activity determination
	Hot kinase enzyme activity assays
	IEF & SDS-PAGE
	Transfection of Agrobacteria → Infection of plants
	Immunofluorescence

Molecular Medicine

Max Perutz Laboratories	The role of N-Glycosylation in ERAD for Ribophorin I
Campus Vienna Biocenter	(ER-associated degradation of proteins)
Aug. & Sept. 2008	sterile cell culture techniques (fibroblasts & HeLa cells)
	Extraction of proteins, SDS-PAGE & Western Blots
	Immunofluorescence
	Pulse-Chase experiments

Microbiology/Immunology

Dept. IFA Tulln	Classification, Characterisation and Identification
Uni. of Natural Resources &	of gut bacteria with probiotic function
Applied Life Sciences	Isolation and Cultivation of MOs
Center for Environmental Tech.	DNA-Extraction, 16S-PCR & RAPD-PCR
& Biomin Holding GmbH	Determination of min. Inhibition Conc. (MIC)
	API-tests (application of carbohydrates)
	Cytotoxicity and Adhesion assays with cell culture

Diploma Thesis

Dept. IFA Tulln	Optimisation of extraction buffer and method
Analytical Center	for allergens from processed food samples
CD-Lab. for Rapid Test Systems	SDS-PAGE and Western Blot analysis of the extracts
for Allergenic Food Contaminants	ELISA analysis of the extracts
& Romer Labs Diagnostics GmbH	Antibody Purification and Characterisation
Nov. 2009 – Nov. 2010	Deglycosylation of allergens

Employment

Biomin Holding GmbH

Konrad Lorenz St. 20	Project 1: Isolation and Identification of Probiotika from
A-3430 Tulln	the intestinal tract from pig, wild boar and chicken
Nov. 2007 – Oct. 2009	Project 2: Nutrigenomics
since Dec. 2010	Species-specific DNA extraction, 16S –PCR and DGGE
	RNA-isolation

Spedition Tres

Brunn am Gebirge	Data management
April 2006 – April 2007	Book-keeping
	Cash register & Outgoing invoices

Glanzstoff Austria GmbH&CoKG

A-3130 St. Pölten	Analysis of the chem. properties of the textile and technical
August 2004 & 2005	fibers, the washing solutions and the spinning bath
	via Titration, Phase separation and Photometry