Component-resolved diagnostics

of food-allergy

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

submitted to the Department of Biology, Chemistry and Pharmacy

of Freie Universität Berlin

by

STEVEN SIEVERS

from Luckenwalde

2016

Die vorliegende Arbeit entstand in der Zeit von Oktober 2009 bis März 2016 unter der Leitung von Frau Prof. Dr. med. Kirsten Beyer in der Klinik für Pädiatrie m. S. Pneumologie und Immunologie an der Charité Berlin.

Teile dieser Arbeit wurden bereits oder werden noch veröffentlich werden.

Erster Gutachter: Prof. Dr. med. Kirsten Beyer (Charité Universitätsmedizin, Berlin)

Zweiter Gutachter: Prof. Dr. rer. nat. Matthias Melzig (Freie Universität, Berlin)

Disputation am: 24. Oktober 2016

Danksagung

Contents

Figures

Tables

Abbreviations

ACN	Acetonitril
APS	Ammonium sulfate
AA	Amino acid(s)
β-ME	beta-Mercaptoethanol
BCA	Bicinchoninic acid
Bidest	Double distilled water, ultrapure
BSA	Bovine serum albumin
CI	Confidence interval
CM	Cow's milk
CRD	Component-resolved diagnostics
CV	Coefficient of variation
DB	Digestion buffer
DBPCFC	Double-blind, placebo-controlled, oral food challenge test
DS	Destain solution
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FA	Food allergy
FI	Fluorescence(s)
g	Gravity of Earth (9.81 m/s^2)
HCL	Hydrochloric acid
HE	Hen's egg
HRP	Horseradish peroxidase
HSA	Human serum albumin
IAA	2-Iodoacetamide
IB	Immunoblot
i.v.	intravenous
kDa	kilo Dalton
kU/l	kilo units per liter (2.42 µg/l)
kU$_A$/l	Allergen-specific kilo units per liter (~2.42 µg/l)
LDS	Lithium dodecyl sulfate
MALDI-TOF	Matrix-assisted laser desorption/ionisation time-of-flight
MES	2-(N-morpholino)ethanesulfonic acid

MeOH	Methanol
min(s)	Minute(s)
MS	Mass spectrometry
MWCO	Molecular weight cut-off
MOPS	3-(N-morpholino)propanesulfonic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nat	native
nm	nano meters (10^{-9})
OD_{600}	Optical density at wave length λ=600nm
PAGE	Polyacrylamide gel electrophoresis
PI	Protease inhibitors
PVDF	Polyvinylidene difluoride
RAST	Radio-Allergo-Sorbent-Test
rec	recombinant
ROC	Receiver Operating Characteristic
SB	Sample buffer
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylene (20) sorbitan monolaurate

1 Introduction

1.1 Food allergy

In Western industrialized nations around 8% of children and 2% of adults suffer from a FA type I (immediate type) [1]. It is assumed that the distribution of food allergies continues to increase worldwide [2,3]. For Europe, calculated on the basis of studies from January 2000 to September 2012, the lifetime prevalence of self-reported FA was 17.3%, and evidence of increasing prevalence was found [4].The main food allergens in children are hen's egg, cow's milk, peanuts, tree nuts, wheat, soy and fish. Of these, cow's milk, hen's egg and nuts are the most important elicitors of severe anaphylactic reactions [5]. Adults usually suffer from persisting food allery to peanut, tree nuts, fish and shellfish. In total, over 160 allergenic foods are known [6]. At present, the only reliable therapy is to prevent the intake of allergen-containing food (elimination diet) and to carry an epinephrine autoinjector. Thereby, FA allergy affects life quality by permanent awareness towards food and its potentially allergenic ingredients. Avoiding strategies to accidental allergen intake may even bear the risk of malnutrition [7]. On the other hand, accidental ingestion of hidden allergens frequently leads to severe reactions [8]. Allergic diseases cause immense direct (ambulant and stationary treatment, pharmaceuticals) and indirect costs (mortality, invalidity, incapacity for work). The annual health care costs for the therapy of asthma and allergies were estimated in 1996 for Germany to 3.447 billion Euros [9].

The cause of FA is unknown to this day. Maternal nutrition during pregnancy, breastfeeding, timing of introduction to food and probiotic supplementation, and influence of microbacterial environment are discussed [10-13]. Since food allergies often occur in more cumulative numbers in the family vicinity, a genetic predisposion is very probable [14,15]. Environmental and genetically predisposed individuals are at risk of developing allergy-related diseases following the pattern: atopic dermatitis (eczema), FA, and asthma / allergic rhinitis. This phenomenon is referred to as atopic or allergic march [16].

1.1.1 Mechanism of type I food allergy

FA type I is an immediate immunological hyperreaction to non-toxic and normally harmless food. The response action takes place after 30 minutes to 2 hours and is reproducibly triggered after the stimulus occurs. It is mediated by immunoglobulin E which shows lowest concentration in serum of all immunoglobulin classes in humans. The typical course comprises sensitization and allergic reaction. The proteinaceous food gets in contact with the mucosa of the gastrointestinal tract and interacts via the epithelial barrier with the immune

system, e.g. antigen-presenting dendritic cells (DCs). DCs process allergens and present peptides on their surface by major histocompatibility complex class-II (MHC-II). Naïve T-cells recognizing this complex differenciate in TH2-cells and secrete interleukins IL-4 and 13 which induce class-switching of respective B-cells. These cells start production of specific IgE which spreads from local to systemic distribution in serum. IgE binds highly specific to FcεRI-receptors on mast cells and basophils where it is susceptible for the allergen. Of lesser importance is the low affinity receptor FcεRII on e.g. lymphocytes. At second contact with the respective allergen, bound IgE directly recognizes the allergen. Two contiguous receptors cross-link and trigger intra-cellular signal cascades which results in degranulation and release of histamine and many other mediators (prostaglandins, cytokins, leukotriens etc.) by the activated cell. Symptoms of the allergic reaction are redness, hives, nausea, vomiting, shortness of breath and under extreme conditions resulting in anaphylactic shock or death. Only small amounts of the respective food in the ppm range (mg/kg) can be sufficient to cause allergic reactions.

Sensitization is the prerequisite for an allergic reaction. In contrast, individuals may have detectable IgE to an allergen but do not suffer from a FA. This phenomenon, referred to as tolerance, may be due to the protective presence of immunoglobulin G4 (IgG_4) whose mechanistic interaction with IgE is subject to recent research [17]. Several studies of HE allergy have suggested that the level of IgG_4 or the ratio of IgE/IgG_4 may be of predictive value [18-20]. However, these findings were not confirmed in other studies and determination of IgG_4 in routine diagnostics is not recommended [10,21].

1.1.2   Nomenclature of allergens

Food allergens causing sensitization via the gastrointestinal tract are referred to class I (e.g. cow's milk, hen's egg, peanut) allergens. Class II food allergens are those which only act after established primary sensitization to an aero-allergen. Their action is caused by structural similarities >70% with the aero-allergen [22]. A well-known example is the cross-reaction between pollen from birch (Bet v 1) and apple (Mal d 1). Data bases like WHO/IUIS (World Health Organization and International Union of Immunological Societies Allergen Nomenclature Sub-committee; www.allergen.org) and Allergome (www.allergome.org) classify allergens according to originating species. The naming of allergenic components uses the first 3 letters of the Linnaean name and the first letter of the epitheton. Components are numbered consecutively with Arabic numbers, e.g. Mal d 1 or Bos d 5. Isoforms are further distinguished by extending the numbers to e.g. Bos d 5.0102. Native and recombinant variants are indicated with "n" or "r". Data bases like e.g. AllFam (http://www.meduniwien.ac.at/allfam/) address biological function of allergens and cluster them in families. Sequences of allergens can be retrieved in Uniprot (www.uniprot.org).

1.1.3 Diagnosis and routine diagnostic platforms

The diagnosis of food allergy is based on the patient's history combined with *in-vivo*-skin tests and/or *in-vitro*-diagnostics (immunoassays) [23]. In case of uncertainty, oral food challenges are conducted.

1.1.3.1 Skin prick test

In the diagnostic course, a skin prick test (SPT) is usually conducted at first. An allergen-containing solution is contacted with a needle in the skin of the patient; an analogous procedure is the prick prick test, where the needle is first engraved in the solid allergen-containing food (e.g. hazel). This method is fast (10 to 20 mins) and inexpensive, but uncomfortable for the patient. The result (the extent of wheal formation and erythrema is measured around the injection site) is also highly variable, due to the skills of the medical staff and the lack of standardized extracts. However, a positive result indicates sensitization to the allergen pricked. Wheal diameters have been applied to predict the outcome of an oral food challenge [24-27]. In their review, Peters, Gurrin, Allen [28] found considerable variations in 95% positive predictive values (PPV) even between pre-selected high-risk populations. These authors limit the value of the reported results (PPV and SPT) due to population bias and lacking standard procedures and extracts. However, SPT are considered similarly sensitive as *in vitro* tests [29].

1.1.3.2 In-vitro tests

In-vitro test methods such as the ImmunoCAP (Thermo Scientific) measure the allergen-specific IgE (sIgE) using the sandwich principle: allergen-containing native or recombinant material is bound to a solid phase (cellulose) and brought into contact with the patient's serum. Specific IgE binds to the allergen, and after washing to remove unspecific serum components, a secondary antibody against IgE is applied which produces a fluorescent signal by an enzymatic reaction. The fluorescent signal correlates with the presence of IgE. The result is quantitative and issued in kU_A/l (1 kU_A/l = 2.42 µg/l). The analytical sensitivity is currently at 0.1 kU_A/l. Sensitized and non-sensitized patients are distinguished using the analytical cut-off value of 0.35 kU_A/l (equal to 0.847 µg/l), and more recent 0.1 kU_A/l (equal to 0.242 µg/l). As a rule, results below this value indicate no clinically relevant food allergy, and on the other hand, values >0.35 kU_A/l are clinically relevant only with corresponding symptoms. The procedure is relatively inexpensive in high-throughput, however, several single allergens (components) or mixtures of allergens (so-called panels) can only be driven sequentially per patient. In addition to the time being consumed, 40 µl serum or plasma of the patient is needed for one determination, which at multiple determinations leads to problems especially in pediatric populations. The three main manufacturers of diagnostic IgE assays on the market are Siemens (Immulite), Thermo Fisher (ImmunoCAP) and Hycor

(Hytec). In spite of similar performance and CVs <15%, these assays measure different IgE-populations with different levels of efficiency, which is why their results are not equivalent [30]. However, these assays provide quantitative results which are used to establish component-specific cut-offs (1.2). Due to high sensitivity and specificity, low CV and convenient handling, ImmunoCAP is regarded as the gold standard in allergen specific IgE testing.

1.1.3.3   *Double-blind, placebo-controlled oral food challenge*

The gold standard is the double-blind, placebo-controlled oral food challenge (DBPCFC). It is applied when patient's history and SPT or sIgE do not correlate well. In DBPCFC, Iincreasing doses of the suspected allergen (or placebo) mixed with a non-allergenic matrix (e.g. pudding) are given to the patient and reactions are observed. Neither patient nor physician know whether verum or placebo is given. Unblinded oral food challenges without placebo (ORF) are also applied, especially in pediatric populations. These procedures are expensive, time consuming and may involve high risks for the patient. They are therefore conducted in specialized centers only. Although false negative (2% to 5%) and positive (5.4% to 12.9%) results were reported in children and adults [31,32], a positive food challenge best verifies or falsifies suspected food allergy, or may even unravel other non-IgE-mediated diseases [33]. However, DBPCFC are conducted with different protocols, and results probably only apply to populations examined [34].

In general, SPT and determination of sIgE are considered as equal sensitive [35].

1.2   Component-resolved diagnostics

1.2.1   Concept

With advances in molecular biology and purification techniques it became feasible to purify targeted proteins either from natural crude extracts or from microorganisms like *E. coli* or yeast. Crude extracts may vary in their allergen content due to naturally low abundance, preparational loss or low solubility. In CRD single allergenic molecules (allergen components) in high purity are used. In this respect, an allergen is typically a mixture of allergenic components. Thereby, CRD can especially assess allergens which are low abundant in crude extracs, and it can target specific IgE by exlusion of potentially cross-reactive allergens. The aim is to dissect the patient's IgE repertoire by concentrating diagnostics on essential components with high specificity and predicative value as concerns clinical reactions [36,37].

1.2.2   The role of native and recombinant proteins in CRD

Native components may be extracted by physicochemical techniques or purified by affinity chromatography from extracts. Provided non-denaturing conditions, the native structure is maintained and as such linear and structural epitopes. Native proteins also provide relevant post-translational modifications. A disadvantage usually is the lack of high purity. In contrast, recombinant proteins can be produced with constant quality and in large amounts, but may be misfolded and thus lack functionality. Possible amino acid alterations, truncations and missing post-translational modifications like glycosylation may also interfere with specific binding of IgE [38]. On the other hand, not the full protein may be necessary to trigger the recognition of especially sequential epitopes. On ImmunoCAP, several recombinant proteins are provided in only fragments, e.g. ω5-gliadin.

Transfer to this study: Native and recombinant CM and HE components will be spotted on a silicon microarray and evaluated whether they are equally recognized by sIgE.

1.2.3   Applications of CRD

CRD was considerably facilitated by the development of protein microarrays (1.2.4). Since then, CRD has been employed to identify immunoreactive components and differenciate multiple sensitizations in the patient. By this approach, sensitization profiles to numerous components can be mapped and correlated with clinical symptoms (Figure 1). CRD has also provided insight in cross-reactions caused by components which share structures and sequences, helping to distinguish primary sensitization to genuine component(s) from

secondary sensitization (1.1.2). At present, more than 120 components are available for use in SPT, quantitative and semiquantitative immunoassays [39].
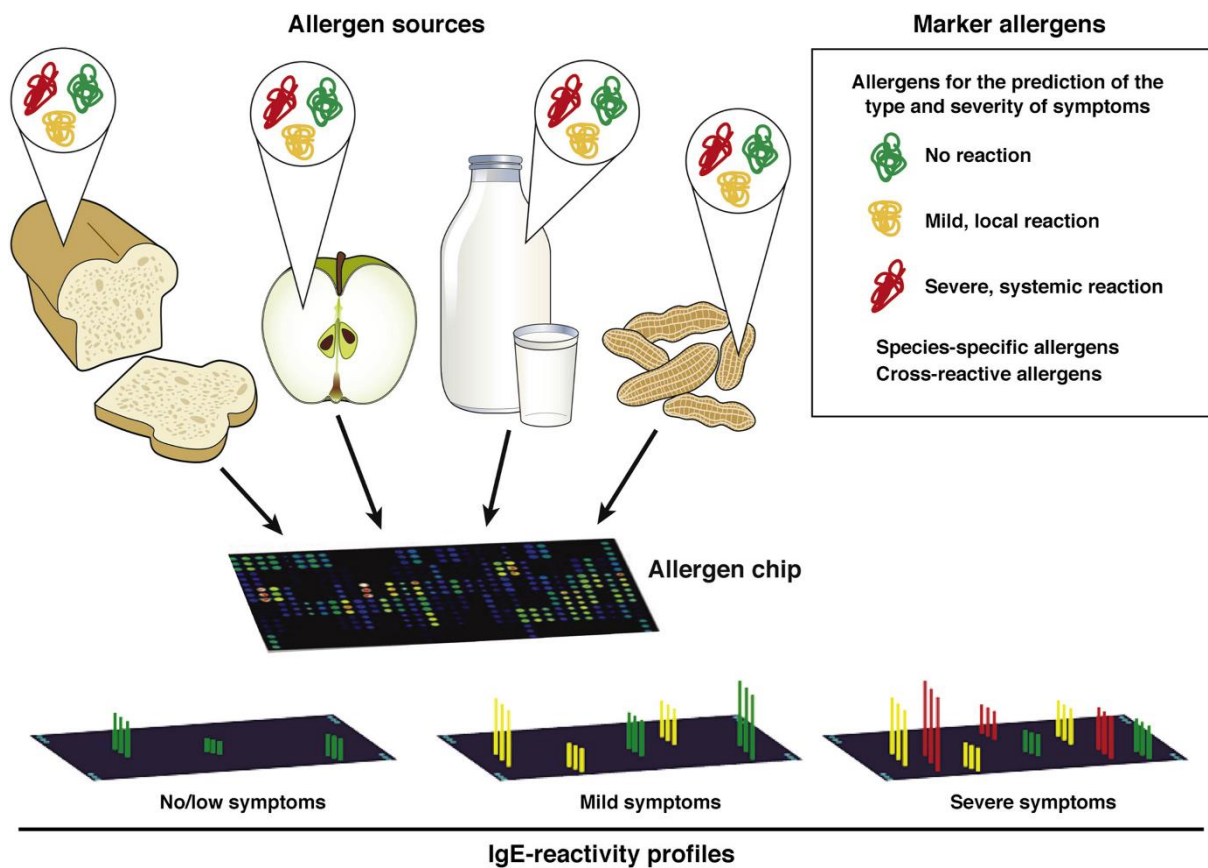
Figure 1: Principle of CRD. Illustration taken from Valenta, Hochwallner, Linhart, Pahr [40].

Physicians try to relate clinical symptoms to components and have started to establish critical threshold levels of quantitative sIgE that allow prediction of allergic reactions. Well studied examples in CRD are peanut and tree nuts (hazel) which most commonly cause anaphylactic reactions in children and persist often lifelong [41,42]. In peanut, 18 allergenic components (Ara h 1 to 17, and Ara h Agglutinin) have so far been identified and linked to clinical reactions in pediatric populations. As the most important component (marker allergen, Figure 1), Ara h 2 was determined in several studies [43-48]. Determination of sIgE correlated best with clinical symptoms as revealed by ROC curve analysis (Figure 2).
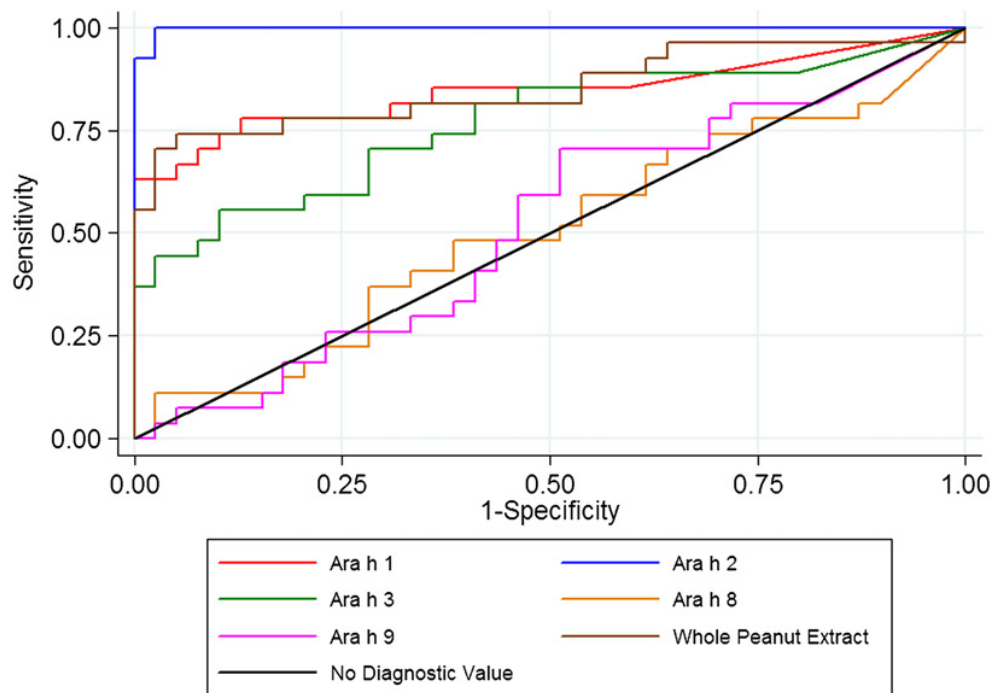
Figure 2: ROC curves of peanut components. The abscissa represents the false-positive-rate (FPR) or fall-out, and the ordinate the true-positive rate (TPR). Illustration tken from Nicolaou, Murray [43]

The high diagnostic value of Ara h 2 over whole peanut extract suggests that components might be more appropriate for a reliable assessment of FA, rendering expensive and risky oral food challenge tests and providing a means of targeted immunotherapie or even vaccination. However, it has to be taken into consideration that these studies used quantitative platforms (e.g. ImmunoCAP) which consume 40 µl serum per determined component. Since 80% of patients are polysensitized [49], microarrays are most suitable for the preparation of the patient-specific allergen profile especially in pediatrics, where only low amounts of blood, e.g. capillary blood, are available.

1.2.4   Microarrays

Protein microarrays have become a well-acknowledged tool in bio-analytical and clinical applications since their introduction [50,51]. Up to thousands of peptides and proteins, immobilized onto a small area, can be simultaneously assessed (multiplexed) with only a small volume containing the ligands of interest to be captured. Assumed to be even more sensitive than macroscopic bioassays [52], these characteristics match the needs of CRD because small concentrations of IgE (ng/ml) against individual allergenic molecules must be detected in limited patient material (serum, plasma). Microarrays have proven to be of immense value for immunological and clinical phenotyping not only in allergy, but also in cancer research, drug screening and antibody binding studies [37,53-56].

1.2.4.1 ImmunoCAP ISAC

The most common microarrayed detection platform in research for CRD, the ImmunoCap ISAC (Thermo Fisher), is based on 112 components (43 are food allergens) from 51 sources spotted on standard glass slides. Native and recombinant components are used. Captured IgE is detected by secondary antibodies conjugated to a fluorescent label and is expressed in semi-quantitative results (Internal Standardized Units, ISU) according to an external calibration serum. Either IgE-, IgG- or IgG_4-specific signals from 30 µl sample volume (serum or plasma) can be detected, diluted 1:2 or 1:50, respectively. The disadvantages of the ISAC are that it is very expensive and only provides semi-quantitative results which cannot be converted into kU_A/l [57]. The analytical sensitivity of the ImmunoCAP (0.1 kU_A/l) is not achieved. Several studies have assessed the ISAC with mixed results from excellent to considerable high CV's for certain allergens [58,59]. In general, CV's <25% are only reported for ISU>1 with a sensitivity of 0.3 to 1.0 ISU. Potential interference between IgE and other isotypes is suspected [60].

1.2.4.2 Coated silicon microarray

Cretich, Di Carlo, Longhi *et al.* [61] introduced a microarray based on silicon substrate, coated with a functional polymer copoly(DMA-NAS-MAPS) [62]. Compared to glass slides, this microarray provides low background, high sensitivity and better reproducibility, shown for sera of 30 adults with sensitization to inhalant allergens [63]. The improved performance of silicon/silicon oxide (Si/SiO_2) slides with controlled oxide layer of 100 nm is due to enhanced fluorescence intensity by optical interference [64]. This microarray is not commercially available, but its surface chemistry is published. Provided technical equipment, this microarray can be self-prepared for customized purposes. A disadvantage is that raw fluorescence data must be processed which may become laborious depending on the number of spotted analytes. Calibration and reference to standards need to be established individually.

Transfer to this study: The performance of the copoly(DMA-NAS-MAPS)-coated silicon slide will be compared with the ISAC glass slide for selected components. Results from both platforms will be referenced to quantitative results from ImmunoCAP. Different types of normalizations and calibrations will be applied. Surface chemistry will not be targeted.

1.3 Wheat

Wheat is, besides rice and maize, one of the most important crops and part of our daily diet. It is also broadly used as ingredient in many food products and thus hard to avoid. Wheat is a potent elicitor of several allergic diseases: baker's asthma, food allergy in infancy and childhood, wheat-dependent exercise-induced anaphylaxis (WDEIA), coeliac disease, food protein-induced enterocolitis syndrome and wheat hypersensitivity [65-67]. An overview of components described as inhalative and/or ingestive is shown in Supplement Table 1.

1.3.1 Food allergy to wheat

Allergy to ingested wheat affects 0.4 to 1% of infants and often co-occurs with allergy to CM and HE [68]. Most children easily outgrow the disease by onset of school age (5 to 8 years), a minority show a resolution during teenage years [69-73]. There has been a considerable research on the complexity of the wheat flour proteome [74-76]. It is clear now that a broad spectrum of proteins, despite variations in wheat sources, cultivars and seed maturation, can trigger allergic reactions with a considerably high variance between patients, populations and relative importance [67,77-79].

For wheat allergy a correlation between specific IgE titer versus clinical reactivity to oral food challenge could not be determined, and clear cut-offs have not been established yet [80-83]. One problem in elucidating the role of IgE titers could be that, due to source and processing, some wheat proteins could be underrepresented in commercial test systems, or that wheat extracts lack sufficient purity [84,85]. As shown by Wan, Gritsch, Hawkesford, Shewry [86], amount and composition of ω-gliadins differ between cultivars which might explain regional differences in sensitization. Cross-reactivity among gliadins and LMW-glutenins as well as sequence homologies with other cereals also complicate specificity [87,88].

The degree of protein solubility was suggested to be a parameter for the various clinical manifestations as water and salt-soluble proteins can be resorbed in the mucosa of the respiratory tract as well as from the digestive system. In contrast, water-insoluble proteins may be only relevant in food allergy or celiac disease [89-91]. A correlation between age, allergen pattern and symptoms was suggested by Battais et al. who found that in children, suffering from AD, the major allergens belong to the water and salt soluble proteins, whereas the storage proteins gliadin affects adults struggling with urticaria and anaphylaxis [92].

1.3.2 Wheat proteins

According to their solubility, wheat proteins can be classified as (i) water (albumins) and (ii) salt soluble (globulins) proteins (~15-20% of the kernel), which comprise enzymes and proteins to maintain structure, metabolism and defence against pathogens. Storage proteins, which account for approx. 80% of the kernel and are to be consumed during germination, are

(iiI) gliadins which can be solubilised with water/alcohol diluents, and (iv) glutenins, partly soluble in mild acidic and alkaline solutions with reducing agents. Gliadins and glutenins are commonly referred to as 'gluten'. In contrast to other food allergens, "gluten free" products exist since a cut-off level of 20 ppm has been defined in regard to celiac disease. Gliadins are further distinguished in monomeric α/ß-, γ- and ω-gliadins, according to their electrophoretic mobility from fast, intermediate to slow [93]. Glutenins are polymeric, consisting of high (HMW) and low (LMW) molecular weight subunits. Despite these classifications, gliadins and glutenins feature a wide polymorphism, and a huge amount of highly repetitive sequences and non-repetitive domains as well as sequence homologies, resulting in cross-reactions and similar immunological recognition patterns [92,94-99]. Food allergy to wheat in infants can be triggered by all storage proteins. The role of proteins of the albumin and globulin fraction, especially α-amylase and α-amylase/trypsin inhibitors (AAI and AATI) is under discussion [92,100].

1.3.3 CRD of wheat food allergy

CRD suggested ω5-gliadin to be a major allergen and useful in the diagnostic workup, also in combination with additional tests [101-103], although differences among populations should be considered [104]. The clinical and prognostic value of ω5-gliadin is well documented but not undisputed, since panels of components correlate better to wheat challenge outcomes and severity of reaction [101,102,104-110]. Most recent results suggest that measurement of IgE to at least 5 components (AAI 0.19, α-, β-, and γ-gliadins, and HMW-glutenin subunits) may improve wheat allergy diagnostics [76]. Still, the association between food allergy or tolerance to ingested wheat and corresponding protein classes is still poorly understood.

Transfer to this study: Water/salt soluble and water insoluble proteins will be seperately analysed in immunoblots with sera of tolerant and allergic individuals in order to compare IgE-binding recognition pattern. Selected protein bands will be assessed by MALDI-TOF MS.

1.4 Cow's milk and hen's egg

CM allergy affects about 2%, and HE about 0.5 - 2.5% of children [111]. They often co-occur. Both have a good prognosis to be outgrown by onset of school age [112]. However, allergic reactions from mild skin symptoms to severe anaphylaxis occur. Major allergens are $αs_1$-, $αs_2$-, β-, and κ-casein, and α-lactalbumin and β-lactoglobulin [113]. Epitopes of these have been identified and linked to clinical symptoms [114-116]. Most patients are sensitized to several components with great variance in their profile [117]. This may be attributed to several characteristics of CM proteins: heterogenity with few functional and structural features, genetic polymorphism with several isoforms, and only partially known secondary

and teriary structures [118,119]. CRD for CM allergy has so far not revealed marker components that separate sensitized from allergic individuals, although severe systemic reactions were found to correlate with the number of recognized components [120]. Several studies showed that casein is the most dominant component individuals are sensitized to, but the predicative value is under discussion. Testing to native components of casein did not improve clinical phenotyping, that's why recombinant variants and peptides were suggested [121-124].

Major food allergens in HE are located in the egg white fraction, they are ovomucoid, ovalbumin, ovotransferrin and lysozyme [125]. In general, higher levels of sIgE to HE correlate with the number of components an individual is sensitized to [126]. High levels of sIgE to ovomucoid correlated with clinical symptoms and persistence of allergy which is attributed to its resistence to heating [127,128].

Transfer to this study: Native and recombinant components of CM and HE will be compared on a silicon microarray to assess functionality of recombinants. Results will also be applied to evaluate the performance of the silicon microarray in comparison to ISAC and ImmunoCAP.

2 Hypotheses and Objectives

Until now, neither a single laboratory parameter can prove the diagnosis of food allergy nor predict the natural time course of the disease.

Commercial test systems may lack sufficient amounts of important elicitors, especially water insoluble proteins. This may be true for wheat because wheat-specific IgE does not correlate well with clinical symptoms. Tolerance or allergy to wheat could therefore be associated with a certain pattern of recognized proteins, especially in the water-insoluble fraction.

To answer this question, 106 sera of clinically tolerant and allergic children were analyzed in immunoblots to water and salt soluble albumins and globulins, and to water insoluble gliadins and glutenins. By comparison of IgE-binding pattern, differences and similarities were analyzed and quantified in a semi-quantitative manner. The identity of recognized protein bands was assessed with MALDI-TOF MS. Of special interest was the significance of $\omega 5$-gliadin which was quantitatively determined in selected sera.

CRD may improve diagnostics by targeting allergens on the molecular level by use of native and recombinant proteins. Recombinant proteins can be produced in large amounts and high purity, but may be misfolded, lack necessary post-translational modifications and thereby lose functionality.

Another issue in CRD is the availability of patient's material, which is limited in especially pediatric populations. Therefore, microarrayed platforms are the matter of choice. The commercially available ImmunoCAP ISAC glass slide covers a large total number of components to food, but lacks sensitivity and reproducibility especially in the low detection range. Its semi-quantitative results are thereore of limited value. Parallel detection of different immunoglobulins is not established, although e.g. IgG_4 might be of diagnostic value.

The aim of this study is the comparison of native and recombinant CM and HE components on a polymer-coated silicon microarray with proposed higher sensitivity and reproducibility compared to the ImmunoCAP ISAC. The parallel detection of specific IgE and IgG_4 is to be established with quantitative results.

By application of a large amount of well-described individual sera, functionality of recombinant proteins will be assessed by correlations of obtained fluorescences with quantitative and semi-quantitative results from ImmunoCAP ad ImmunoCAP ISAC. Biochemical methods and MALDI-TOF MS will be employed to explain differences. The feasibility of parallel detection of specific IgE and IgG_4 will be assessed, and internal and external calibrations applied to generate quantitative results. The performance of the silicon microarray will be evaluated by determination of working range, sensitivity and coefficient of variation.

3 Materials and Methods

3.1 Subjects

The sera used in this study had been collected from patients who were treated in the Department of Pediatric Pneumology and Immunology, Charité Universitätsmedizin Berlin. Stored at -20°C, aliquots from these sera were taken at the time of analyses. Due to limited volumes, the number of assays and tests was limited.

All studies were approved by the local ethical committee and all patients provided written informed consent before entering the study.

3.1.1 Subjects in the wheat study

Retrospectively, the population comprised in total 106 children selected for the study when they were admitted to the clinic of Pediatric Pneumology and Immunology for oral wheat challenge. Criteria for wheat challenges were suspicion of wheat-related symptoms by parents or physicians such as immediate type symptoms (urticaria, vomiting or wheezing) or worsening of eczema after ingestion of wheat containing products. All children with suspected wheat allergy underwent oral wheat challenge using wheat/gluten powder (Kroener, Ibbenbueren, Germany). Seven increasing doses were administered at 30-min intervals using a semi-log scale ranging from 3mg to 3g protein. Both, immediate (e.g. urticaria, vomiting, wheezing) and late reactions (worsening of eczema of at least 10 points increase in SCORAD score within 24 hours) were recorded. SPT was not performed in these children. Sera were obtained from all patients at the time when the i.v.-line was placed before the oral food challenge. In case of insufficient amount, additional serum samples that were collected for medical reason within 3 months before or after the oral wheat challenge were used.

3.1.2 Subjects in the microarray study

Retrospectivly, the populations comprised 82 individuals in the initial microarray assessment, and 91 serum samples of 49 individuals were available for the final microarray. All children were challenged with cow's milk and/or hen's egg, and sIgE to CM and HE was determined. Clinical data on the individuals for the final microarray was published in Ahrens, Lopes de Oliveira, Grabenhenrich *et al.* [129].

3.1.3 Sera

Applied individual sera and determined laboratory parameters are summarized in Table 1.

Table 1: Sets of individual sera used in the microarray study. Allergen names according to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) are given in brackets and are only indicated once for components spotted on ImmunoCAP ISAC. Cap classes and ISU are explained in **Table 3**.

Set	n	extract / component	quantitative sIgE in kU$_A$/l median (range)	quantitative sIgG$_4$ in mg$_A$/l median (range)	component	semi-quantitative sIgE in ISU median (range)
A	82	hen's egg	1.07 (0.01 – 110)	-	-	-
		cow's milk	0.63 (0.01 – 67.2)	-	-	-
B	91	hen's egg	0.8 (0.01 – 102)	-	ovomucoid (Gal d1)	0 (0 - 58.21)
					ovalbumin (Gal d2)	0 (0 - 18.30)
		cow's milk	2.71 (0.01 – 365)	-	αs1-casein (Bos d8αs1)	0 (0 - 30.05)
					β-casein (Bos d8β)	0 (0 - 14.25)
		α-lactalbumin	-	0.2 (0 - 14.2)	κ-casein (Bos d8κ)	0 (0 - 33.56)
		β-lactoglobulin	-	0.12 (0 - 23.6)	casein (Bos d8)	0 (0 - 26.23)
		casein	-	0.48 (0 – 276)	α-lactalbumin (Bos d4)	0 (0 - 15.72)
					β-lactoglobulin (Bos d5.0101)	0 (0 - 15.21)
					β-lactoglobulin (Bos d5.0102)	0 (0 - 9.57)
B.1	14	α-lactalbumin	0.21 (0 – 34)	not used	α-lactalbumin (Bos d 4)	0 (0 - 2.97)
	15	β-lactoglobulin	1.04 (0.01 - 8.87)	not used	β-lactoglobulin (Bos d5.0101)	0 (0 - 5.97)
					β-lactoglobulin (Bos d5.0102)	0 (0 - 5.96)
B.2	15	ovomucoid	1.06 (0 - 29.4)	not used	ovomucoid (Gal d1)	1.48 (0 - 28.45)
		ovalbumin	0.55 (0 – 59)	not used	ovalbumin (Gal d2)	1.86 (0 - 14.95)
B.3	12	α-lactalbumin		0.045 (0 – 0.72)	-	-
		β-lactoglobulin		0.085 (0 – 0.42)	-	-
		casein		0.045 (0 – 0.70)	-	-
C	2	hen's egg	56.80 ; 98.40	-	-	-
		ovomucoid	-	0.39 ; 1.29	-	-
		ovalbumin	-	0.15 ; 2.08	-	-
		cow's milk	727.00 ; 6.60	-	-	-
		α-lactalbumin	-	7.99 ; 0.38	-	-
		β-lactoglobulin	-	4.87 ; 2.41	-	-
		casein	-	8.17 ; 3.15	-	-
D	12	hen's egg	not used	0.09 (0 - 0.67)	-	-

Several individual sera with defined parameters of interest were pooled and applied as positive, negative and atopic controls. Negative and atopic controls were composed of sera determined for specific IgE to CM, HE, wheat, soy, peanut and codfish (fx5 panel on ImmunoCAP) and to house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farina*), cat and dog dander, timothy grass (*Phleum pretense*), common silver birch (*Betula verrucosa*), cultivated rye (*Secale cereale*), mugwort (*Artemisia vulgaris*) and mould fungus (*Cladosporium herbarum*) (SX1 panel on ImmunoCAP). Positve sera used in microarray assays were also measured with ImmunoCAP ISAC, and specific IgE to components was partially determined on ImmunoCAP. Details are shown in Table 2.

Table 2: Composition of HE and CM pool sera. Quantitative and semi-quantitative results, determined on ImminoCAP (in kU$_A$/l) and ImmunoCAP ISAC (ISU). Platform-specific reference codes precede allergen names. HE and CM components in gray were determined, but not spotted. Cap classes and ISU are explained in **Table 3**.

Allergen (unit)	HE low f1 Cap:1-2	HE medium f1 Cap:3-4	HE high f1 Cap:5	CM low f2 Cap:1-2	CM medium f2 Cap:3-4	CM high f2 Cap:5-6
f1 HE (kU$_A$/l)	0,97	18,9	44,4	0,96	12	27,8
f2 CM (kU$_A$/l)	1,56	12,0	63,1	0,94	21	>100
f232 ovalbumin (kU$_A$/l)	1,09	25,7	57,8	-	-	-
f233 ovomucoid (kU$_A$/l)	0,80	26,3	20,0	1,08	-	21,3
f76 α-lactalbumin (kU$_A$/l)	-	5,47	-	0,47	-	99,3
f77 β-lactoglobulin (kU$_A$/l)	-	4,06	-	0,63	-	36,2
f78 casein (kU$_A$/l)	-	11,7	-	0,29	-	-
nGal d1 (ovomucoid) (ISU)	0	3,67	4,93	0	8,51	11,53
nGal d2 (ovalbumin) (ISU)	0	1,28	4,15	0	1,9	8,15
nGal d3 (ovotransferrin) (ISU)	0	1,22	1,05	0	0	0,83
nGal d5 (albumin) (ISU)	0	0,55	0,3	0	0	0,27
nBos d4 (α-lactalbumin) (ISU)	0	0,68	1,56	0	1,33	14,28
nBos d5 (β-lactoglobulin) (ISU)	0	0,54	3,56	0	5,59	7,23
nBos d8 (casein) (ISU)	0	3,28	20,91	0	5,08	20,45
nBos d lactoferrin (ISU)	0	0	3,39	0	0,82	0
nBos d6 serumalbumin (ISU)	0,29	0	0	0	0	0

The atopic control in the wheat study had 0.16 kU$_A$/l in fx5 panel, and 2.16 kU$_A$/l in SX1, but only positive for dust mite (d1 on ImmunoCAP) in order to avoid possible cross-reactions due to primary respiratory sensitization to grass and birch. The negative pool had 0.07 kU$_A$/l in fx5 and SX1, respectively. In the microarray study, the atopic control was 0.05 kU$_A$/l in fx5, and 7.33 kU$_A$/l in SX1. The negative pool was determined with 0.05 kU$_A$/l in fx5 and 0.16 kU$_A$/l in SX1. Negative and atopic pool sera were negative on ImmunoCAP ISAC.

3.2 Determination of specific IgE and IgG$_4$

All quantitative measurements were performed with the ImmunoCAP 100 or 250 FEIA (Fluorescence Enzyme Immuno Assay) system (previously Phadia, Uppsala, Sweden, now Thermo Fisher Scientific, Waltham, Massachusetts, USA). Semi-quantitative results in ISU were obtained by a customized ISAC chip (VBC-GENOMICS, Vienna, Austria; Phadia, Uppsala, Sweden) and published previously [129].

IgE-specific results are reported in kU$_A$/l which corresponds to 0.994 kU/l, calculated from 3 individual sera for each of 5 allergens, according to the WHO International Reference 75/502 which defines 1 U IgE as 2.42 ng. The quantitative concentration of sIgE is also converted in 6 arbitrary CAP classes of different ranges and commonly interpreted for the patient (Table 3), but are not considered in the diagnostic workup anymore.

Table 3: ImmunoCAP and ImmunoCAP ISAC classes and evaluation according to the provider

class	IgE in kU/l	evaluation	ISAC IgE range	ISU	evaluation
0	0.10 – <0.35	negative	0	<0.3	undetectable or very low
1	>0.35 – <0.70	low positive	1	≥0.3 - <1	low
2	>0.70 – <3.50	moderate positive	2	≥1 - <15	moderate to high
3	>3.50 – <17.5	positive	3	≥15	very high
4	>17.5 – <50.0	high positive			
5	>50.0 – <100	very high positive			
6	>100	very high positive			

IgG_4-specific results are reported in mg_A/l and are obtained by automatic dilution of serum in the system. The majority of sera was measured with a lower limit of 0.35 kU_A/l. For statistical reasons, results given as "<0.35" were converted to "0.01".

3.3   Wheat cultivar and additional grains

For immunoblot analyses with single patient serum, only one cultivar of common wheat (*Triticum aestivum*) was used. The cultivar Akteur is a winter wheat and belongs to the quality group E ("elite"). It is the most frequent cultivated group E soft wheat in Germany (https://www.dsv-saaten.de/getreide/winterweizen/sorten/akteur.html;   access   2015-12-21) and was also subject to detailed proteomic research [85]. Peeled grains, harvested in 2009 and stored in a depot, were obtained from a regional supplier in 2010. Additional grains of ancient or ecologically cultivated cultivars were obtained from IGV (Institut für Getreideverarbeitung   GmbH,   Nuthetal,   OT   Bergholz-Rehbrücke,   Germany)   and Getreidezüchtungsforschung Darzau Hof.

3.4   Antibodies and antigens

Cy3-labeled streptavidin	Jackson ImmunoResearch, West Grove, USA
Goat anti-human IgE, labeled with [125]Iodine	DiaMed., Windham, USA
Goat anti-human IgE, ε-chain, serum	Calbiochem, Darmstadt, Germany
Goat anti-mouse IgG, HRP Conjugate (H+L)	Novagen, Darmstadt, Germany
human IgE kappa, monoclonal, from human myeloma serum	Nordic Immunology, Tilburg
human IgG_4 from human myeloma plasma	Calbiochem, Darmstadt, Germany
Human IgG, purified	Invitrogen, Darmstadt, Germany
Mouse anti-human IgE, Fc Fragment specific (HP6029)	Calbiochem, Darmstadt, Germany
Mouse anti-human IgG_4, Fc Fragment specific (HP6025)	Calbiochem, Darmstadt, Germany

3.5 Molecular analyses

3.5.1 Recombinant plasmids

Recombinant plasmids were kindly provided by Dr. Hugh Sampson, Mount Sinai School of Medicine, New York, and prepared by Galina Grishina.

Kappa-casein was in pCMV-SPORT6 vector, other CM clones in pSPORT1 vector. HE clones were in pCR 3.1-Uni vector. For later production of recombinant proteins, all inserts were cloned in pET24b vector from Novagen (now Merck Millipore, Darmstadt, Germany). This vector carries N-terminal a sequence coding for T7-tag of 11 AA (MASMTGGQQMG) and C-terminal a Hexahistidin-Tag. Plasmid DNA was transferred to Charité at .concentration of 30 µg/µl. Accession numbers of cDNA in Table 4.

Table 4: Accession numbers of recombinant proteins in NCBI database.

Protein	cDNA Accession number	entry name
αs1-casein	X00564	Bovine mRNA for pre-alpha S1-casein B
αs2-casein	NM174528	Bos taurus casein alpha-S2 (CSN1S2), mRNA
β-casein	XM_010806178	PREDICTED: Bos taurus casein beta (CSN2), transcript variant X1, mRNA
κ-casein	X00565	Bos taurus mRNA for pre-kappa-casein A
α-lactalbumin	M18780	Bovine alpha-lactalbumin mRNA, complete cds
β-lactoglobulin	X14712	Bovine mRNA for beta-lactoglobulin
ovomucoid	XM_015293764.1	PREDICTED: Gallus gallus serine peptidase inhibitor, Kazal type 7 (putative) (SPINK7), transcript variant X2, mRNA
ovalbumin	NM_205152	Gallus gallus ovalbumin (SERPINB14) (OVAL), mRNA

There are no publications from Dr. Samspon's lab or other labs which asked for those clones (personal communication with Galina Grishina).

3.5.2 Transformation of *Escherichia coli* cells

Stem solutions of plasmid DNA were diluted to a concentration of 3 µg/µl in 50 µl pure water. 2 vials of 25 µl diluted DNA per allergen were mixed with 25 µl BL21(DE3) bacteria cells (Invitrogen) and kept on ice for 30 minutes. After incubation in a water bath at 42°C for 30 seconds, vials were replaced on ice. 250 µl of pre-warmed SOC medium were added and vials were shaken at 300 rpm and 37°C for 1 hour (Eppendorf Thermomixer). 500 µl pre-warmed LB medium with Kanamycin (25 µg/ml) were added to the vials. 50 µl or 500 µl of this transformation reaction were plated onto LB agar plates and incubated at 37°C in inverted position overnight. The following day, 3 clones per plate and allergen were picked and transferred into 2 ml vials with 1ml LB medium with Kanamycin (25 µg/ml). Vials were

incubated at 700 rpm and 30°C overnight and 300 µl autoclaved glycerol (87%) were added. These glycerol stocks were stored at -80°C.

In order to verify successful transformation with plasmid DNA, 3 clones per allergen were picked and grown in 1ml LB with kanamycin (25 µg/ml) at 700 rpm and 30°C (Eppendorf Thermomixer) overnight. 50 µl of the cell suspension were transferred into fresh 1 ml LB with kanamycin (25 µl/ml) and cultivated at 700 rpm and 37°C for 2 hours. Protein expression was induced by adding 10 µl of 0.1 M IPTG (final concentration 1 mM IPTG). One culture remained uninduced and served as negative control. After 2 to 4 hours at 700 rpm and 37°C, cells were harvested by centrifugation at 20800 x g, discarding the supernatant. The cell pellets were transferred in -20°C overnight (lysis). The next day, cells were thawed on ice, mixed with 100 µl 2x sample buffer w/o DTT and heated at 60°C and 1400 rpm (Eppendorf Thermomixer) for 20 minutes. After centrifugation at 20800 x g for 10 minutes, equal amounts of supernatants were loaded on Bis-Tris 4-12% gels and proteins were separated. After electrophoresis, gels were incubated in 20% acidic acid (fixation) and stained with Coomassie Blue R250 solution.

3.5.3   Mini-Prep

Purification of plasmid DNA was performed with EURx GeneMATRIX Plasmid Miniprep DNA Purification Kit according to the manufacturer's instructions.

Per allergen, 8 ml LB medium with Kanamycin (25 µg/ml) were inoculated from glycerol stocks in 15 ml Falcon tubes and incubated with 250 mot/min at RT overnight. Cultures were centrifuged at 1125 x g (Sigma) for 1 minute and the supernatant discarded. Fresh LB medium with Kanamycin (25 µg/ml) was added, cell pellets were resuspended and incubation was continued at 275 mot/min and RT overnight.

1.5 ml of cell suspension were transferred into 2 ml vials and centrifuged at 20800 x g (Eppendorf) and RT for 2 minutes. The supernatant was discarded and the pellet was dried by inverting the vial upside down onto a paper towel. The cell pellet was suspended in 250 µl buffer Cell-R and 200 µl lysis buffer were added. After gently inverting the vial several times, the mixture was kept at RT for 4 minutes. 350 µl neutralization buffer were added and mixed by gentle inversion. After centrifugation at 20800 x g for 7 minutes, the plasmid DNA-containing supernatant was transferred onto the spin column which had been activated with 40 µl activation buffer and kept at RT previously. The loaded spin column was centrifuged at 11000 x g for 1 minute and the flow-through discarded. After two washing steps with 500 µl and 650 µl PX buffer at 11000 x g for 1 minute, a final centrifugation without washing buffer was performed for 2 minutes to completely remove liquid. The spin column was transferred into a new vial and 50 µl elution-PX buffer were added. After two minutes incubation, the

plasmid DNA was eluted by centrifugation at 11000 x g for 2 minutes and the DNA content was determined (NanoDrop). Until further analysis, the plasmid DNA was stored at -20°C.

3.5.4   Sequencing

Both strands of plasmid-DNA was sequenced forward and reverse by Eurofins MWG GmbH (Ebersberg, Germany). Sequences were compared online with target sequences in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3.6   Biochemical analyses

3.6.1   Protein overexpression and purification under denaturing conditions

Per allergenic protein, 30 ml LB medium with kanamycin (final concentration 25 µg/ml) were inoculated from stock solutions and incubated at 150 mot/min and 37°C overnight. From this pre-culture, respectively 7 ml were transferred into 4 culture flasks with 500 ml pre-warmed LB medium with kanamycin, and cultivated at 37°C under constant shaking with 150 mot/min. For the expression of κ-casein, TB medium was used. OD_{600} was observed, and at a value of approximately 0.6 500 µl 1 M IPTG were applied (final concentration 1 mM) to induce protein expression. Growing was stopped when the OD_{600} was between 1.8 and 2, or at latest after 6 hours. Samples of un-induced state and stages after protein induction were taken and analyzed in SDS-PAGE. The cultures were centrifuged at 5445 x g and 4°C for 1 hour. The supernatant was discarded and the cell pellets were weighted. Per 1 g cell pellet, 2.5 ml denaturing lysis buffer pH 8.0 with PI were applied. Cells were re-suspended by gently pipetting, pooled and transferred into a 50 ml Falcon tube to be stored at -20°C.

The lysate was thawn on ice and up to 8 ml were loaded on Poly-Prep columns with 1 ml Ni-NTA resin, pre-equilibrated with lysis buffer pH 8.0. Per allergen, two columns were used in parallel. Binding of the HIS tagged allergens to the Ni-NTA resin was conducted on a "528" testtube rotator at RT for 1 hour.

The columns were placed vertically and the flow-through was collected to be analyzed in SDS-PAGE and repetition of the purification procedure in case of clear target protein remained. Three washing steps at pH 6.3 with each the same volume as the lysate were performed. Four elution steps with 1 ml elution buffer pH 5.9 and another 4 with elution buffer pH 4.5 were conducted. The protein content of wash fractions and elutions was quickly checked with dot blots. Columns were extensively washed with bidest and stored at 4°C. SDS-PAGE was performed with samples from all fractions clearly positive in dot blot analysis. Elutions with high protein content were pooled. Formation of cysteine-mediated inter-protein-bonds and precipitation was prevented by dilution of pooled allergen-specific eluates with 8 M urea and adjustment of pH to 7.4. Dialysis against 2 to 4 l PBS pH 7.4 was

conducted at 4°C under stirring overnight. Depending on the volume of elutions, dialysis was repeated to remove urea completely. Amicons or Centricons with specific MWCO were used for concentration of proteins according to the manufacturer's instruction.

3.6.2 Protein overexpression and purification under native conditions

Protein expression and purification was only applied to α-lactalbumin and ovalbumin. 50 ml LB pre-warmed medium with kanamycin (25 µg/ml) were inoculated with 1 ml pre-culture and treated as described above. Pellets were transferred into -80°C until purification. After thawing on ice, each pellet was resuspended in 5 ml BugBuster solution including 5 µl benzonase (=125 U), 1 µl lysozyme (=30 kU) and 20 µl mix of PI. The suspensions were let on a "528" testtube rotator at 4°C for 30 mins and centrifuged at 5000 x g at 4°C for 40 mins. The supernatants were transferred into fresh tubes, pellets were treated with 100 µl SB/DTT. Poly-Prep columns were loaded with 1 ml Ni-NTA-agarose, corresponding to 0.5 ml resin, and equilibrated with 1 ml BugBuster solution. 5 ml supernatant were transferred into each column and 2.72 mg imidazole were added. Binding to the resin was conducted at 4°C on a rotator for 1 hour. After 5 times washing with 4 ml washing buffer, proteins were eluted with 5 times 0.5 ml elution buffer. SDS-PAGE was performed with samples from all fractions. Elutions 2 and 3 of each protein were pooled and dialyzed against 4 l PBS at 4°C under stirring overnight. Amicons with 3 and 10 MWCO were used to concentrate proteins to a volume of ~300 µl (4045 x g at 4°C for 30 mins). The protein content of the final solutions was determined and proteins were stored at -20°C.

3.6.3 Regeneration of columns

Columns were regenerated by applying 15 ml of 0.2% acidic acid, 30% (w/v) glycerin, bidest, 20% (v/v) ethanol and bidest. Finally, columns were filled with 5% $NiSO_4$ (in bidest) until residual bidest was replaced. Columns were incubated at 4°C overnight and washed with 30 ml bidest.

3.6.4 Native allergenic proteins

Native allergenic proteins were purchased from Sigma Aldrich, Steinheim, Germany except for hen's egg white which was provided by Ovobest, Neuenkirchen-Vörden, Germany. Storage according to the supplier's instructions until use.

Table 5: Native components and extracts.

Component / extract	purity
Albumin from chicken egg white,	Grade VI, ≥98%
α-casein, from bovine milk	≥70%
α-lactalbumin from bovine milk, Type I,	≥85%
ß-casein, from bovine milk,	≥98%
ß-lactoglobulin B from bovine milk,	≥90%
ß-lactoglobulin (A+B) from bovine milk,	≥90%
κ-casein, from bovine milk,	≥70%
Casein	technical grade
Egg white powder	≥80%
Skim milk powder, for microbiology	not given
Trypsin inhibitor from chicken egg white, Type II-0, partially purified ovomucoid containing ovoinhibitor	not given
Trypsin inhibitor from chicken egg white, Type III-0	not given

3.6.5   Determination of protein content

Protein content relative to BSA was determined with a BCA assay (Pierce) according to the manufacturer's instructions. In this assay, protein samples are incubated with a mix of copper sulfate ($CuSO_4$), sodium hydroxide (NaOH), potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6 \cdot 4\ H_2O$) and bicinchoninic acid (BCA). The alkali conditions mediate the reduction of copper ions from Cu^{2+} to Cu^{1+} by peptides of at least 3 AA length, leading to the formation of a chelate complex (Biuret reaction). Two molecules BCA then react with Cu^{1+} ions. The water soluble chelate complex strongly absorbs at 562 nm in a linear way with increasing protein concentration.

9 standard dilutions of provided BSA solution (2 mg/ml) were prepared in the solvent corresponding to that of the samples with unknown protein content. 25 µl of standard dilutions and several dilutions of samples were transferred in two replicates into a 96 well standard plate. 200 µl working solution (a mix of provided Reagent A with Reagent B in relation of 50:1) were added and the plate was shaken for 30 seconds. After incubation at 37°C for 30 mins, the absorbance at 550 was measured on a plate reader. Protein content in the samples was calculated from the standard concentrations using linear regression.

3.6.6   Determination of protein purity

Proteins were separated in SDS-PAGE, stained with colloidal Coomassie and scanned in transmissive light mode on a calibrated imager (GS-800). The optical density (OD) of the gel image was analyzed with Image Lab 5.0 software. Lanes were automatically detected and

manually adjusted with variable lane width. All lanes had equal relative fronts from 0 to 1. Bands were automatically detected with high sensitivity, background was subtracted with disk size 10. Disk size refers to the resolution at which the lane is scanned, sizes ≤10 mm are recommended by the manufacturer. Measured intensity of recognized bands (OD) was integrated and expressed in relative percentages per lane and bands.

3.6.7   TCA precipitation

TCA precipitation was only applied to the supernatant of centrifuged water-insoluble wheat proteins. 4 parts of the supernatant were mixed with 1 part 50% TCA solution to obtain a final TCA concentration of 10%. This mix was vortexed and let at RT for 30 mins. After centrifugation at 16060 x g for 2 mins (Biofuge Pico), the supernatant was carefully removed and discarded. The pellet was resuspended in sample buffer with DTT, heated at 70°C for 10 mins under heavy rotation and stored until use in SDS-PAGE.

3.6.8   Separation of proteins in SDS-PAGE

In general, pre-cast standardized Bis-Tris gels (Invitrogen) were used to guarantee equal conditions and comparability. According to the manufacturer's instructions, LDS sample buffer with freshly added DTT at 50 mM final concentration was used. Samples were heated at 70°C for 10 mins under heavy rotation on a thermomixer (Eppendorf). Electrophoresis was usually performed at 200 V with MES (resolution of medium and small proteins) or MOPS (resolution of large proteins) running buffer, and 500 µl antioxidant solution was added to the cathode chamber in order to avoid re-oxidation of sulfhydryl groups. During the purification process of recombinant proteins, self-cast discontinuous Tris-Glycine gels at different percentages of acrylamide were used and run with Tris-Glycine running buffer containing 0.1% SDS (Table 6). Samples were treated with a self-prepared sample buffer at the same conditions mentioned above. Gels were run at 100 V for 10 mins (focusing of proteins at the border of stacking and separating gel) and then at 180 V until finishing separation.

Table 6: Composition of self-cast Tris-Gylcine gels. Volumes are sufficient for casting 2 gels.

	Stacking gel	Seperating gel			
Component	4%	10%	12%	13%	14%
Bidest [ml]	3.7	2.8	3.1	1.8	1.4
1 M Tris, pH 6.8 [ml]	0.625	-	-	-	-
1 M Tris, pH 8.8 [ml]	-	3.75	3.75	3.75	3.75
Acrylamide [ml]	0.650	3.3	4.0	4.3	4.7
SDS [µl]	50	100	100	100	100
APS 0.1 mg/ml [µl]	25	50	50	50	50
TEMED [µl]	5	5	5	5	5

3.6.9   Staining of SDS-PAGE gels

Gels were stained with solutions based on Coomassie Blue G-250 or R-250 which selectively bind to positively charged and uncharged residues in proteins [130]. Ready-to-use Safe Stain solution or self-prapared Coomassie R-250 were used for initial wheat protein preparations and recombinant proteins. Self-prepared colloidal Coomassie solution was applied to gels to be submitted to MS analysis. Generally, staining was conducted overnight at RT. Gels were destained with bidest until background was low and scanned. Protein bands of interest were excised and stored in 0.5 ml vials at -20°C.

3.6.10 Staining of membranes

PVDF membranes were stained with Amido Black solution for 30 minutes under gentle shaking and treated with destaining solution until background colour was removed.

3.6.11 Documentation of stained SDS-PAGE gels, membranes and X-ray films

Initially, gels were scanned in colour on a HP ScanJet 5317C office scanner. Due to the thickness of gels and the unperpendicular optical path, images obtained were not sharp. Succeeding gels were scanned with the densitometer GS-800 (calibrated transparency, BioRad) and processed with the Quantity One software. X-ray films and membranes were scanned with both HP ScanJet and GS-800.

3.6.12 Protein digestion and identification with MALDI-TOF mass-spectrometry

Matrix-assisted laser desorption/ionization (MALDI), Time-of-Flight (TOF) mass spectrometry is a means to analyze proteins, peptides, sugars and DNA. The sample is mixed with a matrix, e.g. HCCA, and transferred onto a plate where the dried mix forms a crystal. In a high vacuum, this crystal is shot with a pulsed laser beam. The energy of the laser is absorbed by the matrix and transferred on the sample molecules. Ionized molecules start flying in the electric field applied and are detected in a time-dependent manner according to their mass, provided the molecule is singly charged. Large molecules impinge later compared to smaller ones. Results are depicted in *m/z* (mass/charge) and intensity (arbitrary units). In case of multiple charging, the time of flight is reduced and the specific molecule-specific peak shifts to smaller values in the mass spectrum. The resolution is improved by application of a reflector which increases the flight path and is suited for peptides. Proteins are measured without additional reflexion of ions (linear mode). The MALDI-TOF mass spectrometer is calibrated with standard molecules (proteins, peptides), dependent on the samples to be examined.

Tryptic digestion was performed according to the manufacturer's instruction, described in "In-Gel Tryptic Digestion Kit" (Thermo Scientific, Pierce Biotechnology, Rockford, USA) with some modifications. Each band was destained twice with 200 µl DS at 37°C on a Thermomixer at 700 rpm for 30 mins. Reduction with 100 µl 50 mM TCEP in DB at 60°C for 10 mins and alkylation with 100 µl 100 mM IAA in DB at RT for 1 h were conducted. After two additional treatments with 200 µl DS at 37°C and 700 rpm for 15 mins, 100 µl ACN were applied for 15 mins. ACN was discarded and gel bands were air dried. 15 µl activated trypsin in DB (MS grade) with a concentration of 0.01 µg/µl were applied. After 10 mins, 25 µl DB were added and the gel band was disrupted with a fresh tip. The digestion was conducted at 37°C and 700 rpm overnight. After short centrifugation up to 16000 x g and 10 mins sonification treatment, proteolytic digests were collected (first supernatant). Few µl of 1% TFA/ACN (70%/30%, v/v) were added to the remains in the vial, 10 mins sonication and centrifugation were applied. The second supernatant was collected and pooled with the first one. 2 µl of this pool were mixed with 2 µl matrix solution (2 mg HCCA in 100 µl 0.1% TFA/ACN (70%/30%, v/v), diluted 1:2 in 50% ACN, sonicated for 10 mins and shortly centrifuged at 16100 x g) and approximately 1 µl were dotted on an MTP AnchorChip TM 800/384, mounted on an MTP 384 target plate ground steel, and let dry. A mix of peptides 1:2 in matrix solution was also applied to calibrate the system (Table 7).

Table 7: Peptide calibration standard for MALDI-TOF MS.

Peak label ([M+H]+, mono isotopic)	Reference Mass/Da (m/z)
Bradykinin (1-7)	757.399160
Angiotensin II	1046.541800
Angiotensin I	1296.684800
Substance P	1347.735400
Bombesin	1619.822300
Renin Substrate	1758.932610
ACTH clip (1-17)	2093.086200
ACTH clip (18-39)	2465.198300
Somatostatin (28)	3147.471000

Samples and peptide standards were measured in reflector mode in the range from at least 500 to 6000 *m/z* according to settings shown in Supplement Figure 1.

Processing and analysis of measured *m/z* spectra was conducted with flexAnalysis programme using a modified method, S/N was set to 2 for all analyses (Figure 3).
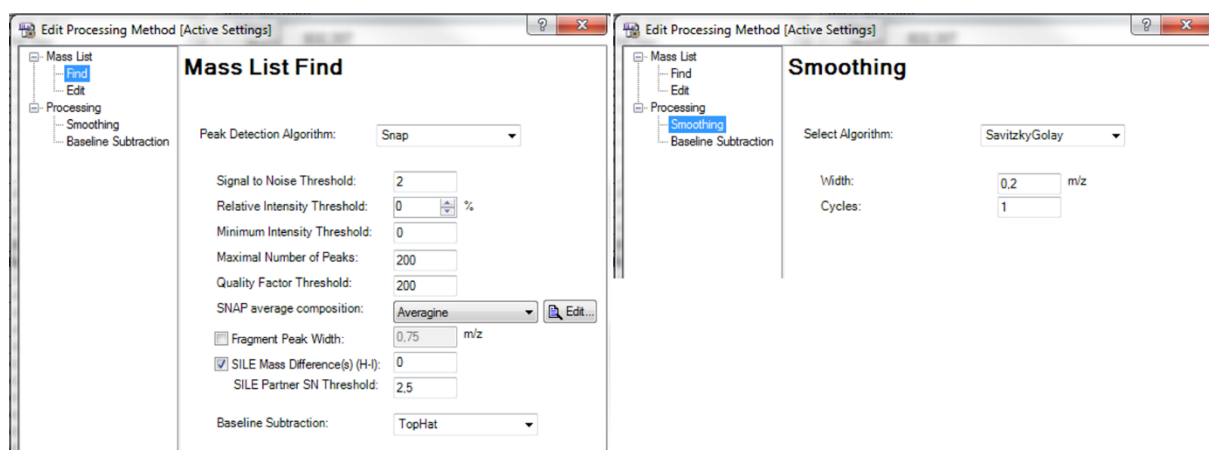
Figure 3: Settings of the method protocol to process and convert an *m/z* spectrum into mass list.

Measured *m/z* spectra were processed and submitted to Mascot search engine in order to identify matches with sequences in SwissProt databases (wheat study: "Other green plants", microarray study: "Other mammalia" for CM, and "Metazoa" for HE proteins). In general, mass tolerances from 50 to 500 ppm and missed cleavage sites (partials) of up to 5 were applied to the spectra until the hit with highest significant score was achieved. As defined in Mascot, "protein score is -10*Log(P), where P is the probability that the observed match is a random event". Protein scores greater than 55, 54 or 63 are significant (p<0.05) in databases "Other green plants", "Other mammalia" and "Metazoa", respectively. Hits with high scores and sequence coverage obtained by lowest ppm and partials are reported. For the application of theoretical *in silico* digested protein sequences to the *m/z* values of the digested peptides, a mass tolerance of maximal 200 ppm and 2 partials were accepted in the wheat study. In identification of purified native and recombinant proteins, also tolerances greater 200 ppm and partials greater 2 were accepted.

3.6.13 Protein extraction of wheat proteins

For extraction of water and salt soluble (albumins and globulins) and water insoluble (gliadins and glutenins) protein fractions, a modified protocol was applied as described elsewhere [84]. Grains were ground (ProfiMixx 47 with grain top element) and 10 grams flour were dissolved in 40 ml 0.5 M NaCl with protease inhibitors cocktail (Roche complete, EDTA-free) and mixed on a testtube rotator at 4°C overnight. After centrifugation with 12000xg for 20 minutes at 4°C, the supernatant with water and salt-soluble proteins was isolated, aliquoted and stored at -20°C. The pellet was manually crushed and two washing steps in with 0.5 M NaCl and one washing step with ultrapure water were performed in a volume of 100 ml/step on a shaker with 350 mots/min at 4°C. Protein content was checked photometrically at 280 nm. Water-insoluble proteins were extracted overnight with 40 ml 80 mM Tris-HCl, pH 8.0,

50% propan-2-ol (v/v) and 20 mM DTT on a shaker with 350 mots/min at 4°C, and recovered by centrifugation with 12000g for 20 minutes at 4°C, aliquoted and stored at -20°C.

Additional grains were either ground manually with mortar and pistil, or with a falling number mill to a size of <0.8 mm. Protein extraction of these cultivars was conducted with modifications of the standard protocol based on suggestions by DuPont, Chan, Lopez, Vensel [131]. Water and salt soluble proteins were extracted with 1.5 ml 0.3 M NaI from 250 mg wheat flour. After 1 hour rotation at RT, albumins and globulins were obtained by centrifugation at 16100 x g for 5 mins. Two washing steps with equal volume of NaI and another two washing steps with Bidest followed. Gliadins were extracted with 1.5 ml 50% propan-2-ol at RT for 1 hour and obtained as described above. The solvent was evaporated by application of nitrogen and proteins resuspended in 1.5 ml 8 M urea by 30 mins sonication. Glutenins and other protein remains were extracted with 1.5 ml LDS SB with 200 mM DTT at RT overnight. This extract was not used in the present study.

3.7 Immunoblots

3.7.1 Dot Blots

Dot blots used in this study were prepared on nitrocellulose membrane which was treated with usually 1 µl of the protein solution.

During all allergen detections with ^{125}iodine labelled anti human IgE from goat, a positive control was used. Droplets of 1 µl containing myeloma IgE at 166.3 (Cap 6), 83.1 (Cap 5), 41.6 (Cap 4), 10.4 (Cap 3), 2.6 (Cap 2), 0.52 (Cap 1) and 0.26 (Cap 0) kU/l were applied onto a nitrocellulose membrane along with PBS (diluent), dried overnight and used the next day or stored at -20°C between Whatman sheets. There was no quantification of the signals obtained.

Dots blots were also used during the purification process of recombinant allergens in order to check the protein content of elutions qualitatively. The treated membrane was therefore incubated with Ponceau-S solution which indicated protein content by displaying red spots.

3.7.2 Western Blot

In the first immunoblot screen of wheat subjects, water and salt soluble proteins and a prestained 10 band molecular weight marker (Fermentas) were separated in a SDS- PAGE on 4-12% Bis-Tris Zoom gels (1.0 mm x IPG well, invitrogen) with 20 µg protein per cm gel under reducing conditions and blotted onto 0.45µm Immobilon-P membrane (Millipore) at 30 V for one hour. Water insoluble proteins were applied with 30 µg protein per cm gel. The membrane was stored between Whatman papers overnight (blocking) and cut into strips of approximately 3 mm broadth. Patient serum with <50 kU$_A$/l was diluted 1:5 in PBS-Tween

0.05% (v/v) with 1% BSA and 10% Goat serum (v/v), or 1:10 if the specific IgE was larger than 50 kU$_A$/l. Negative and atopic (no food allergy, but allergy to house dust mite) sera were also applied and diluted 1:5. The background control was only treated with 1:5 in PBS-Tween 0.05% (v/v) with 1% BSA and 10% Goat serum (v/v). After wetting with PBS-T 0.05%, strips were placed in incubation trays and 300 µl diluted serum per strip were applied. Incubation was conducted on a Duomax rocking shaker at 18/mins at RT for 2 hours. After washing three times with PBS, strips were incubated with [125]iodine labelled anti human IgE from goat (Ringel Immunologische Laboratorien, Aachen) diluted 1:20 to 1:40 in PBS-Tween 0.05% (v/v) with 1% BSA and 10% Goat serum (v/v). A dot blot with myeloma IgE at concentrations corresponding to CAP classes 0 to 6 was also incubated and served as positive control. Strips were dried, arranged on Whatman paper and transferred into an autoradiography cassette with intensifying screen (Thermo Fisher). Kodak BioMax XAR films were exposed at -80°C for up to 15 days (depending on the level of specific IgE and estimated strength of the [125]iodine labelled antibody) and scanned with similar settings among films (highlights 223, shadows 5, midtones 2.3-2.9, resolution 300) and saved as TIF. Each strip image including negative controls was cut from the whole film image and signals were individually amplified to visualize minimal binding reactions. Bands were independently counted by three members of the work group from printed figures.

The second immunoblot screen re-used the serum dilutions from the first screen. Incubation with serum was performed in 15 ml tubes, lying on a rocking platform at 4°C overnight. All subsequent steps were performed as described above in 4 well incubation chambers.

3.7.3   Illustration of protein fractions in SDS-PAGE, determination of Western blot efficiency and handling of immunoblots

The protein content of the fractions obtained during the extraction process was determined photometrically at λ=280 nm for the wash fractions during the extraction process, and with a BCA assay for albumins/globulins and gliadins/glutenins fractions after finishing the extraction. A frozen aliquot of albumins/globulins was used in different dilutions (in 0.5 M NaCl) to determine protein content against a calibrated BSA standard. Due to incompatibility of propan-2-ol and DTT in the extraction buffer of gliadin/glutenin proteins with the BCA assay, these proteins, having precipitated by freezing at -20°C, were pelleted by short centrifugation at 10.000 x g. The supernatant was discarded and the pellet resuspended in 5% SDS. Different dilutions were assayed with 5% SDS as diluent for protein samples and BSA concentration standards.

Table 8: Protein content of wheat extracts and washs

Fraction	Protein content [mg/ml]
albumin/globulin fraction	8.8
wash 1	0.41
wash 2	0.08
wash 3	0.04
gliadins/glutenins fraction	8.5

Samples of water-soluble albumin/globulin proteins and of all washing fractions were diluted 1:2 with LDS SB (x2,) with and without DTT. The high content of propan-2-ol (50%) in the water-insoluble gliadin/glutenin protein fraction impeded proper loading of the gel, thus the supernatant was discarded and the pellet was resuspended in sample buffer (x2) with and without DTT to the final volume equal to the water-soluble protein sample in SB. Equal volumes of water-soluble proteins and water-insoluble proteins were loaded on the gel, the volume of the washing fractions was adjusted relative to the washing volume during protein extraction. The gel was stained with Safe Stain and scanned on a colour laser scanner (238-0-2.2).
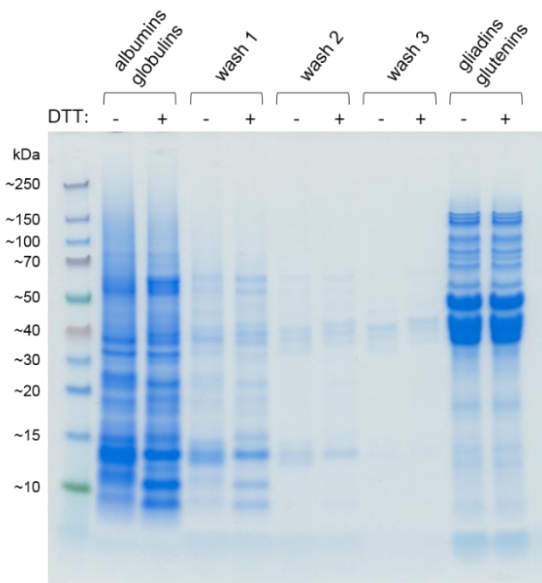
Figure 4: SDS-PAGE of wheat protein extracts and wash fractions (1), stained with Safe Stain. Equal relative volumes (extracts 5 µl, washs 12.5 µl) were loaded and proteins were seperated under reducing (+DTT) and non-reducing (-DTT) conditions on a 4-12% Bis-tris gel with MES buffer. Wash 3 contains protein (at ~ 40 kDa) which corresponds to the big proportion of gliadins and glutenins in the water-insoluble fraction. Albumins and globulins, and wash 1 clearly show different band patterns from ~40 kDa to < ~10 kDa when treated with or without DTT which points to cystein-dependent tertiary protein structures. In the gliadin/glutenin fraction, two bands below ~15 kDa show high similarity to the albumin/globulin fraction and wash 1 to 2, implying insufficient fractionation.

In order to achieve higher sensitivity towards protein content of the washing fractions, and to determine probable protein loss during precipitation of water-insoluble proteins, the

supernatant (200 µl) was treated with TCA to precipitate proteins which were resuspended in 20 µl sample buffer with DTT to obtain a 10fold concentration. SDS-PAGE was repeated and a colloidal Coomassie stain was applied. The gel was scanned on a calibrated scanner and appears in black and white.
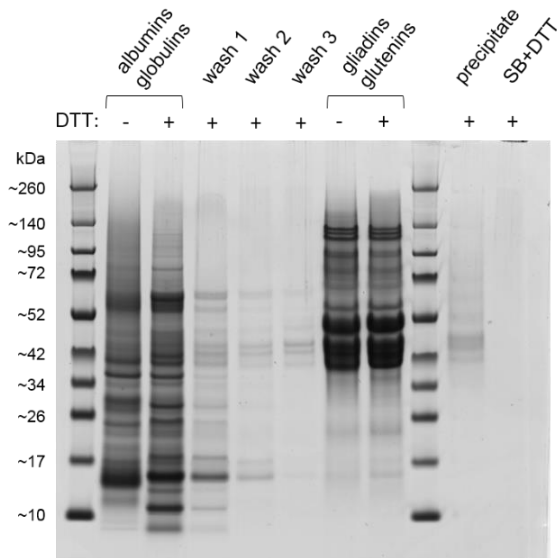
Figure 5: SDS-PAGE of wheat protein extracts and wash fractions (2), stained with colloidal Coomassie. Equal relative volumes (extracts 4 µl, washs 10 µl) were loaded and proteins extracts (albumins/globulins, gliadins/glutenins) were seperated under reducing (+DTT) and non-reducing (-DTT) conditions on a 4-12% Bis-tris gel with MES buffer. Wash 1 to 3 contain protein (at ~ 42 kDa) which corresponds to the big proportion of gliadins and glutenins in the water-insoluble fraction as shown previously. Small proteins below ~17 kDa are clearly visible in Wash 1 and 2. The gliadin/glutenin fraction contains proteins below 17 kDa which correspond to the albumin/globulin fraction and wash 1 and 2. The 10fold concentrated precipitate, applied with 4 µl, contains negligibly little protein of ~72 to ~42 kDa size. The slightly different relative molecular weights of the protein ladder, compared to Figure 4, are due to a different lot provided by the manufacturer.

The extraction of wheat proteins resulted in high yields of albumins/globulins and gliadins/glutenins. However, cross contamination cannot be excluded despite intense washing.

The basis for calculating amounts needed for protein separation on a ZOOM-gel was ~20 µg protein per cm gel which was 7.5 cm in length. The transfer of albumins and globulins proved to be very effective, only few remains of protein were detected in the gel after blotting (Figure 6).

Initial western blots of the gliadin/glutenin fraction showed unequal transfer, the HMW-glutenins being blotted with least efficiency (data not shown). In order to compensate this poor efficiency and the general low abundance of HMW-glutenins and also omega-gliadins, an overloading with a total volume of 60 µl protein in SB with DTT was accepted, resulting in poor separation of proteins between ~40 and ~50 kDa, representing LMW-glutenins and α-, β-, and γ-gliadins. Zoom gels and strips of blotted PVDF membranes were regularly stained after blotting with Safe Stain or Amido Black, respectively, to check blot efficiency (Figure 6).
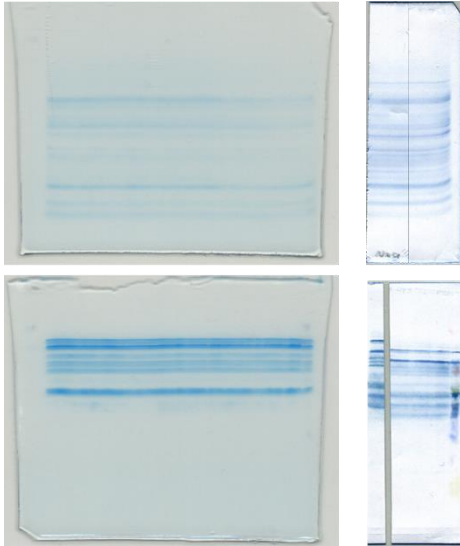
Figure 6: Blot efficiency of albumins/globulins and gliadins/glutenins under standard conditions. Albumins/globulins (top left) and gliadins/glutenins (bottom left) were separated on ZOOM gels (4-12%, Bis-Tris) and blotted onto PVDF membranes (right top and bottom). Gels were stained with Safe Stain and membranes with Amido Black. The inner area of the membranes was cut in strips and used for immunoblots. Outer areas of the membranes were stained with Amido Black. Gliadins/glutenins are blotted askew which was considered in vertical alignment of single strips.

The first immunoblot comprised patients with sIgE to wheat from 0.05 to >100 kU$_A$/l (CAP classes 0 to 6) in order to estimate the optimal exposure time of low and high IgE sera to obtain a sufficient signal to noise ratio with good resolution of single bands. In subsequent immunoblots sera of equal CAP classes were applied along with negative controls (negative and atopic serum, background control). Exposure times ranged from 3 to 14 days. Film and (stained) membrane strips were further processed as described in Figure 7.
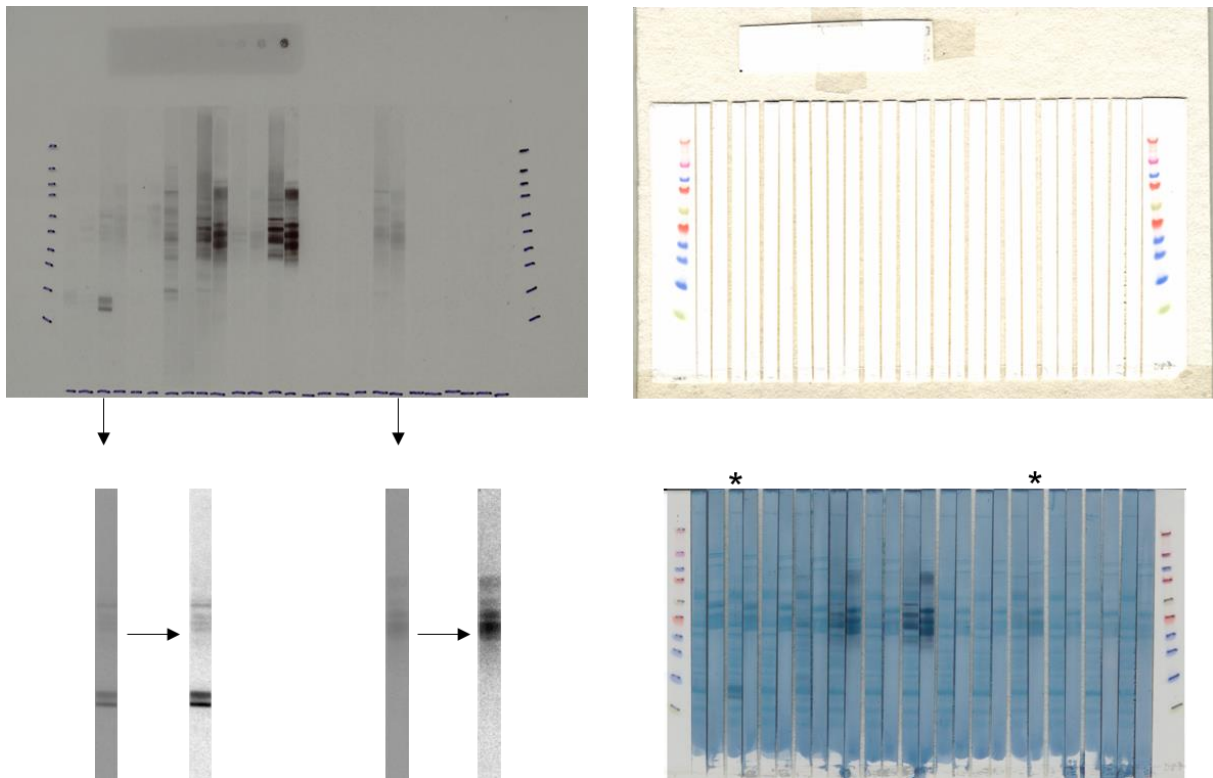
Figure 7: Processing of immunoblots. The exposed film (top left) was scanned and every area corresponding to a strip (top right) was cut from the image and saved. Signals on every strip including negative controls were enhanced by the auto adjustment option in Irfanview software and saved (bottom left). Final vertical alignment of single strips was conducted by overlaying film and Amido Black stained blots (bottom right). Asterisks indicate the strips chosen as an example.

After completion of immunoblots and enhancement of signals, strips were aligned according to protein fraction. Despite using precast gels and standard conditions during electrophoresis, slight deviations among different gels over the period of all immunoblots occurred and could not be fully compensated. This applies to immunoblots with several detected bands where distal bands may be slightly dispositioned in the whole image and the reference gel compared to their actual position. Protein bands of the prestained protein marker <35 kDa are askiew and may deviate by 1 mm, subsequent band counts are therefore to a certain degree arbitrary and only a means of semi-quantitative assessment.

3.8 Microarray assays

3.8.1 Coating of silicon slides

Silicon slides where freshly prepared and stored dry by the lab personnel in Milan as previously described in Pirri, Damin *et al.* [62].

3.8.2 Preparation of recombinant and native proteins

After purification, dialysis and concentration, the protein content was determined. In Table 9, concentrations of spotted recombinant proteins are summarized. Protein purity was assessed by SDS-PAGE (Figure 8). In all downstream applications of recombinant proteins, aliquots of this purification process were used.

Table 9: Concentration of recombinant proteins used in the initial spotting pattern.

rec proteins	rel. stock conc. [mg/ml]	applied rel. conc. [mg/ml]
αs_1-casein	2.22	1.11
αs_2-casein	0.85	0.85
ß-casein	3.51	1.75
κ-casein	0.98	0.98
α-lactalbumin	1.33	1.33
ß-lactoglobulin	1.06	1.06
ovomucoid	2.95	1.47
ovalbumin	1.21	1.21
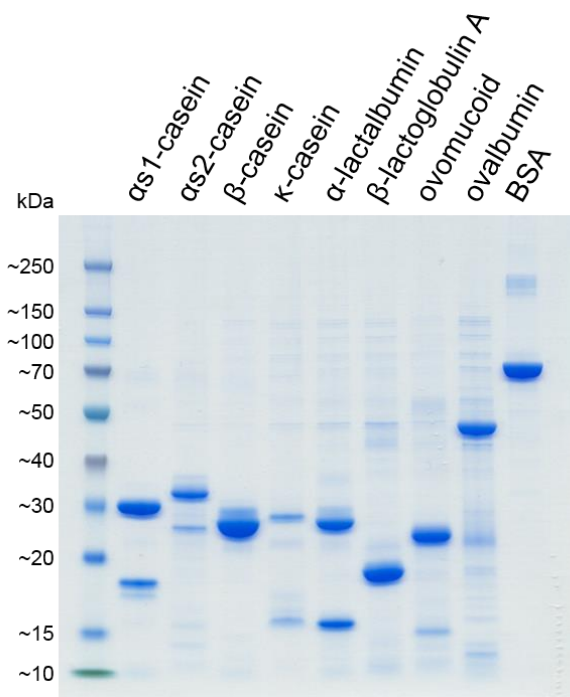
Figure 8: SDS-PAGE of purified recombinant proteins.

2 µg protein were applied on a 4-12% BT gel, stained with Safe Stain.

The set of native allergens was represented by CM components α-, β-, κ-casein, and total casein. As correlates to CM and HE panels on ImmunoCAP, skim milk and egg white were selected. All native allergens were available as powder.

In the initial microarray assays, stock solutions at 10 mg/ml in bidest were prepared. Due to poor solubility, stock solutions of α-, β- and total casein were prepared in 10 mM NaOH. PBS was used for diluting to final concentration at 1 mg/ml. Aliquots of working solutions were repeatedly used.

In the final microarray, all native proteins were diluted in PBS. Total casein was solubilized by sonfication. Working solutions from stocks were aliquoted and stored at -20°C. In contrast to initial applications, aliquots were only used once and discarded after use.

3.8.3   Spotting of proteins

Native and recombinant allergenic proteins of hen's egg and cow's milk were plated in 384-well plates and spotted in replicates of five spots per protein according to a designed pattern using a SciFlexArray spotter. The pattern also included PBS and orientation spots of Cy3-labeled streptavidin at a concentration of 0.1 mg/ml in PBS. Spotting was performed at RT, the volume was 400 pl per drop. Printed slides were stored in a humid chamber at room temperature overnight and used up to two days later.

3.8.4   Assaying with patient sera and scan

The required number of slides was blocked with 50 mM Ethanolamine in 1 M Tris-HCL pH 9 for 1 hour. Each slide was rinsed in distilled water and dried with nitrogen. Slides were transferred in small Petri dishes, placed in a humid chamber, and incubated with 20 µl undiluted serum for 2 hours. After rinsing, the slides were washed in 0.05 M Tris-HCl pH 9, 0.25 M NaCl, 0.05% (v/v) Tween 20 under stirring for 10 min, rinsed and dried again to be incubated in a humid chamber with 100 µl secondary antibody solution which contained a Cy3-labeled anti human IgE monoclonal or polyclonal and a Cy5-labeled anti human IgG$_4$ monoclonal secondary antibody in 50 mM Tris-HCL pH 7.6, 0.15 M NaCl, 0.02% (v/v) Tween-20. The secondary antibodies were applied in 1:200 dilution each. After 1 hour, slides were rinsed, washed in PBS under stirring for 10 minutes, rinsed and dried. All steps were performed at room temperature, incubations and the last washing step in darkness.

Fluorescence was measured the same day using a PerkinElmer Scanarray Lite laser scanner apparatus based on a confocal architecture. After a fast prescan at Cy3-channel with 90% laser power and 70% PMT (photo multiplier) with 50 µm resolution, focus was set by automatic line scan (in final microarrays on Cy3-labeled 35 µg/ml IgE spots) at 80% laser power and PMT. Fine scan was performed at 70, 80 and 90% (Cy3) and 60, 70, and 80% (Cy5) laser power and PMT at a resolution of 10 µm.

3.8.5   Quantification of data

The fluorescence was quantified with ScanArray Express 4.0 software by Perkin Elmer using the following application settings: Method: footprint; Maximum footprint: 100 µm; Spot finding algorithm: new fast spot; Locating range: 2 pixels; Filter data: 3x3 median filter; Protocol Easy Quant; Quantitation method: adaptive circle with minimal spot diameter (% of nominal): 50, and maximal spot diameter (% of nominal): 200; Normalization method: LOWESS. Identification of spots was checked and manually corrected if the quantitation template did not match (a) spot(s). The quantified fluorescence data including median, mean, median minus background, mean minus background and signal-to-noise ratio were exported to MS EXCEL 2003.

3.8.6   Data mining and analysis of the final microarray

Analysis was performed excluding irregularly shaped, smeared, and missing spots. In addition to these visual constraints, in the final microarray spots were also excluded under certain statistical conditions: each spot is characterised by its standard deviation of fluorescence (Ch1 SD), which refers to the homogeneity of the spot, and standard deviation of its surrounding background fluorescence (Ch1 B SD), which is a means to assess lateral influences on the spot fluorescence by outer spots (when too close due to frame shift) or noise. After applying visual control, the values for Ch1 SD and Ch1 B SD were independently ranked. If the highest ranked value exceeded the double of the mean of the remaining 4 (or less, if visual control had led to exclusion) spots, it was excluded. This procedure was repeated until the highest ranked value was within the defined range of the remaining spots. In few cases, this led to formation of two pairs of spots with different sums of SD's. This was accepted as a balance in order to cover the range of the probable specific fluorescence, avoiding bias. This procedure was independently applied to signals detected on Cy3-channel or Cy5-channel.

3.9   Chemicals

4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), research grade	Serva, Heidelberg, Germany
Acetic acid	Merck, Darmstadt, Germany
Acrylamide (prop-2-enamide), 30% / Bis 29:1	Bio-Rad, Munich, Germany
Agar Kobe I	Roth, Karlsruhe, Germany
Albumin bovin serum, Fraction V	Sigma Aldrich, Steinheim, Germany
Albumin human serum, 96-99%	Sigma Aldrich, Steinheim, Germany
Ammonium persulfate (p.a.)	Roth, Karlsruhe, Germany

Ammonium sulfate (p.a.)	Roth, Karlsruhe, Germany
beta-Mercaptoethanol (p.a.)	Roth, Karlsruhe, Germany
Bestatin	AppliChem, Darmstadt, Germany
Coomassie Brilliant Blue G250	Roth, Karlsruhe, Germany
Dipotassium phosphate (K_2HPO_4)	Merck, Darmstadt, Germany
Dulbecco's Phosphate buffered saline (PBS), w/o $CaCl_2$, $MgCl_2$	Biomol, Hamburg, Germany
E-64	AppliChem, Darmstadt, Germany
Ethanolamine (2-aminoethanol)	Merck, Darmstadt, Germany
Goat serum	Gibco/Invitrogen, Washington, USA
Glycerol, 86% (p.a.)	Roth, Karlsruhe, Germany
Glycine	AppliChem, Darmstadt, Germany
Hydrochloric acid fuming 37% (p.a.)	Merck, Darmstadt, Germany
Imidazole	AppliChem, Darmstadt, Germany
Iodoacetamide, >99%	Sigma-Aldrich, Steinheim, Germany
Isopropyl alcohol (2-propanol) (LC-MS grade)	Roth, Karlsruhe, Germany
Isopropyl β-D-1-thiogalactopyranoside (IPTG) >99%	Roth, Karlsruhe, Germany
Kanamycin	Roth, Karlsruhe, Germany
Lectin from Ricinus communis agglutinin	Sigma Aldrich, Schnelldorf, Germany
Leupeptin	AppliChem, Darmstadt, Germany
Methanol	Sigma Aldrich, Steinheim, Germany
	Roth, Karlsruhe, Germany
Monopotassium phosphate (KH_2PO_4)	Merck, Darmstadt, Germany
Nickel-II-sulfat-heptahydrat, 99.999%	Sigma-Aldrich, Steinheim, Germany
Orthophosphoric acid, 85% (p.a.)	Roth, Karlsruhe, Germany
Pepstatin A	AppliChem, Darmstadt, Germany
Phosphate buffered saline (PBS), 10x	Trevigen, Gaithersburg, USA
Phosphoramidon, research grade	Serva, Heidelberg, Germany
Ponceau S	Roth, Karlsruhe, Germany
Potassium carbonate (p.a)	Merck, Darmstadt, Germany
Sodium chloride (p.a.)	Merck, Darmstadt, Germany
Sodium dihydrogen phosphate (p.a.)	Merck, Darmstadt, Germany
Sodium dodecyl sulfat (SDS)	AppliChem, Darmstadt, Germany
N,N,N',N'-Tetramethylethane-1,2-diamine (TEMED)	Bio-Rad, Hercules, USA
Trifluoroacetic acid (Spectroscopy grade)	AppliChem, Darmstadt, Germany
Tris(hydroxymethyl)aminomethane (Tris)	Sigma Aldrich, Steinheim, Germany
Tryptone/peptone from casein	Roth, Karlsruhe, Germany

Tween-20   Roth, Karlsruhe, Germany

Urea   AppliChem, Darmstadt, Germany

Yeast   Roth, Karlsruhe, Germany

3.10 Protease inhibitors for protein extraction and purification

For extraction of wheat proteins, cOmplete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) was used according to the instructions of the manufacturer. Protein purification was conducted with a mix of inhibitors in order to prevent possible degradation during binding of HIS-tagged proteins on the Ni-NTA agarose at RT.

Table 10: Protease inhibitors used during protein purification.

Inhibitor	Concentration of stock solution and diluent	Concentration of working solution	Inhibition of
Phosphoramidon	5mM (MeOH)	10 µM	metallo-endoproteinases
AEBSF	100mM (Bidest)	200 µM	serine proteases, irreversible
Pepstatin A	5mM (MeOH)	10 µM	aspartic acid proteases, reversible
E-64	5mM (50% EtOH)	10 µM	cysteine proteases, irreversible
Bestatin	5mM (MeOH)	10 µM	amino-peptidases, reversible
Leupeptin	25mM (Bidest)	50 µM	serine and cysteine proteases

Stock solutions were aliquoted and stored at -80°C (long term) or -20°C (short term).

3.11 Protein markers and ladders

Spectra Multicolor Broad Range Protein marker,
10-260 kDa   Fermentas, St. Leon-Rot, Germany

Protein marker I, 14.4-116 kDa   Peqlab, Erlangen, Germany

6xHis protein ladder   Qiagen, Hilden, Germany

T7 tag positive control   Merck, Darmstadt, Germany

3.1   Kits

BugBuster plus Benzonase Nuclease   Novagen/Merck, Darmstadt, Germany

EURx GeneMATRIX Plasmid Miniprep DNA   Roboklon, Berlin, Germany
Purification Kit

Pierce BCA Protein Assay Kit   Thermo Fisher Scientific / Life Technologies
                                                  (vendor), Darmstadt, Germany

EasyLink HRP conjugation kit   Abcam plc, Cambridge, UK

3.1   Enzymes

Benzonase nuclease, >90% purity   Novagen/Merck, Darmstadt, Germany
Chymotrypsin, certified MS grade   Dichrom, Marl, Germany
rLysozyme (>95%) solution   Novagen/Merck, Darmstadt, Germany
Trypsin, certified MS grade   Dichrom, Marl, Germany

3.2   Purchased Buffers, media and solutions

Flamingo Fluorescent Gel Stain, 10x   BioRad, Munich, Germany
Gel-Dry solution, 1x   Invitrogen, Darmstadt, Germany
Ni-NTA Agarose   Qiagen, Hilden, Germany
NuPAGE Antioxidants   Invitrogen, Darmstadt, Germany
NuPAGE MES SDS running buffer, 20x   Invitrogen, Darmstadt, Germany
NuPAGE MOPS SDS running buffer, 20x   Invitrogen, Darmstadt, Germany
NuPAGE LDS sample buffer, 4x   Invitrogen, Darmstadt, Germany
NuPAGE Transfer buffer, 20x   Invitrogen, Darmstadt, Germany
Simply Blue Safe Stain   Invitrogen, Darmstadt, Germany
SOC medium   Invitrogen, Darmstadt, Germany
SuperSignal West Dura Ext. Duration Substrate   Thermo Scientific, Rockford, USA

3.3   Self-prepared buffers, media and solutions

Name	Composition	
Blocking buffer, pH 9 with acetic acid	50 mM	Ethanolamin
	1 M	Tris
Wash buffer, pH 9 with acetic acid	250 mM	NaCl
	50 mM	Tris
	0.05% (v/v)	Tween-20
Incubation buffer, pH 7.6 with acetic acid	150 mM	NaCl
	50 mM	Tris
	0.02% (v/v)	Tween-20
Lysis buffer (native), pH 8 with NaOH	50 mM	NaH_2PO_4
	300 mM	NaCl
	10 mM	Imidazole
Wash buffer (native), pH 8 with NaOH	50 mM	NaH_2PO_4
	300 mM	NaCl
	20 mM	Imidazole

Elution buffer (native), pH 8 with NaOH	50 mM	NaH_2PO_4
	300 mM	NaCl
	250 mM	Imidazole
Lysis buffer (denaturing), pH 8 with NaOH	100 mM	NaH_2PO_4
	10 mM	Tris-HCl
	8 M	Urea
Wash buffer (denaturing), pH 6.3 with HCl	100 mM	NaH_2PO_4
	10 mM	Tris-HCl
	8 M	Urea
Elution buffer I (denaturing), pH 5.9 with HCl	100 mM	NaH_2PO_4
	10 mM	Tris-HCl
	8 M	Urea
Elution buffer II (denaturing), pH 4.5 with HCl	100 mM	NaH_2PO_4
	10 mM	Tris-HCl
	8 M	urea
IPTG, sterile filtered, diluted in media to 1mM	1 M	IPTG
Kanamycin, diluted in media to 25µg/ml	25 mg/ml	Kanamycin
LB medium, autoclaved	10 g/l	NaCl
	10 g/l	Tryptone
	5 g/l	Yeast
LB agar, autoclaved	15 g/l LB	Agar
TB medium, autoclaved	12 g/l	Tryptone
	24 g/l	Yeast
	4 ml/l	Glycerol
	12.5 g/l	K_2HPO_4
	2.3 g/l	KH_2PO_4
Coomassie Blue	0.1 g	Coomassie R-250
	10 ml	AcOH
	40 ml	MeOH
	50 ml	Bidest
Coomassie Blue colloidal, Solution A	8 g	$(NH_4)_2SO_4$
	1.6 ml	H_3PO_4
	76.8 ml	Bidest
Coomassie Blue colloidal, Solution B	1.6 ml	0.05% Coomassie G-250
Final stain solution	20 ml	MeOH
	~80 ml	Solution A
	1.6 ml	Solution B

Amido Black Stain	0.1 g	Amido Black
	25 ml	Propan-2-ol
	10 ml	AcOH
	75 ml	Bidest
Amido Black Destain	25 ml	Propan-2-ol
	10 ml	AcOH
	75 ml	Bidest
Ponceau S	0.5 g	Ponceau S
	100 ml	Acidic acid 5% (v/v)
10% SDS solution (1 l)	100 g	SDS
	900 ml	Bidest
SDS sample buffer, 5x	1.25 ml	1M Tris, pH 6.8
	5 ml	100mM EDTA, pH 6.8
	1 ml	β-ME
	1 g	SDS
	ad 10 ml	Bidest
	10 ml	Glycerol
Running buffer, 10x	30.3 g	Tris
	144 g	Glycine
	ad 1000 ml	Bidest
SDS running buffer (1 l)	100 ml	Running buffer, 10x
	10 ml	10% SDS solution
	ad 1000 ml	Bidest
Transfer buffer (Bjerrum), pH 9.2	48 mM	Tris
	39 mM	Glycine
	0.1%	SDS
	10% (1 gel)	MeOH
	20% (2 gels)	MeOH
Blocking solution (Immunoblots)	1%	BSA
	10% (v/v)	Goat serum
	0.05% (v/v)	Tween-20 in PBS, pH 7.0
Destain solution	80 mg	NH_4HCO_3
	20 ml	ACN
	20 ml	Bidest
Digestion Buffer (25 mM)	20 mg	NH_4HCO_3
	10 ml	Bidest

3.4   HRP coupling to lectin

Lectin from *Ricinus communis* has a relative MW of 120 kDa (RCA_{120}). It specifically binds to β-galactosides, e.g. lactose. 100 µl lectin at a concentration of ~4 mg/ml were dialysed against 4 l PBS at 4°C overnight. The binding reaction to 100 µg HRP was performed at RT according to the manufacturer's instructions.

3.5   Hardware

Device	Name by manufacturer	Manufacturer/provider
Blot module	Blot module for XCell II	Invitrogen, Darmstadt, Germany
Centrifugation	BioFuge Pico	Heraeus, Hanau, Germany
	Centrifuge 5417C	Eppendorf, Hamburg, Germany
	3K15	Sigma Laborzentrifugen, Osterode am Harz, Germany
	Megafuge 1.0R	Heraeus, Hanau, Germany
	250ml Dry Spin bottles	Thermo Electron, Langenselbold, Germany
	Sorvall RC-5C Plus	Kendro, Newtown, USA
Chromatography	Poly-Prep Chromatography columns, 2 ml bed volume and 10 ml reservoir	Bio-Rad, Munich, Germany
Detection devices	ChemiDoc MP Imaging System	Bio-Rad, Munich, Germany
	Densitometer GS-800	Bio-Rad, Munich, Germany
	Autoradiography cassette FBCA 810	Fisher Scientific, Pittsburgh, USA
	45 Compact, Type 1120	Protec Gerätebau, Oberstenfeld, Germany
	Scanarray Lite laser scanner	PerkinElmer, Waltham, USA
Dialysis	Dialysis tubular membranes	Roth, Karlsruhe, Germany
	ZelluTrans / Roth locking clips, 65mm	Roth, Karlsruhe, Germany
Drying systems	Dryease minigel drying system	Invitrogen, Darmstadt, Germany
Incubation	CO_2 incubator	Heraeus, Hanau, Germany
	3033 (shaking incubator)	GFL, Burgwedel, Germany
	nunc 4 well dish non-treated, 127.8x85.5mm	Thermo Scientific, Schwerte, Germany

	nunc ELISA 96 well, F-Form	VWR, Darmstadt, Germany
	i12 7cm Rehydration / Equilibration Trays	Bio-Rad, Munich, Germany
Maldi-TOF MS	AUTOFLEX-III LRF200-CID	Bruker Daltronics, Bremen, Germany
	MTP 384 target plate ground steel T F	Bruker Daltronics, Bremen, Germany
	MTP AnchorChip TM 800/384 T F	Bruker Daltronics, Bremen, Germany
Mill	ProfiMixx 47 with grain top element	Bosch, Gerlingen, Germany
pH-Meter	pH-Meter 766 Calimatic	Knick, Berlin, Germany
Photometers	BioPhotometer	Eppendorf, Hamburg, Germany
	ND-1000 Spectrophotometer UV/Vis	NanoDrop Technologies, Wilmington, USA.
	MIOS plate reader	Merck, Darmstadt, Germany
Pipettes	Research	Eppendorf, Hamburg, Germany
Power supplies	Power Pac 300	Bio-Rad, Munich, Germany
	PowerPac 3000	Bio-Rad, Munich, Germany
	Power Pack P25T	Biometra, Göttingen, Germany
Scales	EW1500-2M	Kern & Sohn, Balingen-Frommern, Germany
	BP 211 D	Sartorius, Göttingen, Germany
SDS-PAGE cells	Surelock XCell	Invitrogen, Darmstadt, Germany
	Mini PROTEAN 3 Cell	Bio-Rad, Munich, Germany
Shakers / rotators	MS2 Minishaker	IKA Werke, Staufen, Germany
	RCT basic	IKA Werke, Staufen, Germany
	KS 250 basic	IKA Werke, Staufen, Germany
	KM-2 Akku	Edmund Bühler, Hechingen, Germany
	528 Testtube Rotator	Labinco BV, Breda, The Netherlands
	Thermomixer comfort	Eppendorf, Hamburg, Germany
	Duomax 1030	Heidolph, Schwabach, Germany
Sonification	Elmasonic S 10	Elma, Singen, Germany
Spotting	SciFlexArrayer S5	Scienion, Berlin, Germany
Staining	Gel Staining Trays, mini	Roth, Karlsruhe, Germany

Water bath CS6	MGW Lauda, Lauda-Königshofen, Germany

3.6   Consumables

15 ml Falcon tubes	Sarstedt, Nümbrecht, Germany
50 ml Falcon tubes	Sarstedt, Nümbrecht, Germany
Amicon Ultra Centrifugal Filters, MWCO 3 kDa	Merck Millipore, Darmstadt, Germany
Blotting filter papers, 2.5 mm thickness	Invitrogen, Darmstadt, Germany
Carestream Kodak BioMax MS film, 20x25 cm	Sigma-Aldrich, Schnelldorf, Germany
Cy3 mono-reactive dye pack	GE Healthcare, Little Chalfont, UK
Cy5 mono-reactive dye pack	GE Healthcare, Little Chalfont, UK
Dialysis tubing visking, cellulose, MWCO 14 kDa	Roth, Karlsruhe, Germany
Immobilon blotting filter paper, 7x8.4 mm	Merck Millipore, Darmstadt, Germany
Immobilon-P PVDF-membrane, ø0.45µm, 7x8.4 mm	Merck Millipore, Darmstadt, Germany
Kodak BioMax XAR-5 / X-omat AR5 film, 13x18 cm	Sigma-Aldrich, Schnelldorf, Germany
Mµltiflex round tips 0.5-10 µl	Roth, Karlsruhe, Germany
Mµltiflex E round tips 1-200 µl	Roth, Karlsruhe, Germany
NuPAGE Novex 4-12, 10, and 12% Bis-Tris gel 1.0 mm	Invitrogen, Darmstadt, Germany
NuPAGE Novex 4-12% Bis-Tris ZOOM gel 1.0mm	Invitrogen, Darmstadt, Germany
Pierce Concentrators, MWCO 20 kDa	Thermo Scientific, Waltham, USA
Porous Cellophane backing, 34x44 cm	GE Healthcare, Freiburg, Germany
PreCision-Tips Crystal, 10 µl	Sarstedt, Nümbrecht, Germany
Pure Nitrocellulose Membrane, ø0.45 µm	Bio-Rad, Munich, Germany
Safe-lock tubes, 0.5ml, PCR-clean	Eppendorf, Hamburg, Germany
Safe-lock tubes, 1 and 2 ml	Eppendorf, Hamburg, Germany
Serological pipettes 1, 5, 10 and 25 ml	Sarstedt, Nümbrecht, Germany
Slide-A-Lyzer Dialysis Cassette, MWCO 3.5 kDa	Thermo Scientific, Waltham, USA
Transfer pipette 3.5 ml, length 155 mm	Sarstedt, Nümbrecht, Germany
UVette routine pack	Eppendorf, Hamburg, Germany

3.7 Software

Excel 2007, 2010, 2013	Microsoft, Redmond, USA
Flex-Analysis 3.3.65.0	Bruker Daltronic, Bremen, Germany
Flex-Control 3.6.64.0	Bruker Daltronic, Bremen, Germany
ImageLab 5.0	BioRad, Hercules, USA
IrfanView 4.37	Irfan Skiljan, Wiener Neustadt, Austria
Mascot 2.5.12	Matrix Science Limited, London, GB
Prism 5.0	GraphPad Software, La Jolla, USA
Quantity One 4.6.8	BioRad, Hercules, USA
ScanArray 4.0	PerkinElmer, Waltham, USA
Sequence editor 3.2	Bruker Daltronic, Bremen, Germany
SPSS Statistics 22	IBM, New York, USA

3.8 Statistics

Quantitative IgE- and IgG_4-specific results were tested with Prism 5.0 for Gaussian distribution with 3 different tests: Kolmogorow-Smirnow normality test, D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test. Non-parametric distribution of data was assumed if at least two tests were not passed at 0.05 level. In this study, most quantitative IgE- and IgG_4-specific results failed to pass at least two of three normality tests. Subsequently, correlations of quantitative results from ImmunoCAP or ImmunoCAP ISAC with fluorescent results were conducted with Spearman's rank correlation test ($r_{(s)}$). Significance of correlation was calculated. Comparisons between fluorescent values were calculated with Kruskal-Wallis test and Dunn's multiple comparison test (Prism 5.0). Chi-Square test or Fisher's Exact test (in case of n<5 to be compared) were applied in the wheat study to compare band counts. These calculations were made in SPSS, along with computing Receiver Operating Characteristic (ROC) curves. A Receiver Operating Characteristic-(ROC)-curve relates sensitivity and specificity. In the graphical illustration, the abscissa represents the false-positive-rate (FPR) or fall-out, and the ordinate the true-positive rate (TPR). Significance in all tests was assumed at 0.05 level (two-tailed). Calculated significance levels are indicated with one to three asterisk(s), corresponding to p<0.05, 0.01 and 0.001.

4 Results

4.1 Wheat Allergy study

4.1.1 Characteristics of the wheat study population

Sera of in total 106 children with suspected food allergy to wheat were selected and analysed. Criteria for wheat challenges were suspicion of wheat-related symptoms by parents or physicians such as immediate type symptoms (urticaria, vomiting or wheezing) or worsening of eczema after ingestion of wheat containing products. 62 of these children (WT group) were clinically tolerant and 44 (WA group) suffered from a clinically relevant wheat allergy determined by oral food challenge. Details are depicted in Supplement Table 2 (WT children) and Supplement Table 3 (WA children), and summarized in Table 11. Despite similarities like the rate of present atopic dermatitis (AD) which was approximately equal (89% and 95%), 18% of the WT group suffered from asthma compared to 7% of the WA group. On average, WA children were younger than WT children. 37.1% of the WT children were not sensitized to wheat compared to 13.6% of the WA group.

Table 11: Characteristics of individuals included in the wheat study.

	WT group	WA group
total (n)	62	44
mean age (months)	35.8	24
median age (months)	15	9
female (n)	21	27
male (n)	41	17
sex ratio	1.95	1.59
AD (n / %)	55 / 88.7	42 / 95.5
asthma (n / %)	11 / 17.7	3 / 6.8
wheat-sIgE <0.35 kU$_A$/l (n / %)	23 / 37.1	6 / 13.6
wheat-sIgE >0.35 kU$_A$/l (n / %)	39 / 62.9	38 / 86.4

Clinical reactions during oral food challenge were recorded and are summarized in Table 12. The most frequent symptoms were skin related urticaria (40.9%), redness (22.7) and AD (15.9). Gastrointestinal symptoms like with itching and vomiting (each 13.6%) were relatively rare. No case of anaphylaxis was observed.

Table 12: Clinical symptoms to wheat oral food challenge in WA patients.

symptom	unknown		negative		positive early (<2 h)		positive early+late		positive late (>2 h)	
	n	%	n	%	n	%	n	%	n	%
urticaria	0	0	24	54.5	18	40.9	1	2.3	1	2.3
angioedema	0	0	43	97.7	0	0	1	2.3	0	0
redness	0	0	32	72.7	10	22.7	2	4.5	0	0
itching	0	0	35	79.5	6	13.6	2	4.5	1	2.3
AD	1	2.3	28	63.6	7	15.9	4	9.1	4	9.1
vomitting	1	2.3	35	79.5	6	13.6	2	4.5	0	0
diarrhea	1	2.3	40	90.9	1	2.3	2	4.5	0	0
stomach pain	1	2.3	42	95.5	1	2.3	0	0	0	0
rhinocojunctivitis	0	0	40	90.9	3	6.8	0	0	1	2.3
wheezing	0	0	43	97.7	0	0	1	2.3	0	0
coughing	0	0	42	95.5	2	4.5	0	0	0	0
stridor	0	0	43	97.7	1	2.3	0	0	0	0
anaphylaxis	0	0	0	0	0	0	0	0	0	0

A ROC curve was calculated in order to visualize the correlation of sIgE to whole wheat and clinical reactions (Figure 9).
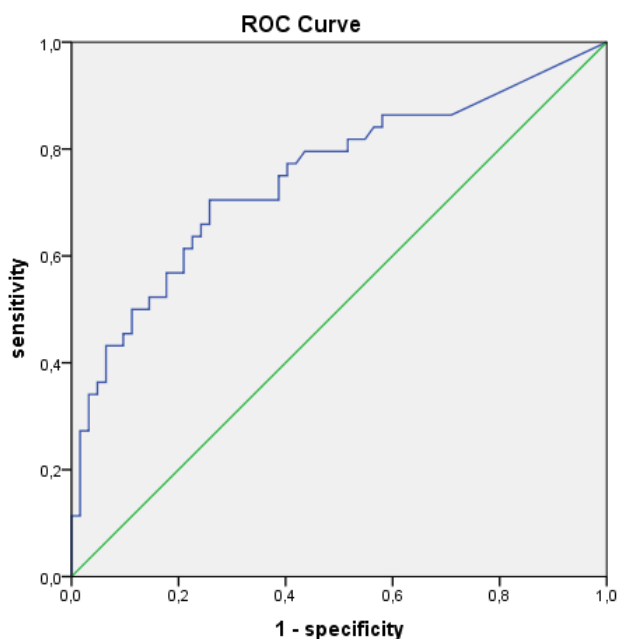
Figure 9: ROC-curve of 106 patients enrolled in the wheat study. The area under the curve (AUC) is 0.749. The asymptotic 95% CI is 0.650 to 0.847. The correlation is highly significant (p<0.000).

Clinical reaction and sIgE to whole wheat do not correlate well, the sensitivity of sIgE is only moderate by the flat slope and the AUC of 0.749.

4.1.2   Protein recognition pattern in the first screen

4.1.2.1   Correlation of wheat-specific IgE and immunoblot results

On immunoblot, arranged with increasing IgE to wheat by ImmunoCAP assay, both groups showed IgE-binding to a broad spectrum of water and salt soluble as well as water insoluble proteins (Figure 10 and Figure 11). Reactions to albumins/globulins a/o gliadins/glutenins were counted and analyzed with Chi-Square test. A detailed comparison of results from ImmunoCAP and immunoblot is documented in the Supplement Table 2 (WT children) and Supplement Table 3 (WA children).
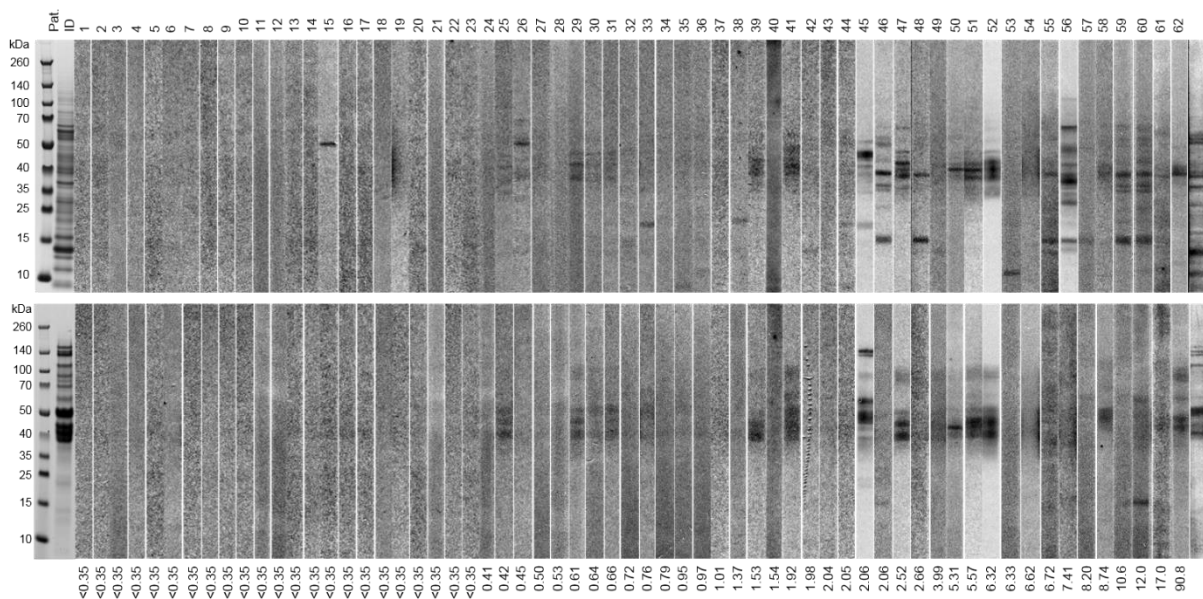
Figure 10: Immunoblots of WT patients, IgE-sorted. On top water and salt soluble albumins/globulins with patient ID number, bottom side water insoluble glutenins/gliadins with wheat-sIgE titer in kU$_A$/l. Protein molecular weight marker and separated proteins, stained with colloidal Coomassie, are shown on the left hand side and were taken from a 4-12% Bis-Tris 12 well reference gel. The last strip on the right shows blotted proteins on an untreated PVDF membrane, stained with Amido Black. Controls are shown in Figure 10.

In the WT group (n=62), there was one patient (no. 15) with sIgE <0.35 kU$_A$/l who showed binding to one protein band of the water and salt soluble (albumin/globulin) protein fraction, but 13 patients (21%; No. 24, 27, 28, 32, 34-37, 40, 43, 49, 54, 61) with sIgE >0.35 kU$_A$/l were negative in IB assays against albumin/globulin proteins, and 25 (40.3%; No. 24, 26-28, 32-38, 40, 42-44, 46, 48, 49, 53-56, 59, 61) against gliadins/glutenins. Of these patients, 11 (17.7%; No. 24, 27, 32, 34-37, 40, 43, 49, 54) are double negative against albumins/globulins and gliadins/glutenins.
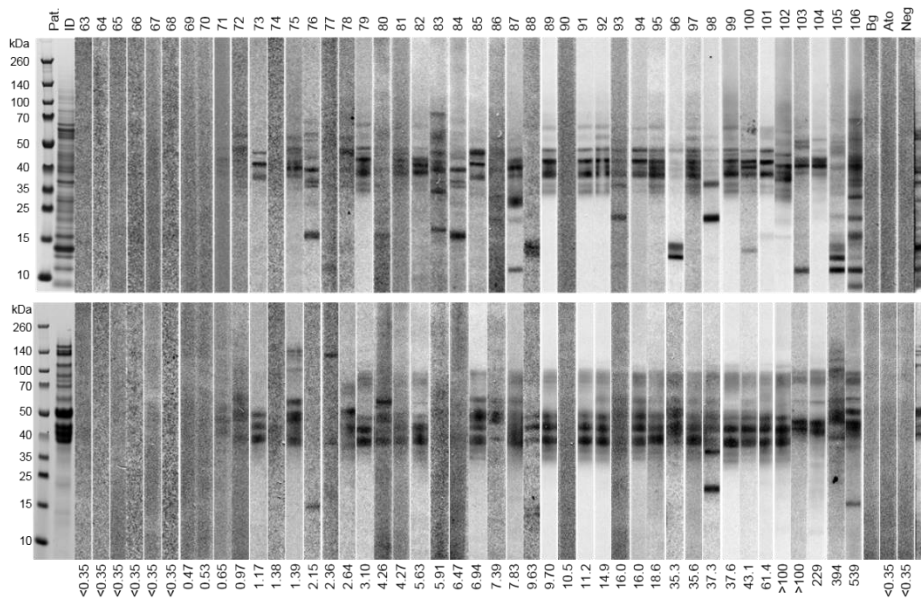
Figure 11: Immunoblots of WA patients, IgE-sorted. On top water and salt soluble wheat proteins (albumins/globulins) with patient ID number, bottom side water insoluble proteins (glutenins/gliadins) with wheat-sIgE titer in kU_A/l. Protein molecular weight marker and separated proteins, stained with colloidal Coomassie, are shown on the left hand side side and were taken from a 4-12% Bis-Tris 12 well reference gel. The last strip on the right shows blotted proteins on an untreated PVDF membrane, stained with Amido Black.

In the WA group (n=44), no patient with IgE <0.35 kU_A/l showed binding to a protein of the albumin/globulin fraction. 6 patients (9.7%; No. 69, 70, 71, 74, 77, 90) with sIgE >0.35 kU_A/l showed no reactivity towards albumins/globulins, and 7 patients (11.3%; No. 69, 70, 74, 83, 84, 90, 93) were negative against gliadins/glutenins. Patients No. 69, 70, 74 and 90 are double negative.

Of note, patients No. 54 (6.62 kU_A/l) and 90 (10.5 kU_A/l) are double negative despite clearly elevated sIgE. If the IB results are specific or due to e.g. misapplication, mismatch of serum and ID etc. was not investigated.

A summary of quantified qualitative IB results is shown in Table 13.

Table 13: Immunoblot reactions of WT and WA individuals in 1st screen.

IB reaction to	WT n=62		WA n=44	
	<0.35 n=23	>0.35 n=39	<0.35 n=6	>0.35 n=38
albumins/globulins pos abs. / %	1 / 8.3	26 / 66.7	0 / 0	32 / 84.2
albumins/globulins neg abs. / %	22 / 95.7	13 / 33.3	6 / 100	6 / 15.8
gliadins/glutenins pos abs. / %	0 / 0	14 / 35.9	0 / 0	31 / 81.6
gliadins/glutenins neg abs. / %	23 / 100	25 / 64.1	6 / 100	7 / 18.4

Related to the total of individuals (n=106), IB analysis correctly showed no positive reaction for 29 individuals with sIgE<0.35 kU_A/l with one exception as mentioned previously. On the other hand, in the WT group for sera with sIgE>0.35 kU_A/l, a high percentage of negative

results for albumins/globulins with 33.3% and for gliadins/glutenins with 64.1% was observed. In the WA group, the percentage was clearly lower with 15.8% and 18.4%, respectively. This discrepancy could be explained by the different distribution of low sIgE. Although the rate of sera >0.35 kU$_A$/l is equal between WT and WA group (Table 11), the percentage of sera with sIgE<2.00 kU$_A$/l is clearly different: 48.7% (n=19) in the WT and 13.2% (n=5) in the WA group. On the other hand, several sera with low sIgE were found to be positive in IB analysis as shown in Figure 10 and Figure 11.

WT and WA group were statistically compared (Chi Square test) as concerns IB reactions to albumins/globulins and gliadins/glutenins. The difference between the groups was highly significant for albumins/globulins (p<0.003, n=106) but failed significance when only individuals with wheat-sIgE>0.35 kU$_A$/l (p<0.074, n=77) were considered. WT and WA group differed in IB reactions to gliadins/glutenins very highly significantly (p<0.000), as well as for the total number of individuals or only those with sIgE>0.35 kU$_A$/l.

4.1.2.2 Correlation of age and immunoblot results

The protein recognition pattern was also considered with regard to patients' age. Immunoblots were re-arranged in order of age (in months) and are depicted in Figure 12 and Figure 13.
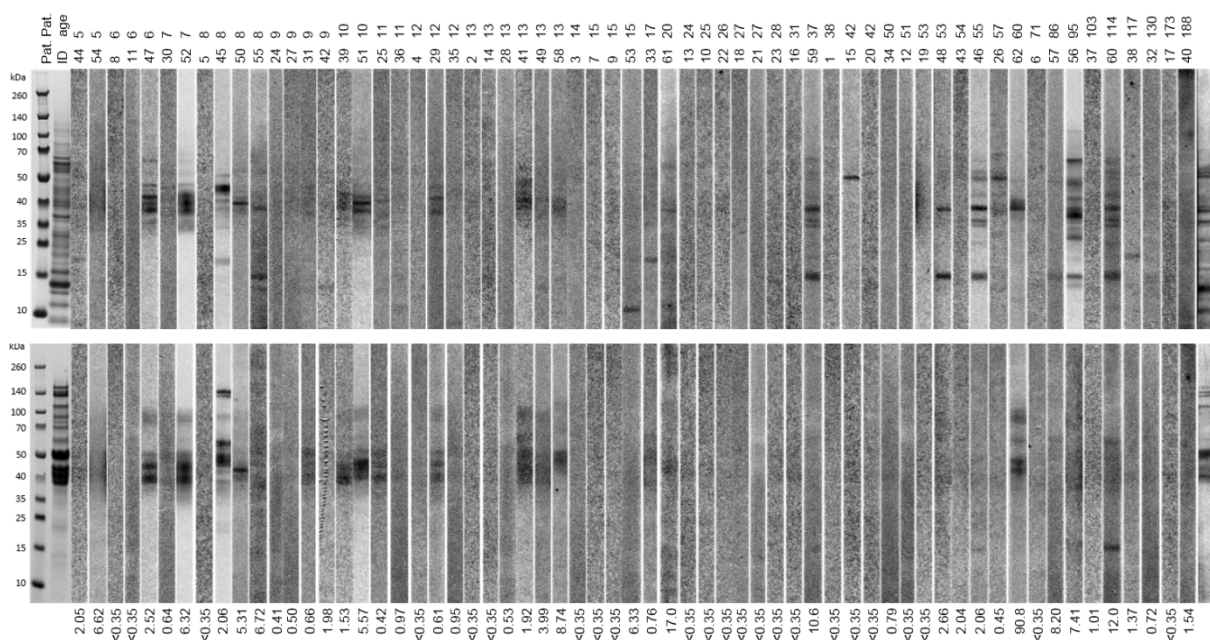
Figure 12: Immunoblots of WT patients, age-sorted (months). On top water and salt soluble wheat proteins (albumins/globulins) with patients' age and ID, bottom side water insoluble proteins (glutenins/gliadins) with wheat-sIgE titer in kU$_A$/l. Protein molecular weight marker and separated proteins, stained with colloidal Coomassie, are shown on the left hand side. The last strip on the right shows blotted proteins on an untreated PVDF membrane, stained with Amido Black. Controls are shown in Figure 13.

For albumins/globulins, with increasing age, a trend towards diversification of recognized bands over a broad range was observed in sera with sIgE>0.35 kU$_A$/l (Pat. 59, 46, 56, 60). On the other hand, positive reactions to gliadins/glutenins clearly decreased with two exceptions (Pat. 62 and 60, although the latter positive result may be unspecific due to insufficient purification of gliadins/glutenins).
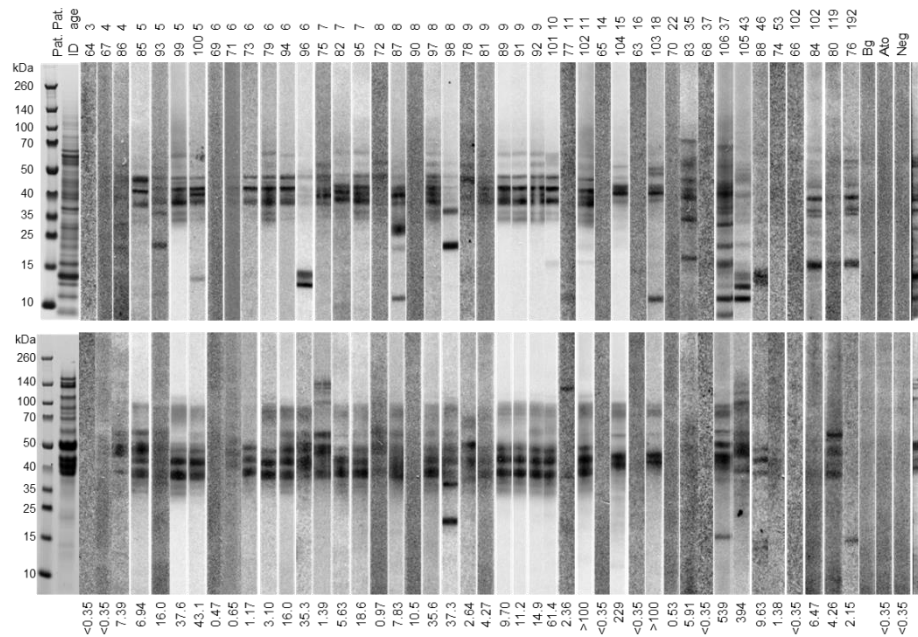
Figure 13: Immunoblots of WA patients, age-sorted (months). On top water and salt soluble wheat proteins (albumins/globulins) with patients' age and ID, bottom side water insoluble proteins (glutenins/gliadins) with wheat-sIgE titer in kU$_A$/l. Protein molecular weight marker and separated proteins, stained with colloidal Coomassie, are shown on the left hand side. The last strip on the right shows blotted proteins on an untreated PVDF membrane, stained with Amido Black. Bg, background; Ato, atopic serum, Neg, negative serum

In WA individuals, no clear trends in recognition pattern neither for albumins/globulins nor gliadins/glutenins were observed. The majority of patients was <12 months, the remaining period of time is only covered fragmentarily. Because of unequal range of age within and between the WT and WA group, and the fact that the blots were assessed only qualitatively for each strip, no statistical correlation between age and recognition pattern is deducible.

4.1.2.3 Semi-quantitative assessment of immunoblot results according to molecular ranges

The general protein pattern of the water and salt soluble albumin/globulin fraction was assessed by counting bands within molecular ranges <10, 10 to 15, 15 to 25, 25 to 35, 35 to 50 and >50 kDa, for the water insoluble gliadin/glutenin fraction ranges <37, 37 to 65, 65 to 100 and >100 kDa were considered. Reference for molecular ranges were the blotted prestained protein marker bands. Patient-specific counts are depicted in Supplement Table 4 (WT children) and Supplement Table 5 (WA children). Absolute counts within each group and relative percentage considering only IB-positive patients are summarized in Table 14.

Table 14: Absolute and relative positive reactions of WT and WA patients' IgE in immunoblot. Bands within indicated ranges (in kDa) were counted independently by three persons. Percentages were calculated to the number (n) of IB-positive reactions, of sera with sIgE>0.35 kU$_A$/l, and the total of patients in each group.

		n	≤10	10-15	15-25	25-35	35-50	>50	n	<37	~37-65	~65-100	>100
			Water and salt soluble albumins/globulins							**Water insoluble gliadins/glutenins**			
WT group	SUM abs.		1	6	4	1	19	10		2	12	7	1
	% of pos in IB	27	3.7	22.2	14.8	3.7	70.4	37.0	14	14.3	85.7	50.0	7.1
	% of >0.35	39	2.6	15.4	10.3	2.6	48.7	25.6	39	5.1	30.8	17.9	2.5
	% of total WT	62	1.6	9.7	6.5	1.6	30.6	16.1	62	3.2	19.4	11.3	1.6
WA group	SUM abs.		4	7	6	13	29	21		3	29	20	3
	% of pos in IB	32	12.5	21.9	18.8	40.6	90.6	65.6	31	9.7	93.5	64.5	9.7
	% of >0.35	38	10.5	18.4	15.8	34.2	76.3	55.3	38	7.9	76.3	52.6	7.9
	% of total WA	44	9.1	15.9	13.6	29.5	65.9	47.7	44	6.8	65.9	45.5	6.8

Considering the number of positive IB reactions as basis for calculations, WA patients recognized ~10 times more bands in the albumin/globulin fraction between 25-35 kDa compared to WT patients, proteins <10 kDa occurred 3.3 times more frequently in the WA group. The WA group also differed in the range >50 kDa by 1.7, and between 35-50 kDa still 1.2. No differences were found for proteins in the range of 10-15 and 15-25 kDa. Similar ratios were determined when sIgE>0.35 kU$_A$/l was taken as reference.

In the water insoluble gliadin/glutenin fraction, bands >100 kDa were recognized only by three WA patients (No. 75, 80, 86) and one WT patient (No. 45). The general IgE-binding pattern towards gliadins and glutenins is very similar between WT and WA children. Based on positive IB reactions, ratios for gliadins/glutenins did not clearly differ. Differences occurred when sIgE>0.35 kU$_A$/l was used as criterion. Then, WA and WT differed in ranges ~37-65 kDa by factor 2.5, and 2.9 for range ~65-100 kDa. Detected bands <37 kDa are probably due to contaminations with albumins a/o globulins because they only apply to patients who also show strong binding in the albumin/globulin immunoblot analysis.

Statistical analysis (Chi-Square or Fisher's exact test) of the frequencies of recognized bands by WT and WA individuals revealed very highly and highly significant differences, depending on the references applied. In the albumin/globulin fraction, very highly significant differences between 25-35 kDa (p<0.000) were calculated for the total number of WT and WA individuals (n=106) and the number of individuals with sIgE>0.35 kU$_A$/l (n=77). Very highly significant was the difference also between 35-50 kDa (p<0.000) for n=106 and highly significant for n=77 (p<0.012), as well as for bands >50 kDa (p<0.000 for N=106 and 0.004 for N=77). Highly significant differences in frequency of recognized bands in the gliadin/glutenin fraction were also calculated for the range between ~37 to 65 kDa and ~65 to 100 kDa (p<0.000 for n=106 and 0.001 for n=77).

Despite statistical differences in frequencies of bands within certain molecular weight ranges, these bands qualitatively occur in the WT as well as in the WA group. As titers of sIgE between WT and WA individuals differed, and a considerable amount of IBs were found negative despite sIgE>0.35 kU$_A$/l, the distinction of WT from WA individuals by the number of recognized bands is questionable.

4.1.3   Protein recognition pattern in the 2nd immunoblot analysis

Patients who reacted positive in the immunoblot were chosen to be re-assessed by applying different gel systems for improved protein separation. These were 12% Bis-Tris gels for analysis of water insoluble proteins, and 10% Bis-Tris gels for water and salt soluble proteins with 12 wells each to provide equally straight separation of proteins. In every second lane, prestained protein marker was loaded. After blotting, a lane with marker and protein was cut from the membrane and used for immunolabeling. The protein content was empirically optimized and checked by colloidal Coomassie stain. Electrophoresis was stopped when the 35 kDa (12%) or 25 kDa (10%) band of the prestained protein marker was reached in order to provide maximal separation for proteins larger than 35 kDa or 25 kDa, respectively. Protein bands in the Coomassie stained gels were numbered and served as reference for individually recognized band(s) by patient's serum (Figure 14).
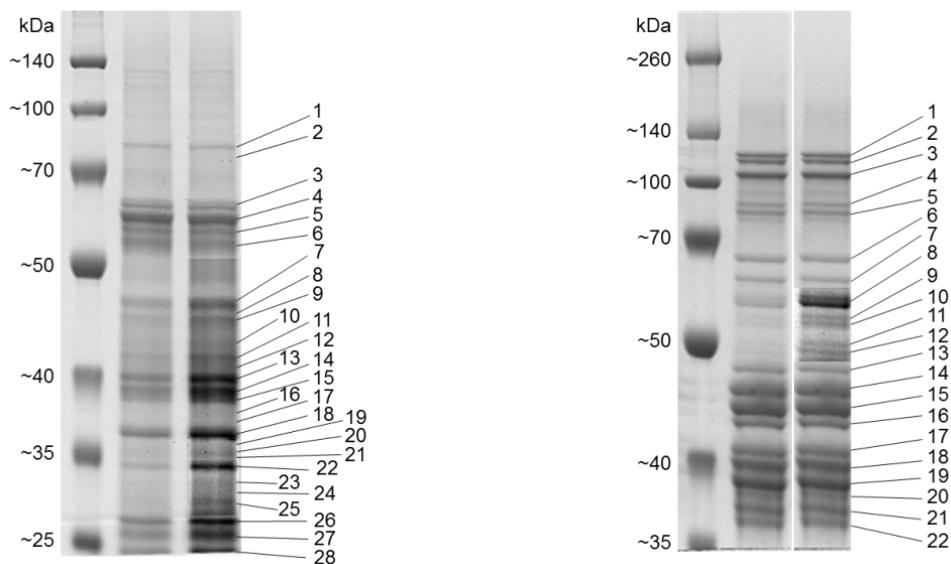
Figure 14: Extended separation of albumins/globulins and gliadins/glutenins in SDS-PAGE with 10% and 12% Bis-Tris gels. Albumins/globulins are shown on the left, gliadins/glutenins on the right. In each second lane, selected areas were enhanced to visualize weak bands in the gel stain. In both applications, MOPS running buffer was used. Indicated bands were used as reference for counting positive signals on immunoblots and cut for subsequent MALDI-TOF MS analysis.

4.1.3.1 Analysis of 2nd immunoblot results after extended SDS-PAGE with water and salt soluble albumins/globulins

For immunoblot analysis of water/salt soluble albumins and globulins, 13 patients of the WT group were compared with 25 WA patients (Figure 15). Single incubated strips were individually amplified as described previously. Detailed results are depicted in Supplement Table 8 (WT children), Supplement Table 9 (WA children) and summarized in Table 15.
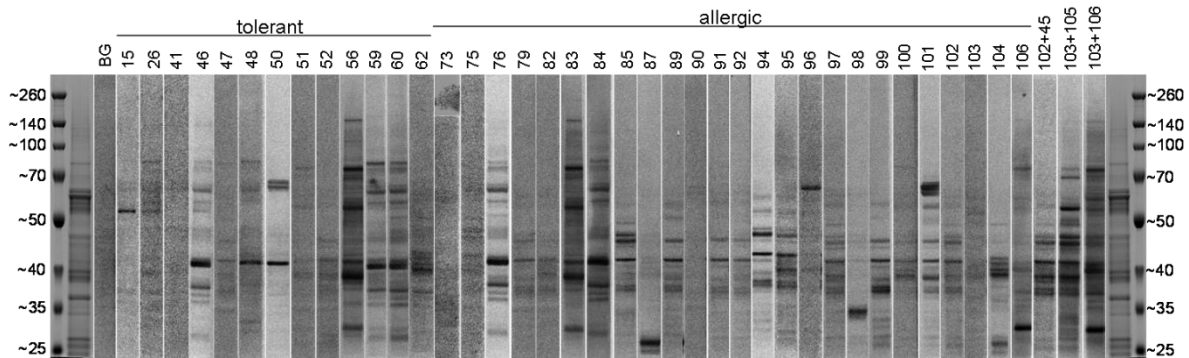
Figure 15: Immunoblots of selected WT and WA patients against albumins/globulins >25 kDa. Electrophoretic separation in 10% Bis-Tris gels and run until 25 kDa was reached. 3 sera were pooled with one reference serum. Molecular weight marker and separated proteins, stained with colloidal Coomassie, are shown on the left and right hand side. One background control (BG) was used.

Unique bands that clearly distinguish by frequency WT from WA patients could not be detected.The finding that more proteins between 35 and 50 kDa are recognized by WA patients (Table 14) could be confirmed by considering counts for bands 7 to 21. In the WT group, highest frequencies were observed for bands 11/12 with 58.3%, and for bands 16, 17 and 19/20/21 with 33.3%, respectively. Except for bands 19/20/21, frequencies of positive reactions to the bands mentioned were higher in the WA group. Bands 7, 8/9, 10 and 13 showed counts in the WA group, but no count in the WT group. However, their frequency was relatively low with 31.8% (band 8/9) as the highest relative percentage (Table 15). Apart from frequencies, the strength of bands 10, 11 and 12 relative to other bands within the strip suggested a biased IgE response to the protein(s) in these bands.

Table 15: Absolute and relative positive reactions of WT and WA patients' IgE to albumins/globulins >25 kDa. Sera of 13 WT and 25 WA patients were re-assessed in a 2nd IB analysis to albumins/globulins >25 kDa and positive signals to protein bands were counted. Some bands were merged and counted as one due to insufficient resolution in IB. Bands with no signal in either group are not shown.

Band ID	WT group					WA group				
	counts in 2nd screen	% of total in 2nd IB (n=13)	% of pos in 2nd IB (n=12)	% of total >0.35 (n=39)	% of total WT (n=62)	counts in 2nd screen	% of total in 2nd IB (n=25)	% of pos in 2nd IB (n=22)	% of total >0.35 (n=38)	% of total WA (n=44)
1	4	30.8	33.3	10.3	6.5	4	16.0	18.2	10.5	9.1
2	3	23.1	25.0	7.7	4.8	4	16.0	18.2	10.5	9.1
3	4	30.8	33.3	10.3	6.5	4	16.0	18.2	10.5	9.1
6	2	15.4	16.7	5.1	3.2	2	8.0	9.1	5.3	4.5
7	0	0	0	0	0	3	12.0	13.6	7.9	6.8
8/9	0	0	0	0	0	7	28.0	31.8	18.4	15.9
10	0	0	0	0	0	2	8.0	9.1	5.3	4.5
11/12	7	53.8	58.3	17.9	11.3	15	60.0	68.2	39.5	34.1
13	0	0	0	0	0	3	12.0	13.6	7.9	6.8
14/15	1	7.7	8.3	2.6	1.6	4	16.0	18.2	10.5	9.1
16	4	30.8	33.3	10.3	6.5	9	36.0	40.9	23.7	20.5
17	4	30.8	33.3	10.3	6.5	6	32.0	36.4	21.1	18.2
18	1	7.7	8.3	2.6	1.6	2	8.0	9.1	5.3	4.5
19/20/21	4	30.8	33.3	10.3	6.5	2	8.0	9.1	5.3	4.5
22	0	0	0	0	0	1	4.0	4.5	2.6	2.3
25	3	23.1	25.0	7.7	4.8	3	12.0	13.6	7.9	6.8
26/27	3	23.1	25.0	7.7	4.8	2	8.0	9.1	5.3	4.5
28	0	0	0	0	0	2	8.0	9.1	5.3	4.5

Chi-Square or Fisher's Exact test conducted for all bands revealed no significant difference in the frequency of recognized protein bands between WT and WA individuals.

4.1.3.2 *Analysis of 2nd immunoblot results after extended SDS-PAGE with water insoluble gliadins/glutenins*

At first, blotting efficiency for water insoluble gliadins/glutenins was optimized by application of different transfer buffers (according to Bjerrum, Dunn, Towbin, and standard Invitrogen transfer buffer) and subsequent stains of PVDF membranes and gels to check remains of protein. Best results were obtained with the transfer buffer according to Bjerrum (48 mM Tris, 39 mM Glycin, pH 9,25) with 10% MeOH (one gel per blotting module) and 0.1% SDS.

For the water insoluble protein fraction, only 7 WT patients could be compared with 25 WA patients (Figure 16). Details are shown in Supplement Table 10 (WT children) and Supplement Table 11 (WA children).
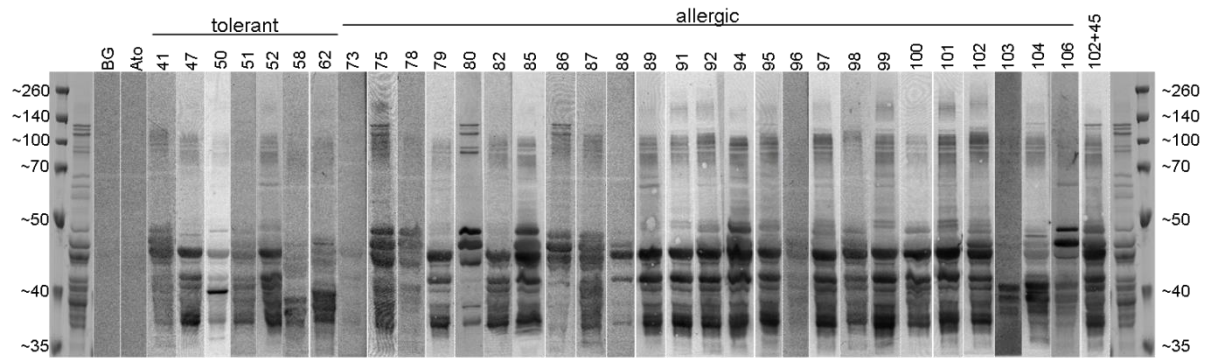
Figure 16: Immunoblots of selected WT and WA patients against water insoluble wheat proteins (glutenins/gliadins). Electrophoretic separation in 12% Bis-Tris gels and run until 35 kDa was reached. 2 sera were pooled. Molecular weight marker and separated proteins, stained with colloidal Coomassie, are shown on the left and right hand side. Due to long running time and despite performing electrophoresis on ice, formation of air bubbles occurred, causing irregular protein separation. One background (BG) and atopic control (Ato) were used.

IgE reactive bands in both groups comprise qualitatively almost all storage proteins <50 kDa (gliadins, LMW-glutenins). All patients show a multi band IgE recognition pattern, there is no patient who recognizes only one band. However, some patients (No. 58 and 103) show slight deviations from the general pattern that comprises bands from ~45 to ~50 kDa (γ-gliadins and LMW-glutenins) as well as ~45 to ~37 kDa (α/β-gliadins and LMW glutenins) with approximately equal intensity. There are also patients (No. 50, 88) with a pattern of tiny bands which strongly differ in intensity.

Table 16: Absolute and relative positive reactions of WT and WA patients' IgE to gliadins/glutenins >35 kDa. Sera of 7 WT and 25 WA patients were re-assessed in a 2nd IB analysis and positive reactions to protein bands were counted. Some bands were merged and counted as one due to insufficient resolution in IB.)* Three individuals of the WT group and one individual of the WA group were classified as "not clear".)** One individual of the WA group was classified "not clear". Details are shown in **Supplement Table 10** for WT and **Supplement Table 11** for WA individuals.

Band ID	total counts in 2nd IB	WT group % of total in 2nd IB (n=7)	% of pos in 2nd IB (n=7)	% of total >0.35 (n=39)	% of total WT (n=62)	total counts in 2nd IB	WA group % of total in 2nd IB (n=25)	% of pos in 2nd IB (n=25)	% of total >0.35 (n=38)	% of total WA (n=44)
1	0	0	0	0	0	3	12.0	12.0	7.9	6.8
2	0	0	0	0	0	3	12.0	12.0	7.9	6.8
3	0	0	0	0	0	3	12.0	12.0	7.9	6.8
4	0	0	0	0	0	1	4.0	4.0	2.6	2.3
5	0	0	0	0	0	1	4.0	4.0	2.6	2.3
6	0	0	0	0	0	1	4.0	4.0	2.6	2.3
7	1	14.3	14.3	2.6	1.6	5	20.0	20.0	13.2	11.4
8	0	0	0	0	0	0	0	0	0	0
9/10	0	0	0	0	0	3	12.0	12.0	7.9	6.8
11/12	0	0	0	0	0	10	40.0	40.0	26.3	22.7
13	3	42.9	42.9	7.7	4.8	13	52.0	52.0	34.2	29.5
14	1	14.3	14.3	2.6	1.6	10	40.0	40.0	26.3	22.7
15	5	71.4	71.4	12.8	8.1	20	80.0	80.0	52.6	45.5
16	4	57.1	57.1	10.3	6.5	15	60.0	60.0	39.5	34.1
17	5	71.4	71.4	12.8	8.1	18	72.0	72.0	47.4	40.9
18	4	57.1	57.1	10.3	6.5	20	80.0	80.0	52.6	45.5
19)*	3	42.9	42.9	7.7	4.8	19	76.0	76.0	50	43.2
20)**	0	0	0	0	0	12	48.0	48.0	31.6	27.3
21	6	85.7	85.7	15.4	9.7	17	68.0	68.0	44.7	38.6
22	7	100	100	17.9	11.3	19	76.0	76.0	50	43.2

Fisher's Exact test or Chi-Square test (for bands 19 and 20 with "not clear" classification) conducted for all bands on the basis of n=7 (WT) and n=25 (WA) revealed significant differences in the frequency of recognized protein bands between WT and WA individuals for bands 19 (p=0.023) and 20 (p=0.019). Band 19 is special in so far that 3 individuals of the WT group and one individual of the WA could not be classified as positive or negative in immunoblot analysis, because of unequal separation of proteins in this molecular range. These individuals were therefore marked as "not clear". One individual of the WT group was also classified as "not clear" for band 20. The merged bands 11/12 showed a trend towards a significant difference (p=0.069).

4.1.4 Protein identification with MALDI-TOF MS and application to immunoblot analyses

4.1.4.1 Assessment of proteins of the albumin/globulin fraction

Protein bands of the reference gels from second screen (Figure 14) and subsequent gels, run under identical conditions, were excised and digested with trypsin. For albumins/globulins, also a reference gel from the first screen (Figure 5) was considered for

protein bands <25 kDa which were numbered from 29 to 35. Digests were analyzed with MALDI-TOF MS. Obtained spectra of *m/z* values of the digested peptides were submitted to plant-specific databases via Mascot search engine in order to identify underlying proteins (untargeted approach). Spectra that could not be directly identified were compared with spectra resulting from *in silico* (theoretically simulated digest of protein sequences with trypsin) digestions of probable proteins (targeted approach). A mass tolerance of maximal 200 ppm and 2 partials were accepted. Scores >55 in Swissprot "Other plants" database were considered significant at p<0.05 level. Criteria for probable identity were a score >40% and a sequence coverage of >20%. For *in silico* digests, two criteria for highly probable identification were applied: (1) a sequence coverage >20% and (2) a match between calculated and measured *m/z* values of 100% for at least one peptide. Detailed results are shown in Supplement Table 12 summarized in Figure 17. Note also CD.
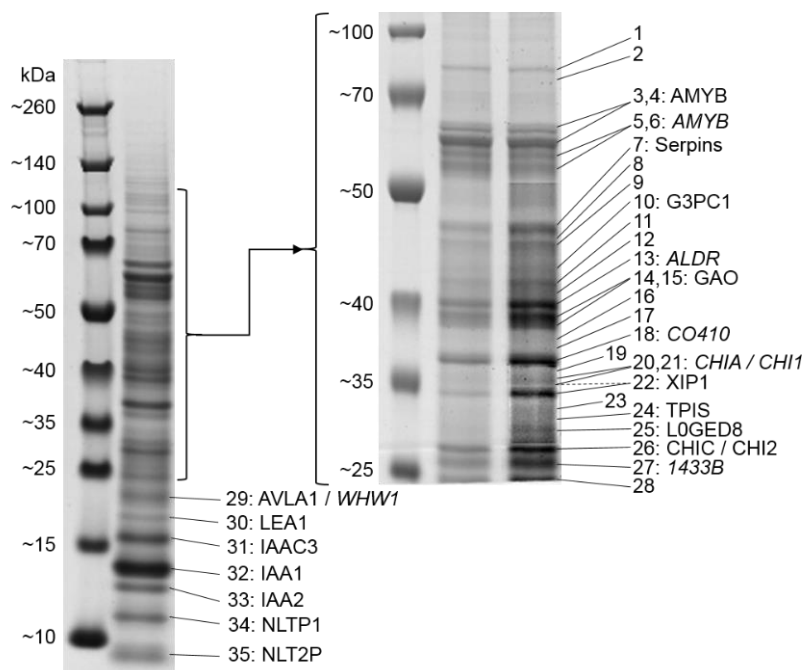
Figure 17: Identified and probable proteins of the albumin/globulin fraction. Directly identified proteins and probable proteins by *in silico* digestion (*in italics*). Abbreviations in capital letters: AMYB, *AMYB*, β-amylase(s); G3PC1, Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic; *ALDR*, aldose reductase; GAO, Gibberellin 20 oxidase 1-A and 1-D; *CO410*, dehydrin COR410; *CHIA / CHI1*, basic endochitinase A / 26 kDa endochitinase 1; XIP1, Xylanase inhibitor protein 1 [Class III chitinase homolog]; TPIS, Triosephosphate isomerase, cytosolic; L0GED8, 14-3-3 protein; CHIC / CHI2, Basic endochitinase C [Rye seed chitinase-c] / 26 kDa endochitinase 2 [CHI-26]; 1433B, 14-3-3-like protein B; AVLA1 / *WHW1*, Avenin-like a1/LMW-gliadin 2482 / Wheatwin-1 [Pathogenesis-related protein 4a]; LEA1, ABA-inducible protein PHV A1; IAAC3, α-Amylase/trypsin inhibitor CM3; IAA1, α-Amylase inhibitor 0.19 / 0.53; IAA2, α-Amylase inhibitor 0.28 [WMAI-0.28]; NLTP1, Non-specific lipid-transfer protein (9k-LTP); NLT2P, Non-specific lipid-transfer protein 2 (nsLTP2). Serpins comprise a family of several isoforms including identified Serpin-Z2B, Z1B and Z2A. Bands 21 and 22 showed significant hits for XIP1, with higher score of band 22.

Altogether, a broad number of proteins could be identified in the albumin/globulin fraction, including ingestive but also inhalational allergens. Several proteins found by Sotkovsky and colleagues using MALDI-TOF MS/MS in the same cultivar Akteur [85] could be retrieved and

were considered as confirmation of own findings (Supplement Table 12). However, 10 bands were not identified, and for several bands only a certain likelihood could be proposed.

4.1.4.2 Application to results of immunoblots to the albumin/globulin fraction

As shown in Table 15, bands 7, 8/9, 10 and 13 only occurred in the WA group, although with low frequency. Band 7 was identified as the protein family serpins which is classified as inhalational and ingestive allergen (Tri a 33) by WHO/IUIS. Bands 3 to 6 represent β-amylase(s), described as food allergen Tri a bA in the Allergome database. The high abundance of β-amylase(s) points to improper storage and onset of germination. Band 10 was found to be a member of the Glyceraldehyde-3-phosphate dehydrogenase family, which so far is only described as inhalational allergen Tri a 34 (C7C4X1_WHEAT). Aldose reductase, the probable protein in band 13, is so far neither described as ingestive nor inhalational allergen. In both groups, the second IB analysis revealed strong signals and high frequency (58.3% of all IB-positive WT and 68.2 % of all IB-positive WA individuals) for bands 11 and 12 (which were merged in counting). These bands could neither be directly identified nor likely proteins were found. Because of approximately equally high frequency in both groups, these bands do not distinguish WA and WT individuals. Thus their diagnostic value is limited. Bands <25 kDa were considered in the first IB analysis (Table 14), later numbered 29 to 35 and identified (Figure 17). Due to their highly significant scores in Mascot and the fact that α-amylase inhibitors (AAIs) and α-amylase/trypsin inhibitors (AATIs) have recently been under discussion, first IBs were re-assessed and single bands were counted according to the protein pattern of the reference gel and <u>not</u> to the blotted prestained protein marker (Figure 17).

Table 17: Absolute and relative positive reactions of WT and WA patients' IgE with albumins/globulins <25 kDa in immunoblot assays. Bands were identified as depicted in Figure 17. Mapping of IB signals to selected bands in Coomassie stain without regard to the blotted prestained protein marker. Counts are shown in detail in **Supplement Table 6** (WT children) and **Supplement Table 7** (WA children). Percentages were calculated to total number of individuals per group, to individuals with sIgE>0.35 kU$_A$/l, to the number of positive IB to albumins/globulins (**Table 13**) and the number of individuals with positive IB result for albumins/globulins <25 kDa.

		Band						
		35	34	33	32	31	30	29
WT group	SUM abs.	0	1	0	1	6	3	0
	% of total WT (n=62)	0.0	1.6	0.0	1.6	9.7	4.8	0.0
	% of >0.35 kU$_A$/l (n=39)	0.0	2.6	0.0	2.6	15.4	7.7	0.0
	% of pos in IB (n=26)	0.0	3.8	0.0	3.8	23.1	11.5	0.0
	% of pos<25kDa in blot (n=10)	0.0	10.0	0.0	10.0	60.0	30.0	0.0
WA group	SUM abs.	1	4	3	3	4	1	4
	% of total WA (n=44)	2.3	9.1	6.8	6.8	9.1	2.3	9.1
	% of >0.35 kU$_A$/l (n=38)	2.6	10.5	7.9	7.9	10.5	2.6	10.5
	% of pos in IB (n=32)	3.1	12.5	9.4	3.1	12.5	3.1	12.5
	% of pos<25kDa in blot (n=12)	8.3	33.3	25.0	25.0	33.3	8.3	33.3

Differences in frequencies had been proposed only for proteins <10 kDa which was labeled as band 35 and identified as Non-specific lipid transfer protein 2 (NLT2P). According to the first counting, WA individuals reacted 3.3 more often compared to WT individuals. This factor could not be confirmed, only one WA individual recognized this band.

For the protein range 10-15 kDa, no clear difference had been determined. Bands in this range are 34, 33 and 32, identified as Non-specific lipid transfer protein (NLTP1), α-Amylase inhibitor 0.28 (IAA2) and α-Amylase inhibitor 0.19 (IAA1). All three proteins were more frequently recognized by WA individuals. Fisher's Exact test with reference to the total number of patients (n=106) revealed no significant differences between WT and WA group. For band 33, however, a tendency towards significance was determined (p=0.069).

In the range of 15-25 kDa, a slight difference of 1.5fold more recognized bands in the WA group had been stated. Bands involved are 31, 30 and 29, identified as α-Amylase/trypsin inhibitor CM3 (IAAC3), ABA-inducible protein PHV A1 (LEA1) and Avenin-like a1/LMW-gliadin 2482 (AVLA1) a/o probably Wheatwin-1 (WHW1). WT and WA differed highly significant only in band 29 (p=0.027). With reference to only the number of individuals with sIgE>0.35 kU$_A$/l (WT n=39, WA n=38), the difference slightly failed significance (p=0.055). Comparisons of bands 35 to 30 according to this reference remained not significant.

4.1.4.3 *Assessment of proteins of the gliadin/glutenin fraction*

The direct identification of gliadins and glutenins in MALDI-TOF MS was difficult despite repetitive tryptic digests. Sequence homologies, simultaneous extraction and few peptides with high masses complicated identification. Therefore, results from DuPont, Chan *et al.* [131] were consulted and served as reference for *in silico* digests. Detailed results are shown in Supplement Table 13 and summarized in Figure 18. Note also CD.
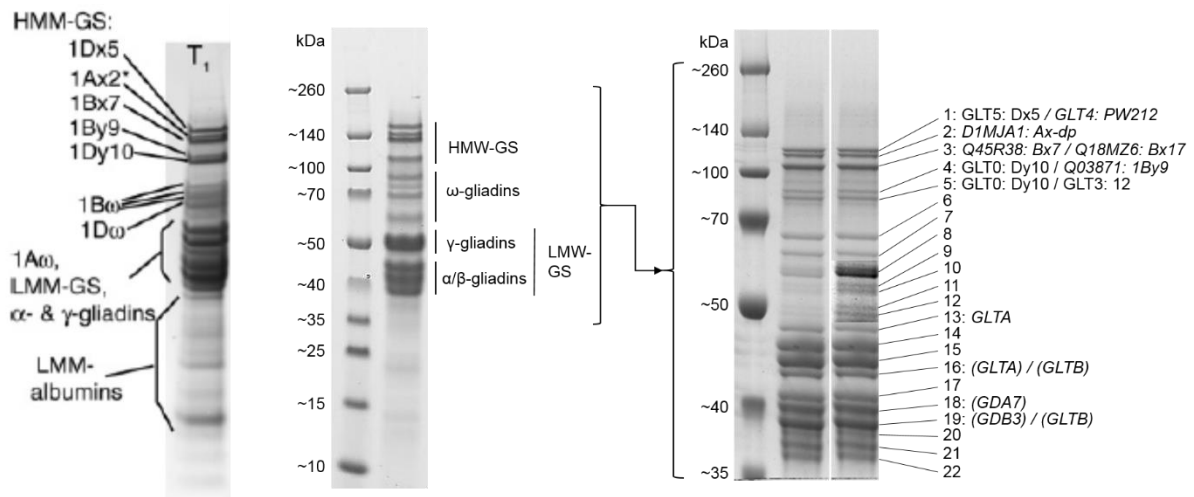
Figure 18: Probable proteins of the gliadin/glutenin fraction. Commassie-stained protein bands of the gliadin/glutenin fraction in 4-12% Bis-Tris (middle) and 12% Bis-Tris (right, with additionally amplified bands) gel systems were assigned according to findings from DuPont, Chan *et al.* [131] in the cultivar *Triticum aestivum* L. "Butte86" (left, modified). Directly identified proteins, probable proteins by *in silico* digestion (*in italics*) and possible proteins (in brackets and *in italics*). Abbreviations in capital letters: GLT5, Glutenin, high molecular weight subunit DX5; GLT4, Glutenin, high molecular weight subunit PW212; D1MJA1, High molecular weight glutenin subunit Ax-dp; Q45R38, HMW glutenin x-type subunit Bx7; Q18MZ6, High-molecular-weight glutenin subunit Bx17; GLT0, Glutenin, high molecular weight subunit DY10; Q03871, HMW glutenin subunit 1By9; GLT3, Glutenin, high molecular weight subunit 12; GLTA, Glutenin, low molecular weight subunit; GLTB, Glutenin, low molecular weight subunit 1D1; GDA7, Alpha/beta-gliadin clone PW8142; GDB3, Gamma-gliadin (fragment).

Three HMW-glutenins could be directly identified (Dx5, Dy10, 12). Other HMW-glutenins were determined *in silico* according to suggestions of DuPont, Chan *et al.* [131]. Neither LMW-glutenins nor gliadins could be directly identified, only one LMW-glutenin (GLTA) could be determined with certain probability by *in silico* digest. Proteins shown in brackets and *in italics* failed to match defined criteria for direct or probable identification, however, there is evidence for their existence by position in SDS-PAGE and relative molecular weight (Supplement Table 13).

4.1.4.4   Application to results of immunoblots to the albumin/globulin fraction

Significant differences in frequencies were only found for bands 19 and 20. Neither of them could be identified, but some evidence was found for LMW-glutenin 1D1 (GLTB) a/o gamma-gliadin (GDB3) to be existent in band 19, encouraged by comparison to DuPont, Chan *et al.* [131]. For bands 11/12, which were not identified, only a trend towards significant difference between WT and WA group was observed. As their position in SDS-PAGE is near to those of omega-gliadins, of which omega5-gliadin has been suggested to be highly specific for WA patients, some further investigations were performed.

4.1.4.5   Attempts to locate ω5-gliadin

As shown by *in silico* digests, tryptic digestion of omega5-gliadin (UniProt: Q402I5) leads to large fragments >6000 *m/z* and to small fragments <600 *m/z* which cannot be properly measured in reflector mode and therefore cannot contribute to identification (Table 18).

Table 18: Fragments of omega5-gliadin from tryptic in silico digests. Digests were applied to the full form of UniProt sequence Q402I5 with signal peptide (+SP) and the mature form (-SP) allowing for up to 2 partials

partials					
2		**1**		**0**	
+SP	-SP	+SP	-SP	+SP	-SP
38.288.678	38.288.678	26.025.826	26.025.826	17.969.620	17.969.620
31.980.764	31.980.764	23.924.558	23.924.558	14.382.131	14.382.131
26.210.942	26.210.942	20.337.068	20.337.068	11.661.706	11.661.706
26.025.826	26.025.826	17.969.620	17.969.620	5.972.948	5.972.948
23.924.558	23.924.558	14.382.131	14.382.131	2.068.121	584.365
20.337.068	20.337.068	11.846.822	11.846.822	584.365	261.144
17.969.620	17.969.620	11.661.706	11.661.706	277.146	203.127
14.382.131	14.382.131	5.972.948	5.972.948	203.127	
12.413.176	12.413.176	2.634.475	827.498		
11.846.822	11.846.822	2.327.257	769.481		
11.661.706	11.661.706	2.068.121	584.365		
5.972.948	5.972.948	769.481	261.144		
2.893.611	1.012.614	584.365	203.127		
2.819.592	827.498	277.146			
2.634.475	769.481	203.127			
2.327.257	584.365				
2.068.121	261.144				
769.481	203.127				
584.365					
277.146					
203.127					

Therefore, tryptic digests of bands in the range of 55 kDa in SDS-PAGE were also assessed in linear mode from 5 to 22 kDa to identify ω5-gliadin-specific fragments (Figure 19, Figure 20). The system was not re-calibrated with protein standards.
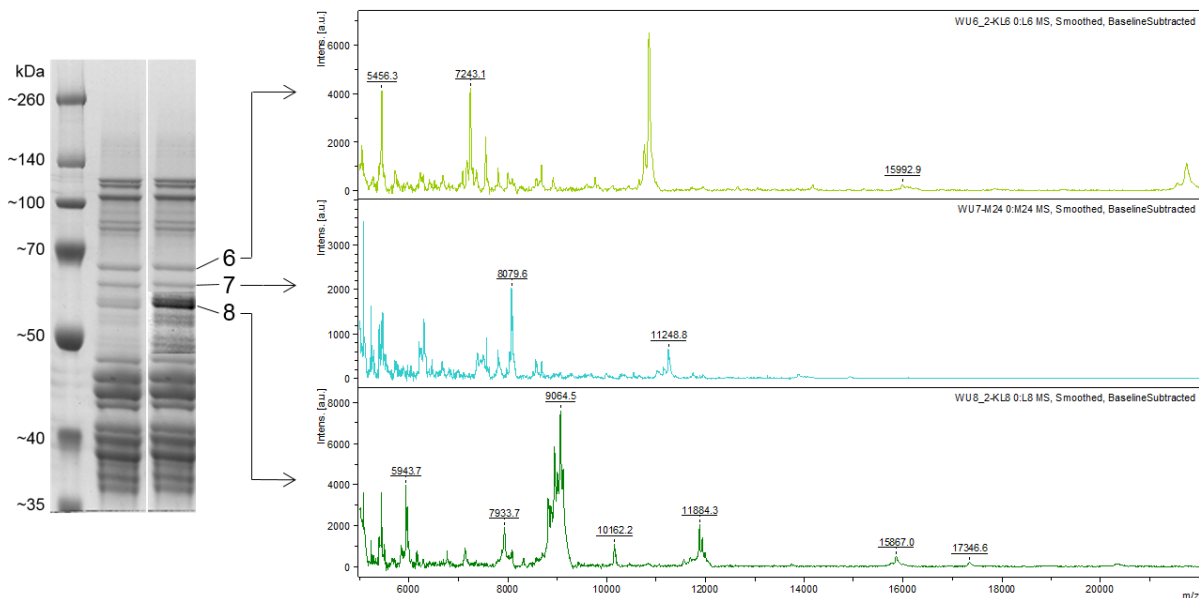
Figure 19: Measurement of tryptic digests from bands 6, 7 and 8 in linear mode. Band 8, consisting of 2 bands which could not be excised separately, contains fragments of 5943.7, 11884.3 and 17346.6 *m/z* which could correspond to omega5-gliadin-specific fragments of 17.969.620, 11.661.706 and 5.972.948 *m/z*. The proposed fragment with 14.382.131 *m/z* is missing. *m/z* spectra were smoothed and baseline was subtracted.

Some evidence was found that ω5-gliadin is part of band 8 which consists of two tiny bands. As *m/z* spectra measured in the linear mode are not applicable to Mascot database search,

no other procedures to match the recorded *m/z* spectrum with entries of ω5-gliadin peptide sequences were conducted.

In order to locate the position of probable ω5-gliadin from bands of lower range, bands 9 to 12 were also examined (Figure 20).
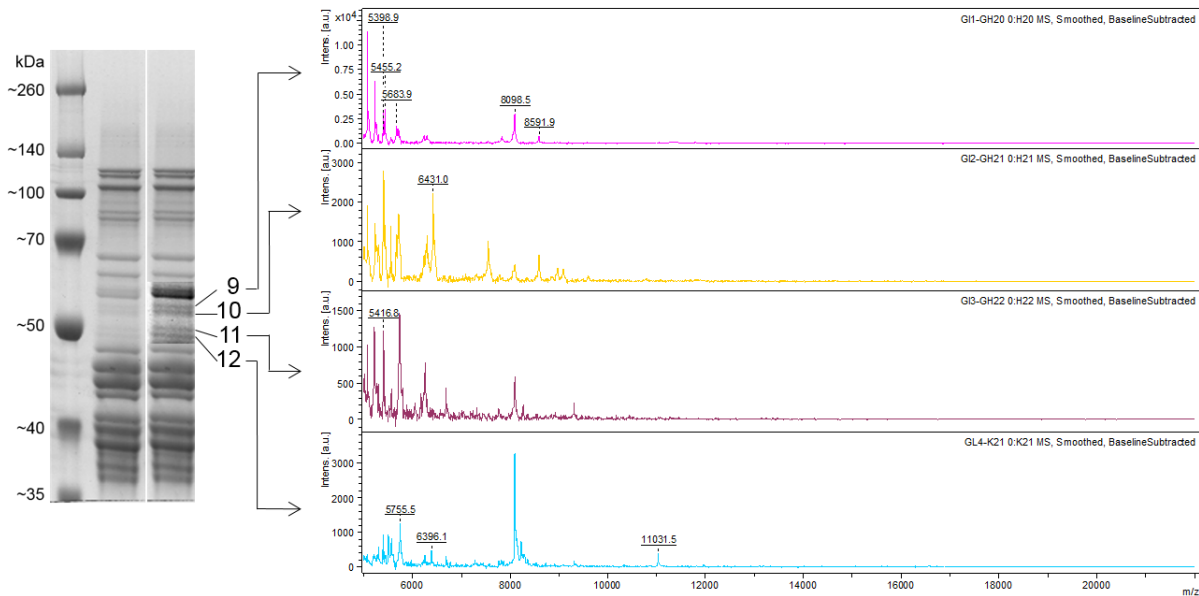
Figure 20: Measurement of tryptic digests from bands 9-12 in linear mode. Bands 9 to 11 do not contain fragments between 10000 and 22000 *m/z*. Only band 12 contains a fragment of 11031.5 *m/z* which is not specific for omega5-gliadin. *m/z* spectra were smoothed and baseline was subtracted.

Despite some fragments larger than 6000 *m/z*, bands 9 to 12 were found to be unspecific for ω5-gliadin. Repetitions of digests and application of protein standards for calibration did not improve specificity of signals.

Since no relevant components in the gliadin/glutenin fraction, which were recognized by IgE in immunoblots, could be directly identified, quantitative component-testing was performed. Levels of measured specific IgE were used for statistics, but also for re-investigating immunoblots and Coomassie stained gels in order to approach the position of the ω5-gliadin-specific protein band.

4.1.5 Quantitative determination of specific IgE to ω5-gliadin

Specific IgE to ω5-gliadin for 5 individuals of each group with wheat-specific IgE <0.35 kU$_A$/l, and for 16 individuals of the WT group and 14 of the WA group with wheat-specific IgE >0.35 kU$_A$/l was determined quantitatively on ImmunoCAP. Individuals within each group were chosen randomly. Results are listed in Supplement Table 14 and plotted in Figure 21.
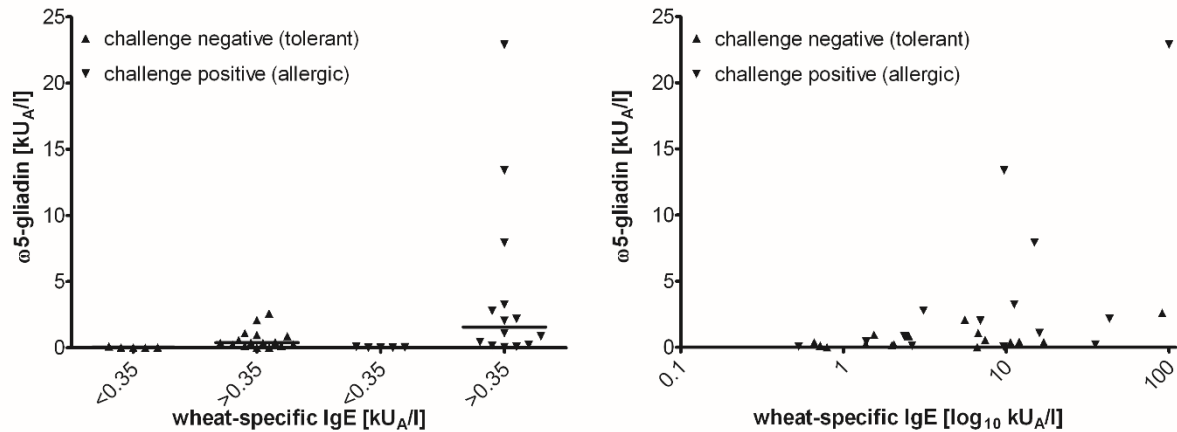
Figure 21: Quantitative determination of specific IgE to omega5-gliadin. 21 WT and 19 WA individuals were randomly selected and sIgE to omega5-gliadin was determined. No significant difference was found in levels of sIgE between WT and WA children (left). Vertical bars indicate median value. The correlation of wheat- and ω5-gliadin-sIgE is shown for 16 WT and 14 WA individuals with sIgE to wheat >0.35 kU_A/l on the right hand side.

Levels of sIgE to omega5-gliadin in WA and WT individuals with wheat-sIgE <0.35 kU_A/l were equally low <0.1 kU_A/l. 16 WT and 14 WA individuals with wheat-sIgE >0.35 kU_A/l differed in the mean level of sIgE to omega5-gliadin. However, this difference was not significant (p=0.0844) as revealed by two-tailed Mann Whitney U test. The distribution of sIgE to ω5-gliadin and whole wheat across selected WT and WA individuals was also tested and found to be not significant (p<0.294 and p<0.361, respectively). The correlations between sIgE to wheat and ω5-gliadin sIgE were assessed with Spearman's rank correlation (r_s) and were positive in both groups (r_s=0.5121 for WT and r_s=0.5297 for WA), and only significant in the WT group (p=0.0425) compared to the WA group (p=0.0514).

In order to visualize the diagnostic value of quantitatively determined sIgE to wheat and ω5-gliadin, ROC curves for the selected individuals was computed (Figure 22).
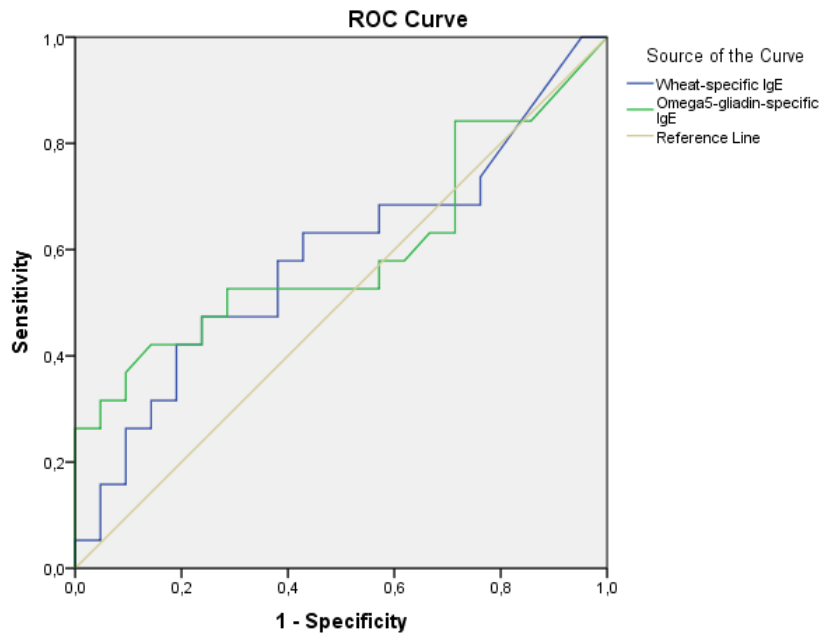
Figure 22: ROC-curves of 21 WT and 19 WA individuals determined for specific IgE to wheat and omega5-gliadin. The area under the curve (AUC) is 0.586 for wheat-specific IgE, and 0.598 for omega5-gliadin-specific IgE. The correlations are neither significant for wheat-specific IgE (p<0.350) nor omega5-gliadin-specific IgE (0.291).

For the selected individuals, the level of sIgE to neither whole wheat nor to ω5-gliadin is of value with AUC of 0.586 and 0.598, respectively. Despite random selection of individuals, the low total number of 40 patients could have contributed to this result. In order to unravel a population based bias, detailed characteristics of the selected individuals were brought together and are depicted in Table 19.

Table 19: Analysis of selected WT and WA patients for determination of omega5-gliadin specific IgE. Results "<0.35" were set to "0.35" in order to perform calculations.

parameter	WT group		WA group	
	<0.35 kU$_A$/l	>0.35 kU$_A$/l	<0.35 kU$_A$/l	>0.35 kU$_A$/l
sera of individuals (n)	5	16	5	14
wheat-specific IgE (kU$_A$/l)				
Minimum	0.05	0.66	0.35	0.53
Median	0.35	4.05	0.35	9.67
Maximum	0.35	90.80	0.35	100.00
Mean	0.29	10.52	0.35	18.34
ω5-gliadin specific IgE (kU$_A$/l)				
Minimum	0.00	0.00	0.00	0.05
Median	0.01	0.38	0.00	1.56
Maximum	0.10	2.59	0.06	22.90
Mean	0.03	0.65	0.02	4.09
age (months)				
Minimum	13.0	5.0	3.0	5.0
Median	25.0	43.5	16.0	9.0
Maximum	53.0	188.0	102.0	53.0
Mean	26.6	57.0	34.4	14.7
sex				
female (n)	2	6	4	6
male (n)	3	10	1	8
sex ratio	0.7	0.6	4.0	0.8
AD (n)	4	15	4	14
Asthma (n)	1	6	0	1

The clearest difference between selected WT and WA was in age. Individuals with wheat-specific IgE <0.35 kU$_A$/l differed only slightly in median age (WT 25.0 versus WA 16.0 months). On the contrary, the median age of WT individuals >0.35 kU$_A$/l was 43.5 months and thus clearly higher compared to 9.0 months of WA individuals. This finding reflects the usual correlation of increasing tolerance with age. No difference in the rate of AD or sex ratio was determined. In the WT group with <0.35 kU$_A$/l, more individuals suffered from asthma. For this aspect, no further analysis was performed.

Except for the low total number of selected individuals, differences in age or other characteristics reported could account for the poor predictive value of sIgE to ω5-gliadin. Therefore, immunoblots were re-considered in order to locate ω5-gliadin. Especially patients determined with a high titer were of interest because an ω5-gliadin-specific band at ~65 kDa was expected. These were 2 WT individuals (No. 51 and 62), and in the WA group 7 individuals (No. 79, 85, 89, 91, 62, 100 and 102). Neither in Figure 10, Figure 11 nor Figure 16 a clear band at the expected position was detected for all these patients.

4.1.6   Summary of wheat study results

62 WT and 44 WA individuals were compared in immunoblots to albumins/globulins and gliadins/glutenins. The immunoblot technique proved to be reliable as shown by significant correlation of positive immunoblot results against albumins/globulins and gliadins/glutenins with levels of sIgE>0.35 kU$_A$/l. Semi-quantitative analyses of recognized protein bands within molecular ranges suggest that WT and WA individuals do not qualitatively differ in their IgE recognition pattern, but for albumins/globulins between 25-35, 35-50 and >50 kDa positive signals in immunoblot are highly significantly more frequent. No qualitative but highly significant quantitative differences for gliadins/glutenins were observed between <37-60 and 60-100 kDa in the gliadin/glutenin fraction.

Assessment of albumins/globulins >25 kDa with selected sera showed slight qualitative differences which are not significant. By means of MALDI-TOF MS, Serpins, Glyceraldehyde-3-phosphate dehydrogenase and Aldose reductase were identified. They only occured in WA individuals but with low frequency compared to the total of WA individuals. There is some evidence that the frequency of α-amylase (AAI) and α-amylase/trypsin inhibitors (AATI) is increased in WA individuals. Some bands with differences in frequencies remained unidentified by MALDI-TOF MS.

Identification of bands in the gliadin/glutenin fraction was successful for HMW glutenins which were recognized only by one WT and three WA individuals. In the population examined, HMW glutenins did not represent major allergens. The IgE-response to gliadins/glutenins comprises bands between ~37-50 kDa which involve α-, ß-, γ-, and gliadins, and LMW glutenin subunits. The role of ω5-gliadin remains unclear as IB analysis did not show clear signals in the expected range nor in the suggested band, determined by MALDI-TOF MS. Randomly selected sera used for quantitative determination of sIgE to ω5-gliadin show no significant difference between selected WT and WA individuals.

4.2   Additional immunoblots with related cultivars

Recent discussions suggest old cultivars to be less allergenic compared to modern ones [132]. For apple, which is source of the Bet v 1 homologue Mal d 1 and important in secondary FA, there is evidence that certain cultivars are more compatible [133]. Legume crops (peanut, soy) have also been object to intense research in this regard as reviewed by Riascos, Weissinger, Weissinger and Burks [134].

Therefore, several old grain cultivars and related species were selected for comparative electrophoresis of albumins/globulins and gliadins (Figure 23, Figure 26).
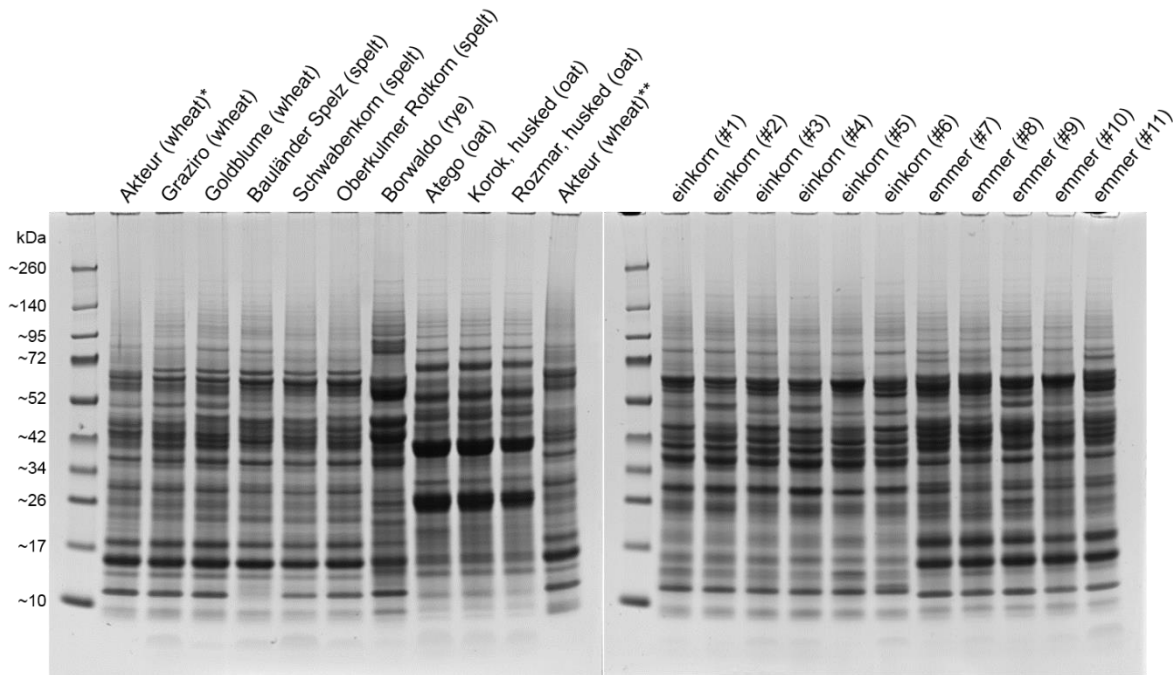
Figure 23: SDS-PAGE of albumins/globulins in different grain species. Albumins and globulins of different grains were extracted with 0.3 M NaI. 5 µl per sample were loaded on 4-12% Bis-Tris gels, run with MES-buffer and stained with colloidal Coomassie. The cultivar Akteur is illustrated with 0.3 M NaI (*) and 0.5 M NaCl (**) extract. Emmer and einkorn cultivars are labelled with running numbers due to missing proper names.

The albumin/globulin protein band pattern varies clearly between different species, especially oat cultivars differ considerably from all others. Within a species, slight differences occur concerning more or less one protein band which is less clear or completely missing. Bauländer Spelz lacks a clear band at approximately 12 kDa which is unique among all spelt cultivars applied. In einkorn (1-6), bands below ~17 kDa are less intense compared to wheat, spelt, rye and emmer. In the wheat cultivar Akteur, these bands were identified as α-amylase/trypsin inhibitors (AATI's). In einkorn, ATI's seem to be less abundant, but not missing.

The different protein band pattern in the range of approximately 40 and ~52 kDa in the cultivar Akteur revealed different capabilities of the solvents NaI and NaCl a/o the molarity applied.

Blotting of albumins/globulins was conducted with Bjerrum transfer buffer for 70 mins on ice. Immunoblots were performed with pool serum of 6 patients (age 5-15, mean 7.6 years), sensitized to wheat, along with negative serum as control. Wheat-sIgE was 22 kU$_A$/l for the positive pool and 0.06 kU$_A$/l for the negative pool. Arranged membranes were fixed on transparency foil with an IgE dot blot, and BioMax MS films were exposed up to three times. The first film is shown in Figure 24.
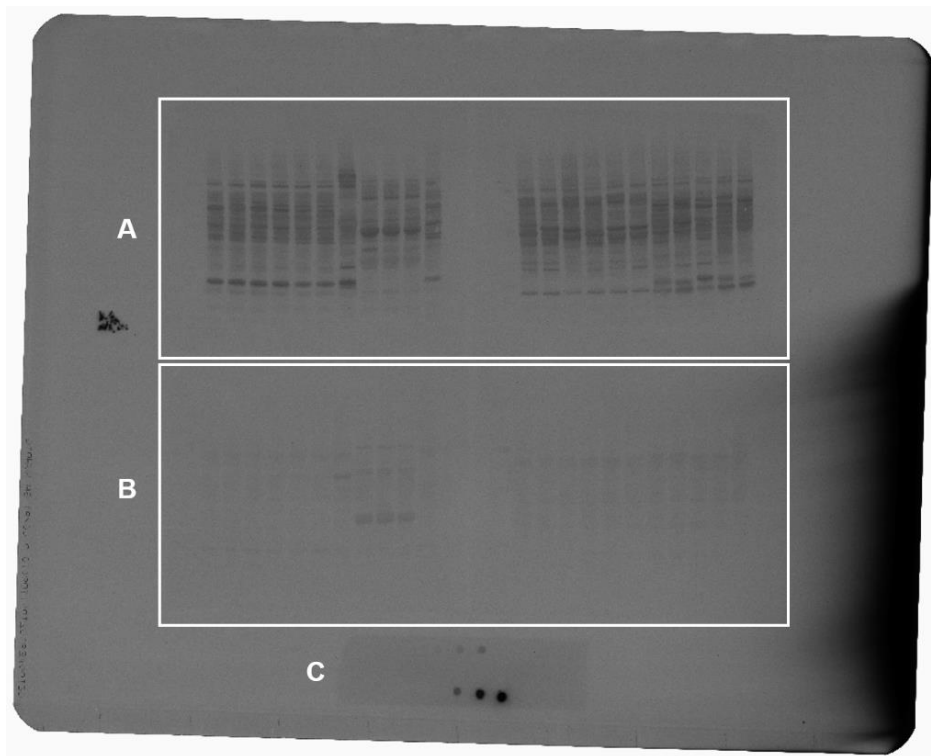
Figure 24: Immunoblots with albumins/globulins of several grains and species. First film after 7 days exposition. Immunoblots were incubated with positive (A) and with negative pooled serum (B). A clear difference is seen in signal strength, however, unspecific background shows in negative controls. Dot blot positive control at the very bottom (C). The image was processed with Quantity One software: a Median (3x3) noise filter was applied and OD was set to 4 (High) and 2 (Low) using the calibrated quantity option.

Despite application of two further films to be exposed for shorter duration, the signal to (unspecific) noise ration could not be improved. Additional digital processing of the immunoblots incubated with positive serum was applied (Figure 25).
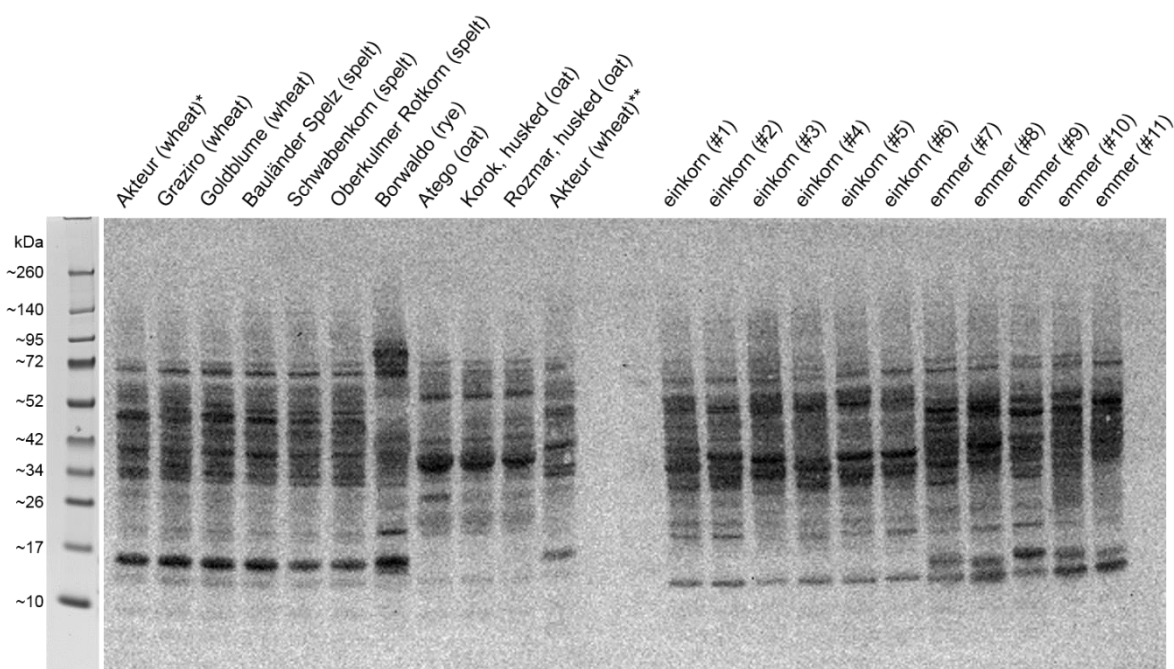
Figure 25: Detail of additional immunoblots. Area A in **Figure 24** was cut and signals enhanced with IrfanView software.

As shown in Figure 25, all cultivars gave positive signals in IB analysis. Wheat and spelt do not differ in the IgE recognition pattern. The reduced number of positive bands below ~17 kDa suggest a lower abundance of IgE-reactive AATI's in oat and einkorn in comparison to wheat, spelt, rye and emmer. Wheat extracted with 0.5 M NaCl only shows a clearly weaker signal for a band at ~17 kDa. Of note, emmer cultivars (7-11) show two clearly positive bands below ~17 kDa whereas wheat and spelt only show one clearly strong band and a weak second one. The majority of positive signals in all lanes occurs in the range between ~35 kDa and ~72 kDa and thus replicates findings with single patient sera in the cultivar Akteur.
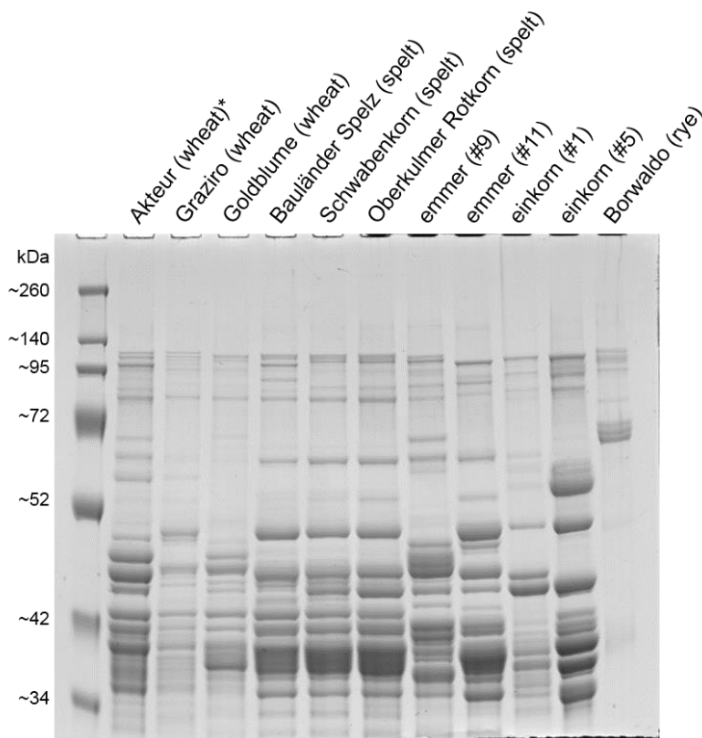
Figure 26: SDS-PAGE of gliadins in different grain species. Gliadins of different grains were extracted with 50% propan-2-ol. After lyophilizing, proteins were resuspended in 8 M urea. 4 to 6 µl per sample were loaded on 12% Bis-Tris gels and run with MOPS-buffer. Electrophoresis was performed on ice and stopped when the ~34 kDa band was reached. The gel was stained with colloidal Coomassie.

The gliadin protein band pattern varies clearly between different species. Also within species clear differences occur, e.g. emmer or einkorn. Oat samples were not used because no proteins >~34 kDa were extracted (data not shown). Despite no DTT was applied, HMW-glutenins are also present, visible in the range of ~72 to approximately 110 kDa. Subsequent gels to be used for western blotting were loaded with slight adjustments of volumes for cultivars Grazio, Goldblume and Borwaldo, adding 2 (Grazio) and 1 µl sample volume, respectively.

Blotting of gliadins was conducted with Bjerrum transfer buffer for 90 mins. Immunolabeling was performed with the pool sera of previously labeled albumin/globulin blots. BioMax MS films were exposed two times with immunoblots.
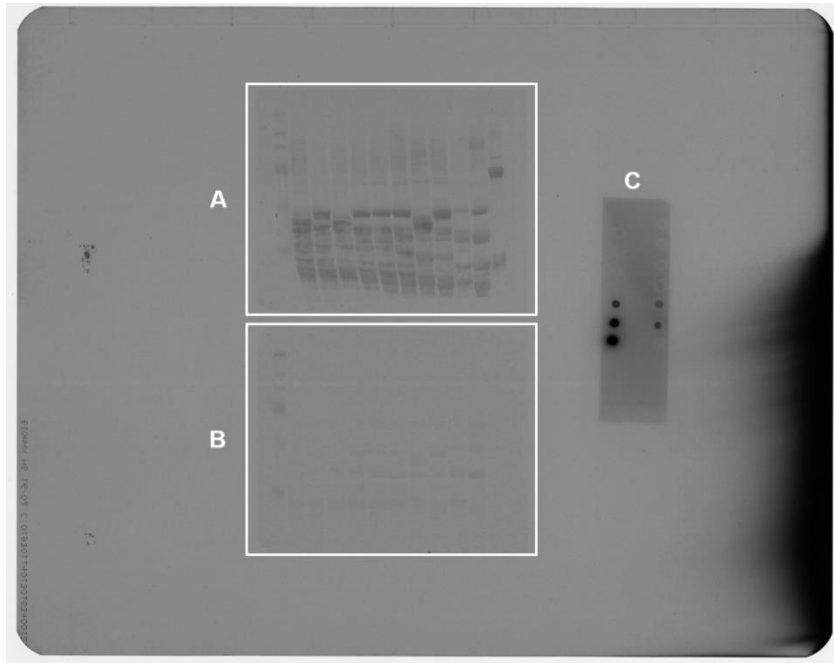
Figure 27: Immunoblots with gliadins of several grains and species. Second film after 12 days exposition. The immunoblots on top (A) was incubated with positive serum, the one below (B) with negative serum. A clear difference is seen in signal strength, however, positive signals are weaker compared to those in albumin/globulin immunoblots (Figure 24). The dot blot positive control (C) is strongly positive and excludes a failure of secondary antibody application. The image was not processed with a software.

Digital processing of the IB incubated with positive serum was applied (Figure 28).
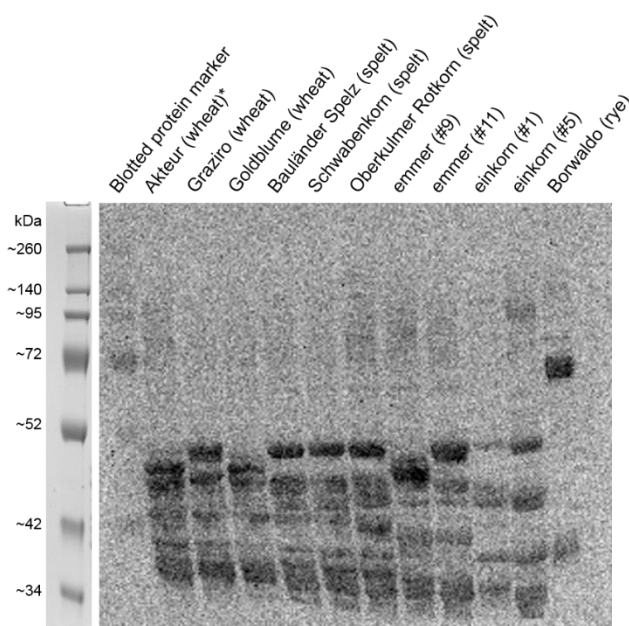
Figure 28: Detail of immunoblots in Figure 27. Area A in **Figure 27** was cut and signals automatically enhanced with IrfanView software. Due to the long duration of exposition, the blotted prestained protein marker is visible.

As shown in Figure 28, all cultivars gave positive signals in immunoblot analysis. Wheat cultivars slightly differ in in the IgE recognition pattern, spelt IgE pattern show no difference. Relative to other cultivars and species applied, emmer No. 1 shows weakest signals. Rye is exceptional as it does not contain gliadins, however, binding to proteins of ~ 72 kDa occurs. Of note, HMW-glutenins, which were not targeted, but extracted to a certain extent, are not clearly recognized. The IgE recognition pattern of bands below ~52 kDa resembles previous findings with single patient sera in the cultivar Akteur (Figure 16).

4.3 **Microarray study**

Practical operations were performed in the lab of Dr. Marcella Chiari in assistance with Dr. Marina Cretich at CNR, Ist. di Chimica del Riconoscimento Molecolare, Milano, Italy.

4.3.1 Sequencing and verification of clones

Sequences of forward and reverse strand were aligned in BLAST. Coverages showed a high similarity and ranged from 96 to 99%. Forward strand sequences were used for alignment with clone nucleotide sequences (BLAST:blast). Sequences agreed to 100% for β-casein and α-lactalbumin, to 99% for αs1-, αs2-, κ-casein, β-lactoglobulin and ovalbumin. 98% were achieved for ovomucoid. Finally, forward strand sequences were also submitted to "Translated BLAST:tblastx" in order to query for protein sequences that would fit the translated nucleotide sequence other than the related clones. Uncultured/environmental sample sequences were excluded from query. Results given as predicted, synthetic constructs, crystal structures/lattices were not considered. Given this, all recombinant nucleotide sequences could be aligned with AA sequences of the targeted allergens. Details of sequence alignments are shown in section 9.3.

4.3.2 Initial microarray

4.3.2.1 Spot pattern of the initial protein array
Besides allergens, several concentrations of IgE and IgG_4 were to be applied in order to establish a calibration curve. Due to temporal limitations, only three concentrations of each IgE and IgG_4 in PBS could be spotted. Extracts, components and PBS were spotted with 5 replicates each (Figure 29).
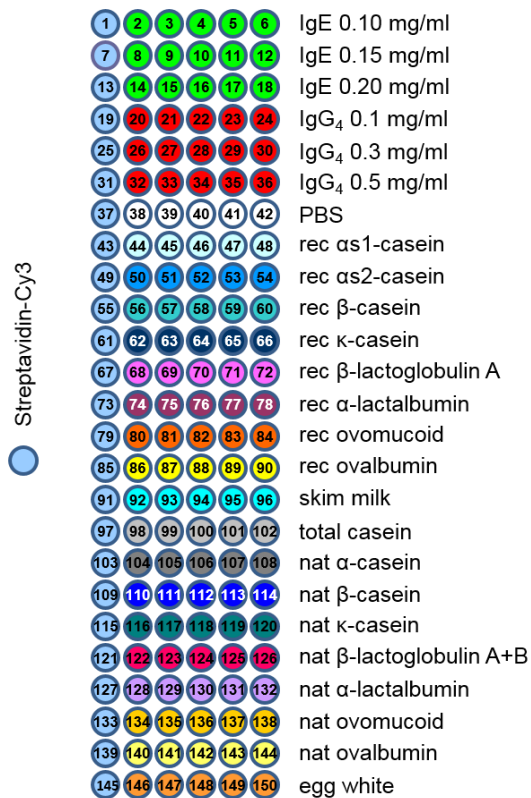
Figure 29: Initial protein pattern.

IgE-specific fluorescences were scanned at 70%, 80% and 90 % laser power/PMT, IgG₄-specific fluorescences were scanned at 50%, 60% and 70% laser power/PMT.

With this array of allergenic proteins and controls, several assays were conducted to (1) establish the parallel detection of IgE-and IgG₄-specific fluorescence, (2) set the optimal laser powers/PMTs for IgE and IgG₄ at Cy3 and Cy5-channel, (3) determine an allergen-specific baseline for IgE-specific fluorescence, and to (4) assess internal and external calibration.

4.3.2.2 *Sequential and parallel incubation with mAB's against IgE and IgG₄*

In order to reliably detect human IgE and IgG₄ in parallel, it was necessary to exclude cross reactions between mAB's to be applied. Therefore, HE high and low pool sera were assayed and cross-sequential application versus co-application of secondary mAB's was conducted (Figure 30).
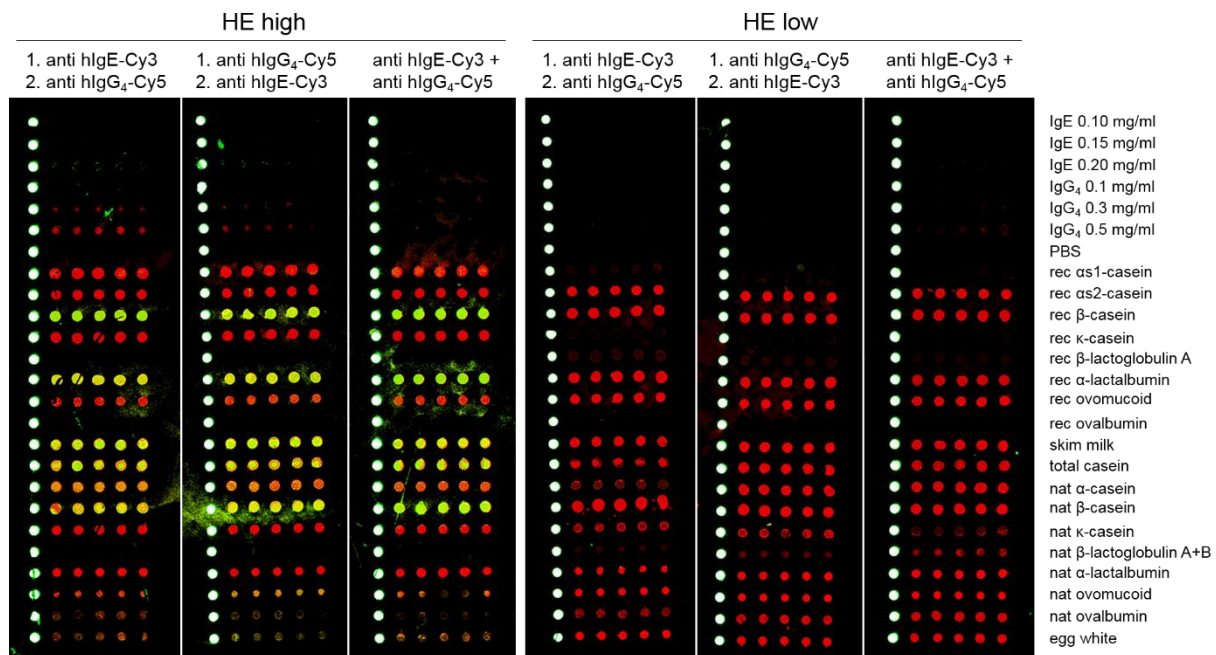
Figure 30: Comparison of IgE- and IgG₄-specific fluorescnces after sequential and parallel incubation with secondary mAB's. IgE-specific fluorescence (green) and IgG₄-specific fluorescence (red) were detected at 80% and 60% laser power/PMT and merged using auto adjustment by Scan Array software. Spots with defined concentrations of IgE and IgG₄ were missing.

Merged images of IgE-specific and IgG₄-specific fluorescences showed neither differences between cross-sequential nor parallel application of secondary mAB's. Microarrays incubated with HE low serum showed only IgG₄-specific (red) fluorescence, whereas HE high serum gave a complex mix of fluorescences specific for IgG₄ (red), IgE (green) and both (yellow). Unfortunately, different concentrations of IgE and IgG₄ were not or only insufficiently detected which was ascribed to misspotting. Lack of coverage with secondary AB solution was found to be unlikely due to the high number of microarrays with missing spots. Some spots also showed irregular fluorescence (native k-casein, β-lactoglobulin and ovalbumin), indicating unequal deposition of protein due to e.g. impurity of protein or hydrophobic interactions with the surface. Of note, recombinant ovalbumin neither showed binding to IgE nor IgG₄.

Scans on Cy3-channel at 70% and 90% laser power/PMT led to very low fluorescence (70%) or enhancement of unspecific background (90%). On the contrary, 70% laser power/PMT applied on Cy5-channel resulted in saturation of several allergens, whereas 50% only showed low fluorescence. However, scans at these laser powers/PMT were also quantified and submitted to statistical analysis. Fluorescences of allergens and IgE/IgG₄-calibration spots of each microarray were summed up and compared with each other. Kruskal-Wallis test statistic revealed no significant differences between differently incubated microarrays at 80% and 90% laser power/PMT for IgE-specific fluorescences, and 50% and 70% laser power/PMT for IgG₄-specific fluorescences (range of P values 0.1523 to 0.9817). A statistical difference was only found for IgE-specific fluorescences at 70% laser power/PMT. Cross-sequentially incubated microarrays were not different from each other, but when compared to

parallel incubation, a significant difference was calculated (P<0.0001). IgE-specific fluorescences at 80% and IgG$_4$-specific fluorescences at 60% were also log-transformed in order to repeat statistics with a different test based on Gaussian distribution of data. One-way analysis of variance with Bartlett's test for equal variances could be applied to IgE-specific fluorescences and revealed no statistical difference. IgG$_4$-specific fluorescences could not be normalized by log-transformation. Subtraction of background fluorescence was not statistically considered.

It was concluded that there was no unspecific cross reaction between the two mAB's and that parallel incubation with mAB's anti human IgE-Cy3 and anti human IgG$_4$-Cy5 could be applied. Standard laser powers/PMTs for quantification were set to 80% for IgE- and 60% for IgG$_4$-specific fluorescences.

4.3.2.3 Assessment of sensitivity

Assays with negative, atopic and positive controls with low, medium and high levels of sIgE were conducted and IgE-specific fluorescences were compared (Figure 31, Figure 32). Sensitivity to IgG$_4$ could not be examined because no quantitative data was available for the pool sera.
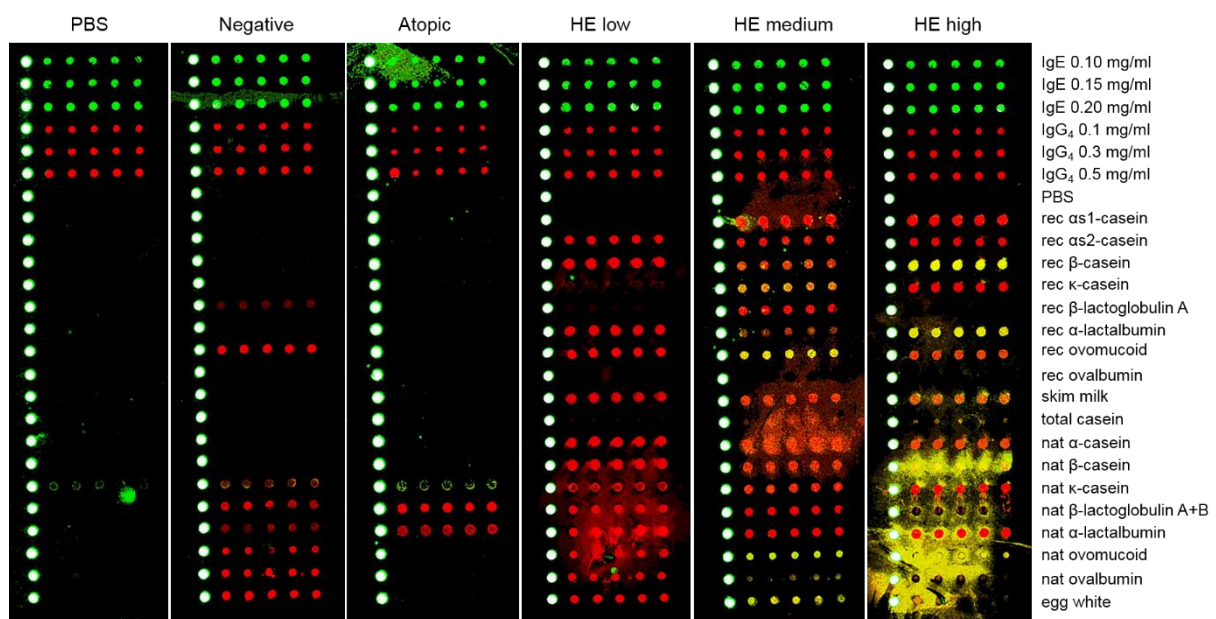
Figure 31: Comparison of PBS and negative with positive HE sera. IgE-specific fluorescence (green) and IgG$_4$-specific fluorescence (red) were detected at 80% and 60% laser power/PMT and merged using auto adjustment by Scan Array software.

In PBS, negative and atopic controls unspecific binding to native κ-casein was observed. No further IgE-specific fluorescences of allergens were clearly visible. HE sera showed an increase of IgE-specific fluorescence from low to high, which was accompanied by IgG$_4$, resulting in (bright) yellow. HE low pool serum showed an equal fluorescent pattern dominated by IgG$_4$ with no IgE-specific fluorescence (Figure 30). Except for recombinant

ovalbumin, HE medium pool serum was positive for all allergens with either IgE-, IgG$_4$- or both IgE/IgG$_4$-specific fluorescences. For that reason, it was later selected as intra and inter slide control serum. In HE pool serum, strong IgE binding to several allergens was observed (especially recombinant and native β-casein, recombinant α-lactalbumin). In general, the pattern shown in Figure 30 was reproduced. However, strong background occurred, impeding analysis of spotted allergens below native α-casein.
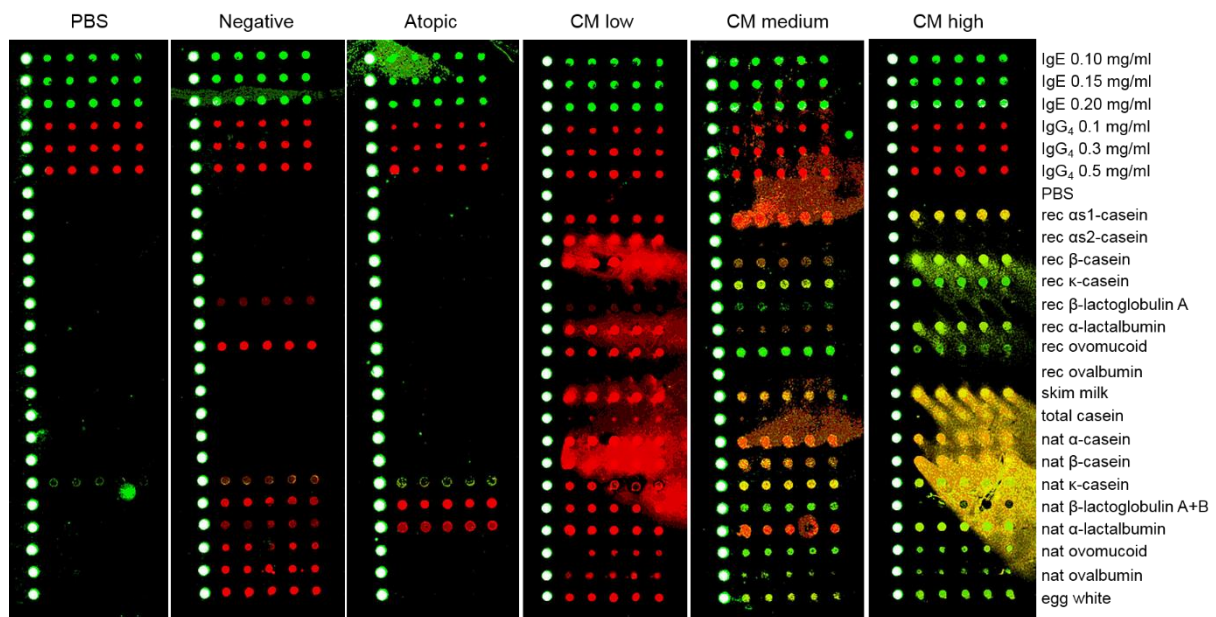
Figure 32: Comparison of PBS and negative with positive CM sera. IgE-specific fluorescence (green) and IgG$_4$-specific fluorescence (red) were detected at 80% and 60% laser power/PMT and merged using auto adjustment by Scan Array software. Microarrays with PBS, negative and atopic pool serum are identical to those in **Figure 31**.

CM sera also showed an increase of IgE-specific fluorescence from low to high, which was less accompanied by IgG$_4$, resulting in more green than yellow spots. Despite high levels of IgE, some allergens were not clearly detected (recombinant αs2-casein, recombinant β-lactoglobulin) in CM high pool serum. On the other hand, considerable background occurred, smearing over some spots. However, spot morphology and spot-specific fluorescence were preserved as seen for native κ-casein and native β-lactoglobulin. CM low pool serum showed an equal fluorescent pattern like HE low pool serum with dominance of IgG$_4$-specific fluorescence. CM medium pool serum showed a similar recognition pattern like HE medium pool serum with a shift to IgE-specific fluorescence, however, recombinant αs2-casein was not clearly detected and total casein only showed very small spots.

In all applications, spotted concentrations of IgE and IgG$_4$ were positive and appeared not different between microarrays.

Extensive statistical analyses were applied. IgE-specific median and mean fluorescences at 70%, 80% and 90% laser power/PMT were compared without and with subtraction of background fluorescence. Total fluorescences of allergens and PBS (19 analytes) of all

microarrays were calculated. Log-transformations were performed to convert data into Gaussian distribution. Especially PBS, negative and atopic pool sera were not transformable due to few relatively strong unspecific fluorescence of native κ-casein and low remaining fluorescence from other allergens. Therefore, Kruskal-Wallis statistic and Dunn's Multiple Comparison tests were applied. Selected results at 80% laser power/PMT are shown in Table 20, detailed results of 70% and 90% laser power/PMT are depicted in Supplement Table 15.

Table 20: Comparison of total median and mean fluorescences from microarrays incubated with PBS, negative, atopic and positive CM and HE pool sera at 80% laser power/PMT. Differences between median and mean fluorescence are marked red, different pairs of results with and without log-transformation are marked green. Fl, fluorescence; BG, background

Incubation	Median FL at 80%				Mean FL at 80%			
	Fl	log(Fl)	Fl-BG	log(Fl-BG)	Fl	log(Fl)	Fl-BG	log(Fl-BG)
PBS vs Negative	ns	ns	ns	ns	ns	ns	ns	ns
PBS vs Atopic	ns	ns	ns	ns	ns	ns	ns	ns
PBS vs CM high	***	***	***	***	***	***	***	***
PBS vs CM med	***	***	***	***	***	***	***	***
PBS vs CM low	**	***	*	**	***	**	*	**
PBS vs HE high	***	***	ns	***	***	***	ns	***
PBS vs HE med	***	***	***	***	***	***	***	***
PBS vs HE low	*	**	ns	ns	**	*	ns	*
Negative vs Atopic	ns	ns	ns	ns	ns	ns	ns	ns
Negative vs CM high	***	***	***	***	***	***	***	***
Negative vs CM med	***	***	**	***	***	***	**	**
Negative vs CM low	ns	ns	ns	ns	ns	ns	ns	ns
Negative vs HE high	***	***	ns	**	***	***	ns	**
Negative vs HE med	***	***	**	***	***	***	**	***
Negative vs HE low	ns	ns	ns	ns	ns	ns	ns	ns
Atopic vs CM high	***	***	***	***	***	***	***	***
Atopic vs CM med	***	***	***	***	***	***	***	***
Atopic vs CM low	*	*	*	**	*	*	*	**
Atopic vs HE high	***	***	ns	***	***	***	ns	***
Atopic vs HE med	***	***	***	***	***	***	***	***
Atopic vs HE low	ns	ns	ns	ns	ns	ns	ns	ns
CM high vs CM med	ns	ns	ns	ns	ns	ns	ns	ns
CM high vs CM low	**	**	ns	*	**	**	ns	*
CM high vs HE high	ns	ns	ns	ns	ns	ns	*	ns
CM high vs HE med	ns	ns	ns	ns	ns	ns	ns	ns
CM high vs HE low	***	***	ns	**	***	***	ns	**
CM med vs CM low	ns	ns	ns	ns	ns	ns	ns	ns
CM med vs HE high	ns	ns	ns	ns	ns	ns	ns	ns
CM med vs HE med	ns	ns	ns	ns	ns	ns	ns	ns
CM med vs HE low	ns	ns	ns	ns	ns	ns	ns	ns
CM low vs HE high	ns	ns	ns	ns	ns	ns	ns	ns
CM low vs HE med	ns	ns	ns	ns	ns	ns	ns	ns
CM low vs HE low	ns	ns	ns	ns	ns	ns	ns	ns
HE high vs HE med	ns	ns	ns	ns	ns	ns	ns	ns
HE high vs HE low	*	*	ns	ns	*	*	ns	ns
HE med vs HE low	ns	ns	ns	ns	ns	ns	ns	ns

Variations between median and mean fluorescence occurred in 6 pairs of results (in red). Log-transformation of fluorescence altered differences only in two cases (PBS vs. CM low,

and PBS HE low, mean fluorescence). Log-transformation of fluorescence minus background resulted in significant differences in 6 pairs of values (in green). Then calculated significances were similar to those obtained from fluorescence with no subtraction of background. Except for HE high and low, positive pool sera were not calculated as statistically different. Also PBS, negative and atopic pool sera did not differ from each other. PBS was different from HE and CM low sera, but subtraction of background decreased significance levels. On the contrary, negative pool serum was not different from low positive sera. Apart from HE low, atopic serum differed from all positive sera in fluorescence. Compared to HE high serum, subtraction of background led to no difference, which only became statsistically significant when log-transformation was applied. The same pattern was observed between CM high and low pool serum.

Of note, negative controls and PBS were not different from HE high pool serum when subtraction of background was applied. The high unspecific background fluorescence reduced specific fluorescences of proximal spots, and total specific fluorescence dropped. This was considered a major disadvantage of this mode of processing. However, these results indicated a limited sensitivity of the microarray which was to be assessed by application of a large number of individual patient sera.

4.3.2.4 Pool sera as a means of converting quantitative IgE into fluorescence and vice versa

Specific IgE to CM, HE and selected components was quantitatively determined in pool sera (Table 2). Atopic pool serum had been determined in fx5 with 0.05 kU$_A$/l, and was considerd as such for calculations. The CM result ">100 kU$_A$/l" could not be considered. However, the minimum of 5 data points with quantitative results was available for CM, HE and ovomucoid. Absolute and log-transformed IgE-specific fluorescences were plotted against quantitative results. Linear and logarithmic regression was applied (Figure 33, Figure 34).
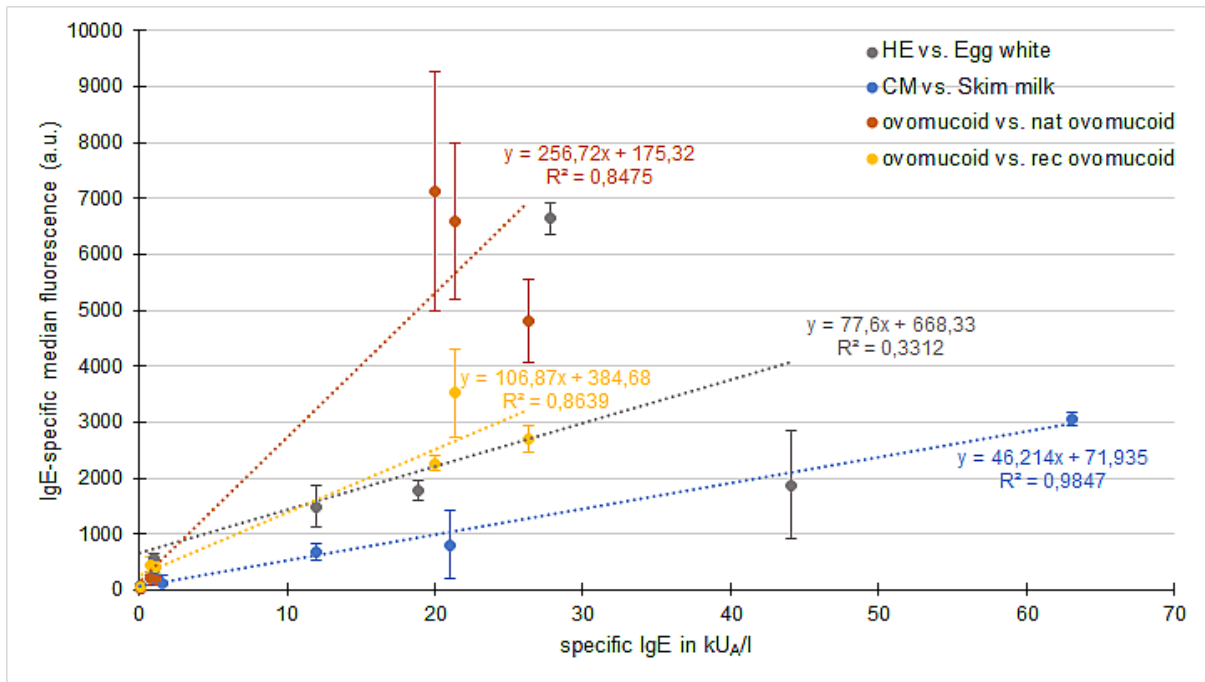
Figure 33: Linear regression of quantitative sIgE to CM, HE and ovomucoid against IgE-specific median fluorescences at 80% laser power/PMT.

The linear regression model was most appropriate for CM vs. skim milk with R^2 0.98. Recombinant and native ovomucoid were also fitted with good R^2 of 0.8475 and 0.8639. HE specific IgE and fluorescence could not be sufficiently fitted. Mean fluorescences were not significantly different.
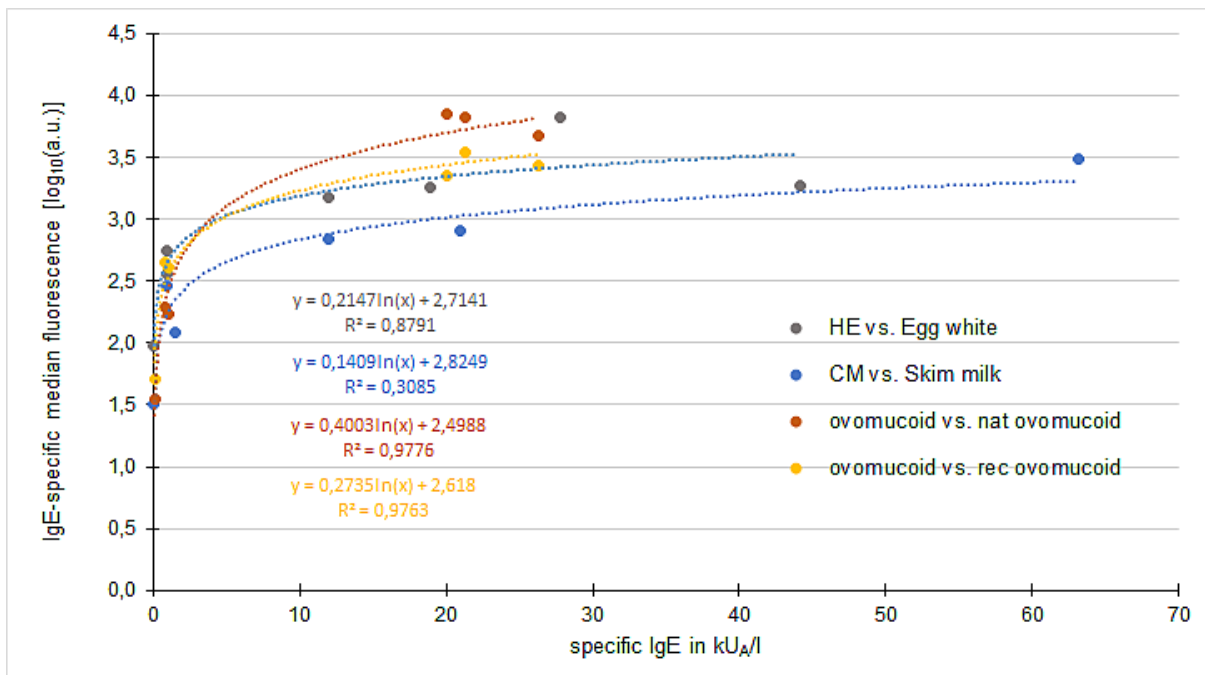
Figure 34: Logarithmic regression of quantitative sIgE to CM, HE and ovomucoid against IgE-specific median fluorescences at 80% laser power/PMT.

Contrary to linear regression, CM vs. skim milk could not be sufficiently fitted, whereas logarithmic regression was appropriate for HE vs. egg white. Recombinant and native ovomucoid were fitted with higher R^2 compared to linear regression model. Mean fluorescences were not different.

Equations for CM/Skim milk and HE/Egg white were applied to fluorescences obtained from a large numer of individual patient sera (4.3.2.6). Ovomucoid could not be considered due to missing quantitative data for individual sera.

4.3.2.5 Correlation of quantitative results from individual patient sera with IgE-specific fluorescences

Sera of 82 patients (set A) with determined sIgE to CM and HE were assayed. IgE-specific median and mean fluorescences were correlated with spotted extracts (skim milk, egg white) to assess sensitivity and linearity of the assay (Figure 35). Native and recombinant components and combinations of components were correlated with CM and HE results to identify significant individual components. These correlations were also calculated with subtraction of allergen-specific fluorescences measured with PBS, negative and atopic pool serum which were considered as blanks.
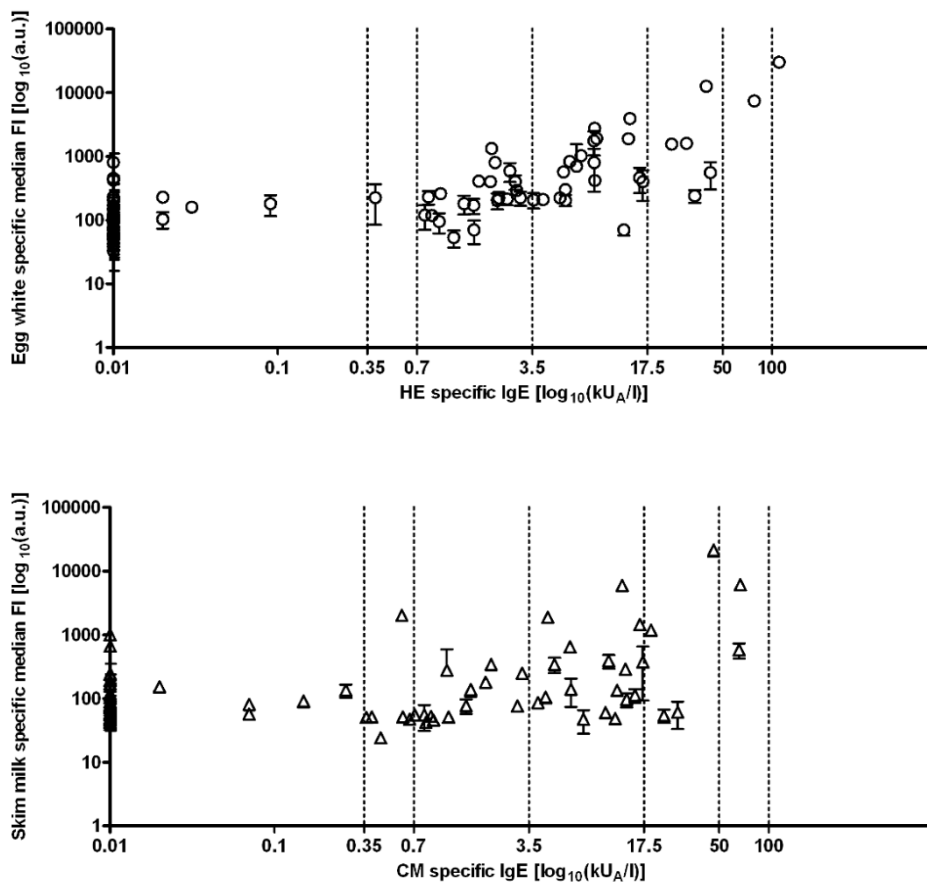
Figure 35: Correlation of quantitative HE and CM results with fluorescences of egg white and skim milk. Median fluorescence at 80% laser power/PMT was used. Results given as "<0.35 kU$_A$/l" were set to 0.01. Dashed bars indicate CAP classes from 0 to 6 (**Table 3**).

Plots revealed a positive correlation between HE and egg white, but only a weak correlation between CM and skim milk. Clearly visible was a lack of sensitivity, IgE titers <0.35 kU_A/l were far from being distinguished from those >0.35 kU_A/l.

Table 21: Calculated correlations of quantified IgE to CM and HE with IgE-specific fluorescences on the extended allergen microarray. Correlations were calculated for median and mean FI obtained from 82 sera at 80% laser power/PMT.

quantified allergen	Median FI	Mean FI	Median FI - BG FI	Mean FI - BG FI	spotted allergen
	0.476**	0.486**	0.524**	0.518**	rec αs1 casein
	-0.096	-0.094	-0.201	-0.189	rec αs2 casein
	0.296**	0.300**	0.367**	0.353**	rec β-casein
	0.363**	0.357**	0.371**	0.361**	rec κ-casein
	0.304**	0.335**	0.431**	0.390**	rec α-lactalbumin
	0.165	0.187	0.160	0.140	rec β-lactoglobulin A
	0.481**	0.485**	0.544**	0.536**	sum of rec CM components
CM	0.351**	0.375**	0.429**	0.424**	skim milk
	0.391**	0.388**	0.298**	0.292**	nat total casein
	0.606**	0.600**	0.624**	0.609**	nat α-casein
	0.230*	0.264*	0.249*	0.320**	nat β-casein
	0.305**	0.351**	0.315**	0.359**	nat κ-casein
	0.469**	0.482**	0.416**	0.443**	nat α-lactalbumin
	0.199	0.224*	0.184	0.231*	nat β-lactoglobulin A+B
	0.678**	0.689**	0.701**	0.709**	sum of α-,β-,k-casein, α-lactalbumin, β-lactoglobulin A+B
	0.573**	0.574**	0.579**	0.580**	sum of total casein, α-lactalbumin, β-lactoglobulin A+B
HE	0.579**	0.565**	0.581**	0.561**	rec ovomucoid
	0.107	0.111	0.079	0.033	rec ovalbumin
	0.567**	0.530**	0.555**	0.527**	sum rec HE components
	0.638**	0.637**	0.664**	0.643**	nat ovomucoid
	0.582**	0.563**	0.605**	0.580**	nat ovalbumin
	0.687**	0.677**	0.701**	0.690**	sum nat HE components
	0.733**	0.695**	0.753**	0.713**	egg white

Calculated results confirmed a positive correlation between sIgE to HE and egg white specific fluorescence. Skim milk fluorescence and sIgE to CM correlated weak.

Correlations of sIgE to CM with fluorescence of recombinant proteins showed divergent results. No significant correlation was observed for αs2-casein and β-lactoglobulin A. Other correlations were highly significant but ranged between 0.296 and 0.476, indicating only a weak to moderate positive correlation. However, the sum of all recombinant proteins ($r_{(s)}$=0.481**) exceeded the result of skim milk ($r_{(s)}$=0.351**) which was considered the correlate to CM on ImmunoCAP. Subtraction of background fluorescence clearly increased correlations for median fluorescence of αs1- and β-casein as well as for α-lactalbumin. On the other hand, the correlation for αs2-casein clearly decreased, indicating a different impact of subtraction of background fluorescence.

Native CM components showed comparable significant and highly significant correlations between 0.230 and 0.606. The sum of native components α-, β-, κ-casein, α-lactalbumin and β-lactoglobulin A+B showed highest correlation ($r_{(s)}$=0.678), clearly different from the sum of total casein, α-lactalbumin and β-lactoglobulin ($r_{(s)}$=0.573). Of note, median and mean fluorescences for native β-lactoglobulin A+B differed in statistical significance. This was

considered an indicator that both median and mean fluorescence needed to be analyzed and compared.

The correlation of quantified sIgE to HE with fluorescences of egg white and components was highly significant with the exception of recombinant ovalbumin. Contrary to CM results, the sum of native HE components did not exceed the correlation value of egg white.

Comparison of paired recombinant and native components revealed similar correlations for β-casein and κ-casein. Other comparable pairs of CM and HE components revealed higher correlations of native proteins to recombinant ones. No significant differences were observed between median and mean fluorescence except for native β-lactoglobulin A+B as mentioned above. Subtraction of background fluorescence slightly altered correlations, and was not suitable for all analytes, e.g. recombinant αs2-casein and native total casein, which were surrounded by high local background compared to other analytes.

Subtraction of blanks (PBS, negative and atopic pool sera) did not alter correlations.

4.3.2.6 *Conversion of IgE-specific fluorescences in quantitative results by means of equations*

Calculated linear and logarithmic equations were applied to fluorescences of egg white and skim milk. Deviations between given result (in kU_A/l) and calculated result were determined.

Neither median fluorescence of egg white nor skim milk could be sufficiently converted into given sIgE in kU_A/l to CM and HE. Despite the goodness of fit in linear regression, these equations led to implausible results. Logarithmic equations produced results in the dimension of kU_A/l, but these were too high, at average 10 units. In few cases, a good fit was observed. If these were specific or random events could not be determined. Details are shown in Supplement Table 16.

4.3.2.7 *Inter slide and intra slide variation*

With a delay of 30 sera that been already assayed, HE medium pool serum was introduced as inter slide control for IgE- and IgG_4-specific fluorescences. It was applied to one of each 10 slides (considered as one batch), and to one complete batch. Coefficient of variation was calculated by the equation: CV% = SD / mean * 100. Results are shown in Table 22.

Table 22: Intra and inter slide variation of IgE- and IgG$_4$-specific fluorescences. Calculations on the basis of mean fluorescences obtained at 80% (IgE) and 60% (IgG$_4$) laser power/PMT. Basis for inter slide CV n=7, and for intra slide n=10.

spotted analyte	IgE-specific mean FI		IgG$_4$-specific mean FI	
	intra-assay CV (%)	inter-assay CV (%)	intra-assay CV (%)	inter-assay CV (%)
IgE 0.1 mg/ml	28.1	79.5	58.7	57.8
IgE 0.15 mg/ml	33.5	52.1	43.2	52.7
IgE 0.2 mg/ml	26.4	47.7	72.0	32.9
IgG$_4$ 0.1 mg/ml	35.1	94.6	34.7	52.6
IgG$_4$ 0.3 mg/ml	27.1	41.5	24.7	36.4
IgG$_4$ 0.5 mg/ml	21.5	37.5	20.9	21.3
PBS	19.1	46.4	45.0	80.6
rec αs1-casein	13.7	18.7	15.8	24.4
rec αs2-casein	10.9	32.4	10.7	22.0
rec β-casein	24.5	33.1	17.2	26.8
rec κ-casein	15.3	24.8	15.1	14.8
rec β-lactoglobulin A	13.6	29.2	14.8	25.6
rec α-lactalbumin	15.1	24.0	14.7	21.1
rec ovomucoid	14.6	27.0	15.0	19.7
rec ovalbumin	15.1	14.8	16.1	13.3
skim milk	29.9	45.0	24.0	41.7
total casein	10.8	58.0	18.2	32.2
nat α-caseins	16.6	32.5	19.4	31.3
nat β-casein	20.6	42.5	19.3	47.8
nat κ-casein	17.1	22.9	17.0	18.3
nat β-lactoglobulin A+B	19.5	22.3	15.9	36.3
nat α-lactalbumin	17.1	19.3	18.5	25.7
nat ovomucoid	22.7	41.6	15.5	27.9
nat ovalbumin	15.9	51.6	18.4	48.8
egg white	15.7	27.9	19.0	37.5

Intra slide variation was clearly lower compared to inter slide variation for both IgE- and IgG$_4$-specific fluorescences. Spotted concentrations of IgE and IgG$_4$ varied considerably even in intra slide comparison, being inacceptable as a means of calibration. High results for PBS were probably due to unspecific noise or low mean fluorescence with high SD. IgE-specific CV's of allergens ranged from 10.8 (total casein) to 29.9% (skim milk) and were very similar to IgG$_4$-specific CV's. At average were calculated 17.1% for IgE-, and 16.9% for IgG$_4$-specific fluorescence. Results of inter slide variation were clearly higher and with 31.5% (IgE) and 28.6% (IgG$_4$) at average twofold as high as intra slide variation. Several reasons were taken into account for these results: irregular deposit of protein on the microarray surface, hydrophobicity of some proteins (causing the "donut-effect"), highly manual processing (especially washing procedures), and suboptimal setting of focus prior to scan. Therefore, optimization and standardization strategies were developed.

4.3.2.8 Interim conclusions

Correlations of skim milk and egg white fluorescences with sIgE to CM and HE were moderate to weak. Sensitivity was not satisfactory. Native components correlated higher with sIgE to CM and HE than recombinant components. Subtraction of background slightly increased correlations, but was inappropriate when serum remains caused high unspecific

background. Spots were detected even when surrounded by strong background, making subtraction of background superfluous. A baseline that clearly distinguishes sera corresponding to the 0.35 kU$_A$/l cut-off was not found. External calibration by means of pool sera was applied and failed to to deliver comparable values to quantitative results. This was assigned to the high inter slide variations.

These results implied further optimizations of sample preparation and deposition, and extension of internal calibration for IgE and IgG$_4$ with at 5 least five concentrations. HE medium pool serum was selected as inter assay control.

4.3.3   Establishment of calibration curves

4.3.3.1   Calibration curve for IgG$_4$

Previous assays had shown that the IgE- and IgG$_4$ internal calibration curves needed to be adjusted and extended. For IgE, ImmunoCAP uses calibrators in the range 0.1 to 100 kU/l (0.242 to 242 µg/l) which are incubated with calibration caps. This application resembles the 3-level sandwich assays with patient serum (cap – patient serum – secondary AB). In this study, an alternate approach for IgE was applied: myeloma-derived IgE was directly spotted to be then recognized by the secondary ABs. Since this two-level sandwich lacks one step of amplification, higher concentrations of antigen were applied with reference to IgG$_4$ calibrators on the ImmunoCAP, where concentrations of 300, 120, 35, 3 and 0.001 mg/l are assayed during calibration. With these concentrations, IgG$_4$ and IgE were prepared in different solutions and spotted on 5 microarrays according to the pattern shown in Figure 36.
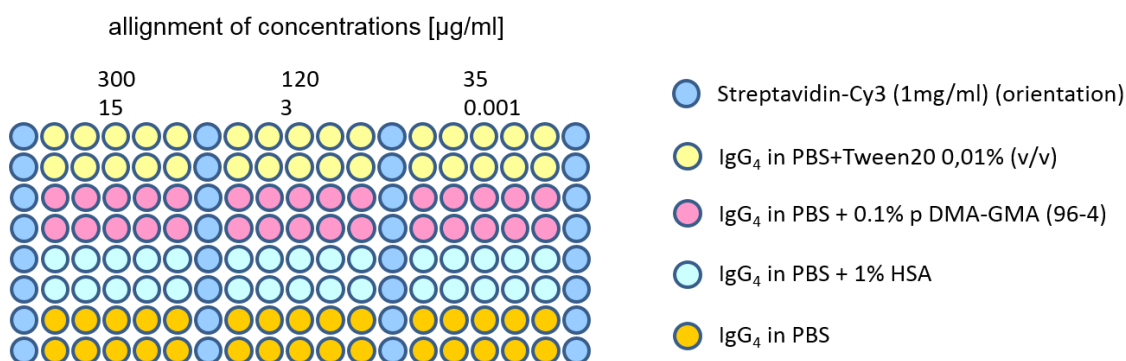
Figure 36: Spotting scheme of IgG$_4$ prepared in different solutions.

After incubation with mAB anti-human IgG$_4$-Cy5 (1:500) and scans at several laser powers/PMT, fluorescence at 70% was selected for comparison of diluents. One microarray was treated with Flamingo stain after detection of IgG$_4$ to visualize spotted protein (Figure 37).
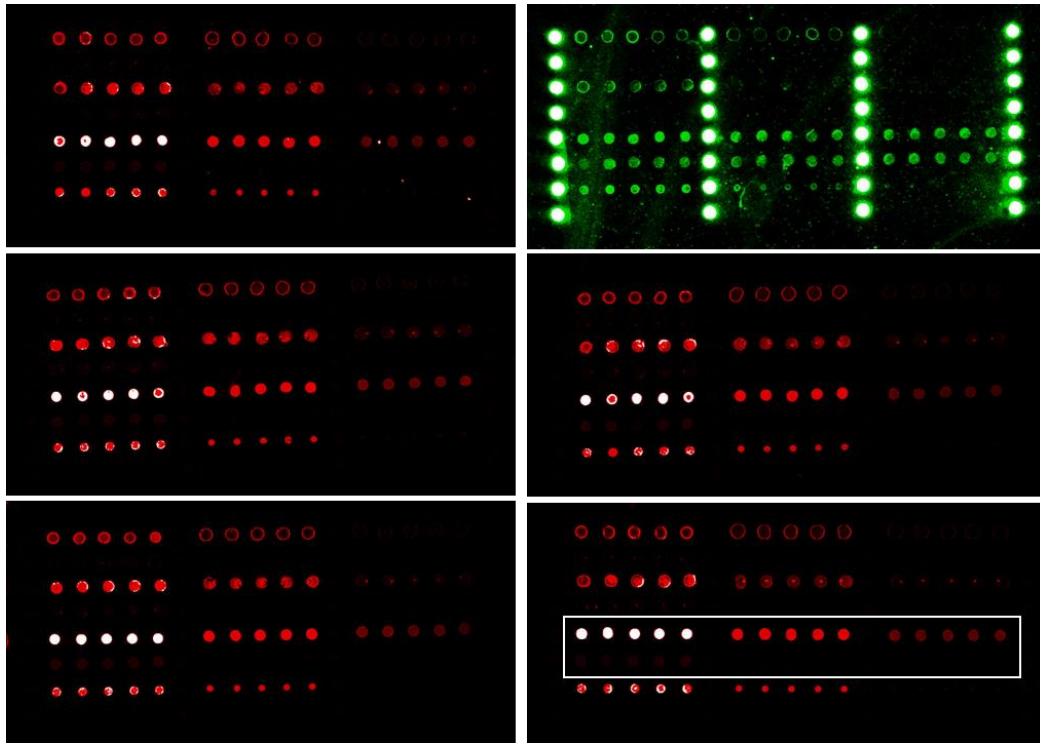
Figure 37: IgG₄-specific fluorescence from 5 arrays with different diluents for IgG₄. After scanning on Cy5-channel, specific for IgG₄ signals, one array (left top) was stained with Flamingo Pink and scanned on Cy3-channel (right top). One complete area of IgG₄ in 1% HSA/PBS is framed (right bottom).

IgG₄ in 1% HSA/PBS showed clearly visible fluorescence down to 15 µg/ml on all 5 arrays. Other diluents failed to provide specific fluorescence below 35 µg/ml. In the protein stain, IgG₄ in PBS was not detected below 35 µg/ml concentration.

IgG₄-specific fluorescence was quantified to calculate the range of detection (Figure 38).
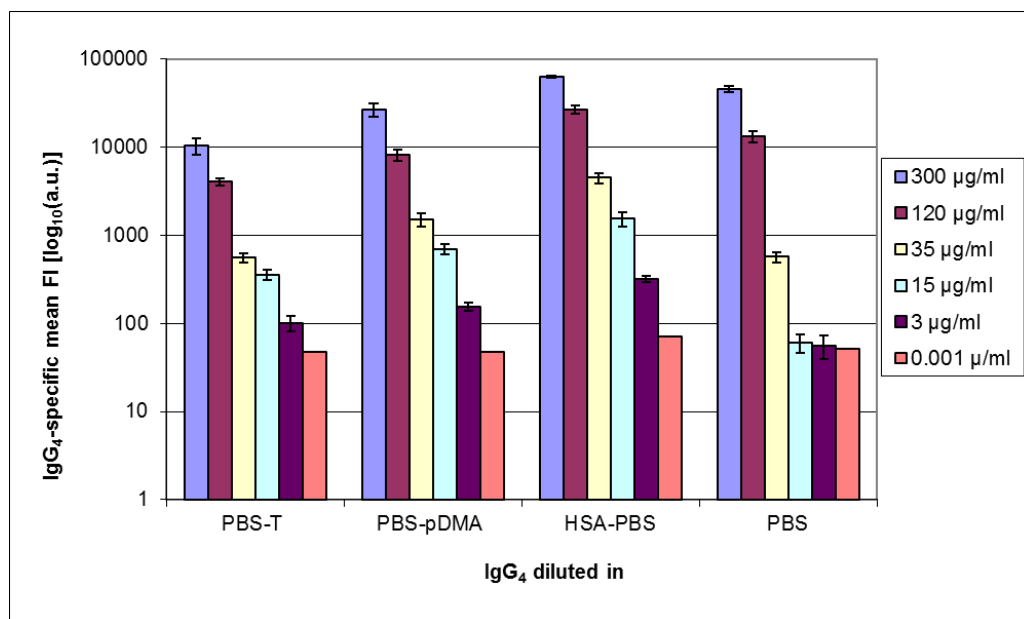
Figure 38: Quantified mean fluorescence of IgG₄ diluted in different solvents. Results represent averaged mean fluorescence of 5 arrays with 5 replicates per spotted concentration in linear scale (top) and logarithmic scale (bottom). Bars indicate SD.

From the selected diluents, 1% HSA in PBS showed superior performance over PBS-pDMA-GMA (96-4), PBS-Tween and PBS, and was chosen as standard diluent for IgG$_4$ in subsequent applications. At 70% laser power/PMT, spotted concentrations of IgG$_4$ from 3 to 300 µg/ml were clearly and distinguishably detected. However, 300 µg/ml IgG$_4$ were saturated while the lowest concentration of 3 µg/ml just became detectable. This indicated a limited working range on high and low end at the concentrations applied when scanned with only one laser power/PMT.

4.3.3.2 *Calibration curve for IgE*

The IgE calibrators of the ImmunoCAP comprise concentrations of 100, 50, 17.5, 3.7, 0.7, 0.35 and 0.01 kU/l. corresponding to 242, 121, 42.35, 8.47, 1.694, 0.847 and 0.00242 ng/ml. These concentrations were prepared in the dilutions tested with IgG$_4$, and 6 microarrays were spotted with an extended spotting scheme. A mAB anti human IgE-Cy3 was applied at 1:500 dilution (Figure 39).
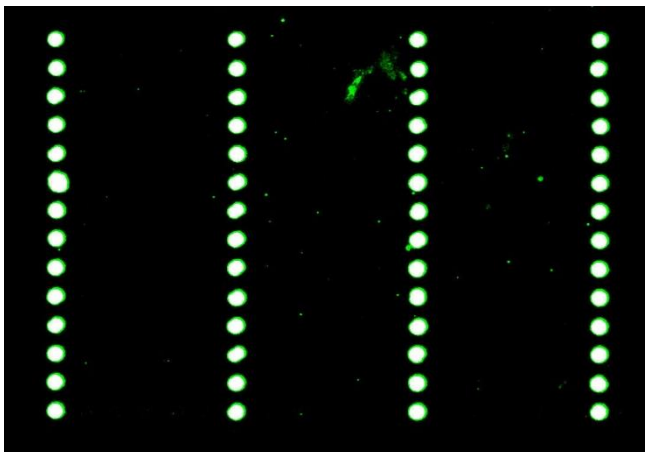
Figure 39: Scan for IgE spotted in kU/l range on Cy3-channel at 80% laser power/PMT.

No specific signals were observed. The repetition with reconstituted IgE from a new vial was also negative. Subsequently, the number of drops per spot was increased from 1 to 5 and spot distance was shifted from 500 to 620 nm. However, these settings did not improve the detection of IgE. Therefore, the concentrations of IgG$_4$ in 1% HSA/PBS were applied to IgE and both were spotted on 6 arrays. The IgE- and IgG$_4$-specific fluorescences were assessed independently by application of mABs in sequence, and simultaneously by co-application of mABs. This design of assays and scans would allow to detect crosstalk between mABs.
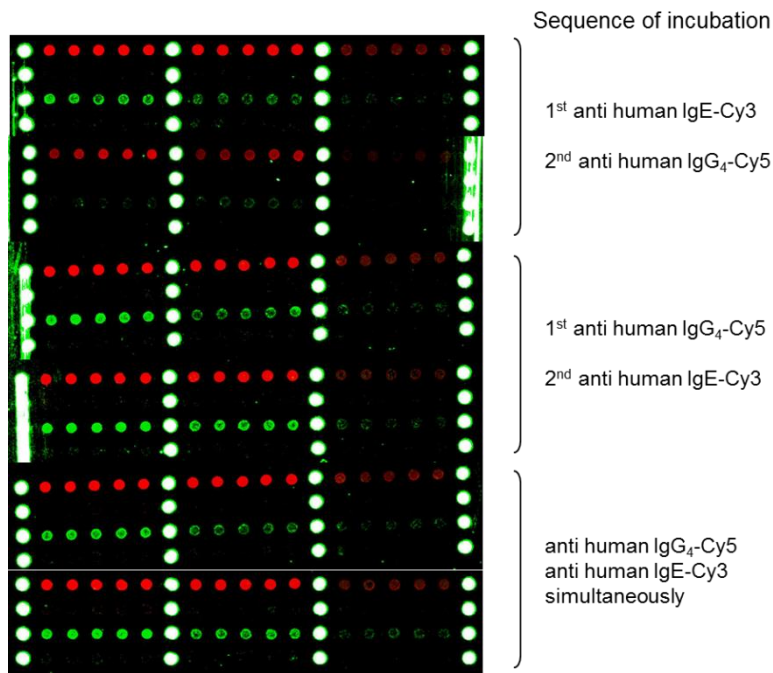
Figure 40: Sequential and simultaneous application of mABs against IgE and IgG₄. Overlays of scans at 90% laser power/PMT for IgE and 70% for IgG₄ (auto adjusted image).

Scans for IgE at 90% laser power/PMT were used for quantification (Figure 41).
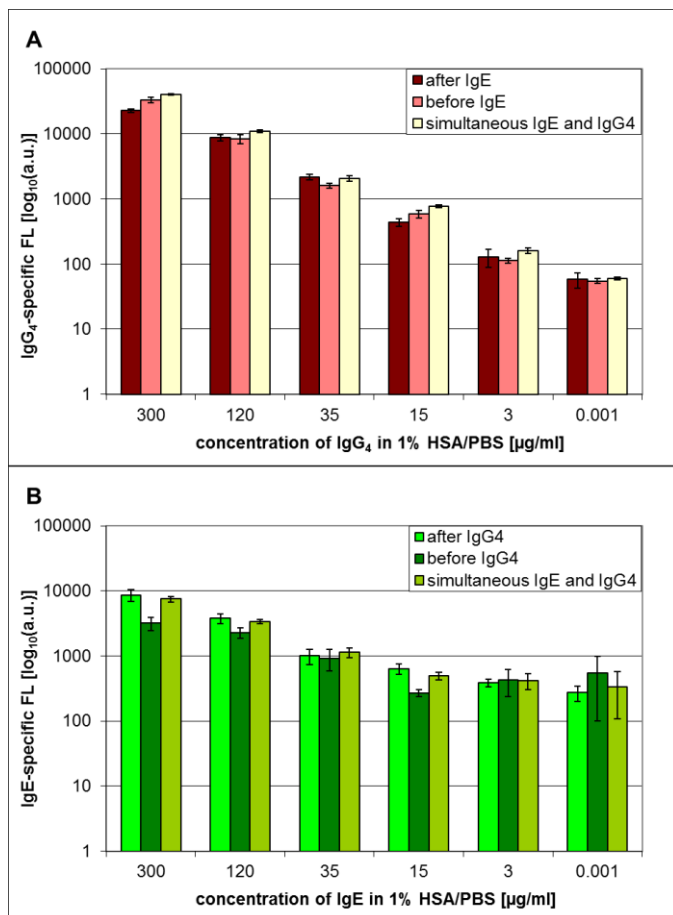
Figure 41: Quantification of sequential and simultaneous application of mABs against IgE and IgG₄. Results represent averaged mean fluorescence of 2 arrays per incubation (**Figure 40**). IgE-specific signals were quantified from 90% scans, IgG₄ from 70%. Bars indicate SD.

Sequential and parallel detection with mABs showed no evidence for systematic alterations of kinetics in detection of IgG$_4$-specific signals. On one hand, presence of mAB anti human IgE coincided with maximal fluorescences at concentrations 300, 120, 15 and 3 µg/ml. On the other hand, application of mAB anti IgG$_4$ after mAB anti IgE did not result in increase of fluorescence except for concentration 35 µg/ml.

On the contrary, clear alterations in detection of IgE were observed at concentrations 300, 120 and 15 µg/ml. Preceeded incubation with mAB anti IgG4 and parallel application of mABs coincided with an increase in fluorescence. This was taken as evidence to apply a different AB for detecting IgE-specific fluorescence (4.3.3.7).

These results also showed a large discrepancy between spotted concentrations and obtained fluorescences. Even at 90% laser power and PMT, IgE-specific fluorescences were clearly lower compared to corresponding concentrations of IgG$_4$.

4.3.3.3   *Optimizations of protein preparation*

Repeated freeze and thaw cycles were to be avoided. Skim milk protein solution was prepared freshly and NaN$_3$ was added to a concentration of 0.1%. This solution was stored at 4°C and used over a period of max. 2 weeks. Aliquots of native components, egg white, 1% HSA and IgG$_4$-calibrators in 1% HSA were derived from stock solutions and stored at -20°C. IgE-calibrators in 1% HSA were prepared in 2 stock solutions. Every aliquot was only used once with the exception of one lot of 20 microarrays which had to spotted with IgE calibrators from a previous application, stored at -20°C. In contrast to previous assays, ovomucoid without ovoinhibitor was used to directly compare native and recombinant component.

4.3.3.4   *Design of the final spotting frame*

An array of cow's milk and hen's egg allergens was spotted in combination with 6 concentrations of purified myeloma-derived IgG$_4$ and IgE. PBS and 1% HSA were used as negative controls. Five replicates of each allergen, HSA and PBS were spotted within a frame of orientation spots of Cy3-labelled streptavidin. The first series of arrays contained two concentrations of skim milk (1 and 2 mg/ml). On all subsequent arrays, skim milk was spotted with ~2 mg/ml and the remaining spots were used as bank area. The IgE calibrator with 300 µg/ml was reduced to 240 µg/ml from lot 2 on due to saturation at this concentration (Figure 42).
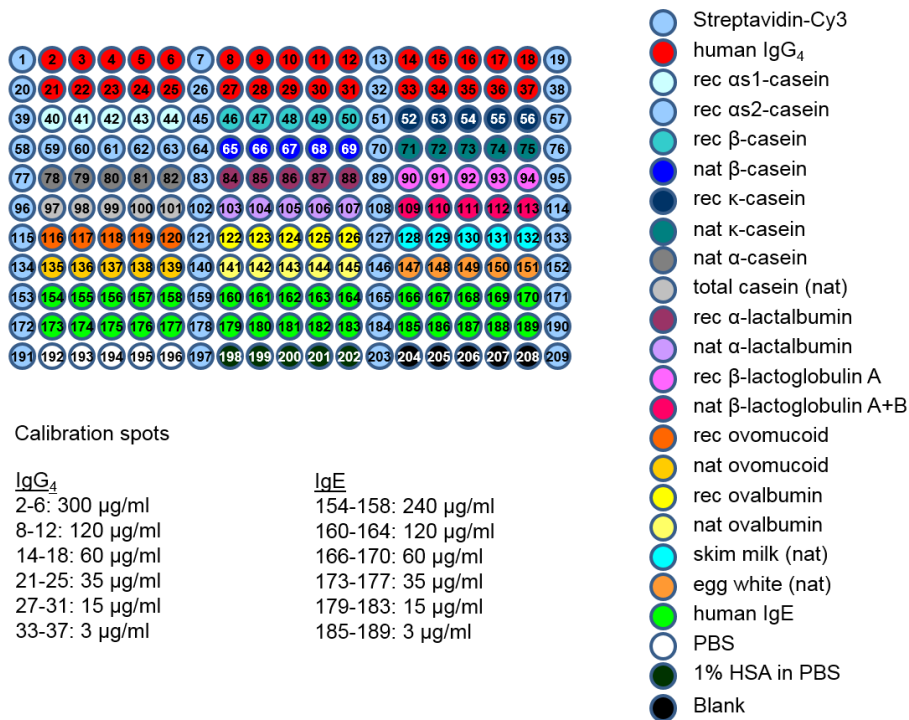
Figure 42: Spotting scheme and concentration of proteins. Initial arrays had a 300 µg/ml IgE calibrator. This was modified from lot 1 on along with the omission of skim milk at 1 mg/ml concentration. Instead, the Blank area was introduced.

As recombinant proteins were not lyophilized prior to use, the relative protein concentration to BSA was used. The concentration of native and recombinant proteins was pairwise adjusted to achieve approximately equal concentrations. Due to the fact that not all spots contain allergens, the term "analyte(s)" is used when technical aspects are considered.

4.3.3.5   Auto fluorescence of spotted proteins

To exclude unspecific fluorescence of spotted allergens or blank background, two microarrays were incubated with CM low and high positive pool serum but no secondary antibodies were applied (Figure 43).
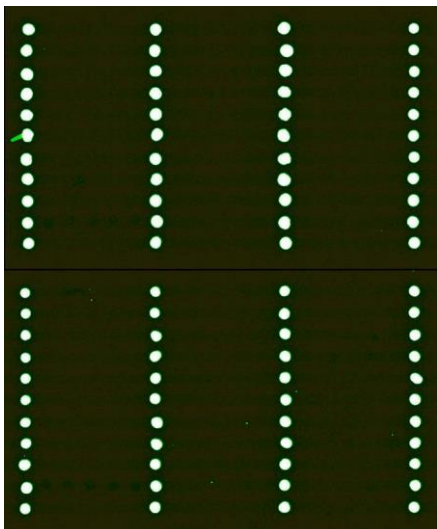
Figure 43: Assessment of auto-fluorescence on two microarrays.

The microarrays were scanned on Cy3-channel at 80% laser power/PMT and on Cy5-channel at 60%. The overlays show neither Cy3- and Cy5-specific signals, nor unspecific background fluorescence. Only Cy3-labelled Streptavidin used as orientation spots is detected (white).

4.3.3.6 Spot morphology and protein content

The first batch of spotted microarrays was analyzed with protein-specific staining, and one array of subsequent batches was assessed by light microscopy (Figure 44).
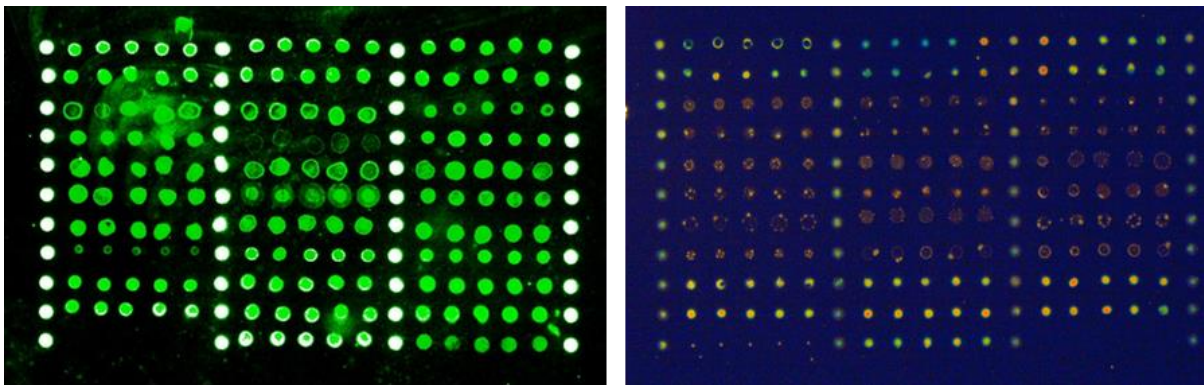
Figure 44: Protein-specific stain of spotted analytes and light microscopy. The Flamingo Pink stain (left), scanned on Cy3-channel with 80% laser power/PMT, and a slide of a subsequent lot, seen under a light microscope. On the Flaimingo stained slide, spots 128 to 132 contained skim milk at 1 mg/ml and spots 204-208 at 2 mg/ml. Subsequent arrays only contained skim milk at 2 mg/ml on spots 128 to 132, and spots 204 to 208 were left as blank area. Orientation spots appear white due to saturation.

Irregular spot morphology was observed for αs1-casein, recombinant and native β-casein and recombinant α-lactalbumin. These proteins showed a strong circle and a weak inner area ("donut effect"). Native α-lactalbumin appeared smeared and native ovomucoid was very densed. The light microscopy shows a different array with corresponding morphology of spotted analytes.

The protein-specific fluorescence by Flamingo stain (on Cy3-channel) was quantified, and mean and median fluorescence from the average of all five spots per analyte were calculated (Figure 45).
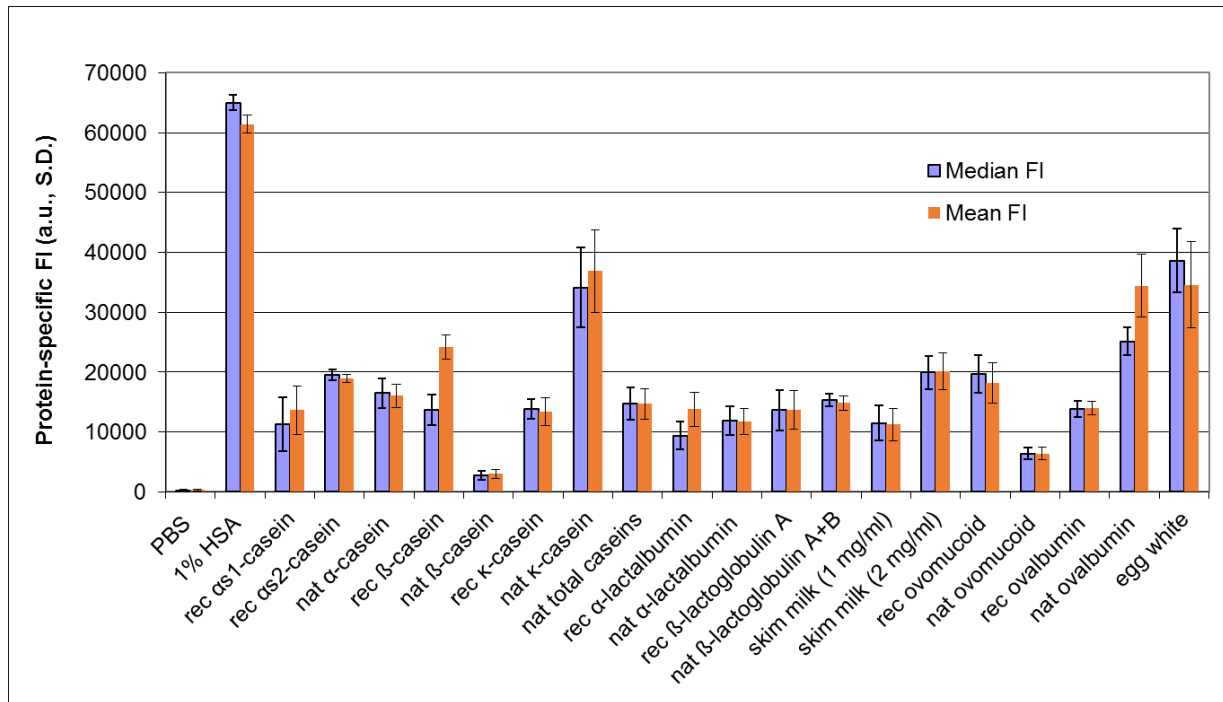
Figure 45: Protein-specific fluorescence of spotted analytes with Flamingo Pink total protein stain. A clear difference between median and mean fluorescence was only observed for recombinant β-casein. The Streptavidin-Cy3 orientation spots were not quantified as they are saturated.

The quantification revealed that the mean and median fluorescence values did not clearly differ with the exception of recombinant β-casein and native ovalbumin. Pairs of recombinant and native α-lactalbumin and β-lactoglobulin showed approximately equal fluorescences and thus protein content. Least fluorescences were measured for native β-casein and ovomucoid. Despite diluting native κ-casein to 0.54 mg/ml, it exceeded the mean fluorescence of all components twofold.

4.3.3.7 Cross-labeling and comparison of monoclonal and polyclonal AB against IgE

As shown in chapter 4.3.3, the mAB against IgE was less strong compared to that against IgG$_4$ at same concentrations of calibrators, respectively. Therefore, cross-labeling of fresh mABs was applied to address the question whether the labeling with Cy3 interfered with the binding of the mAB to IgE (Figure 46).
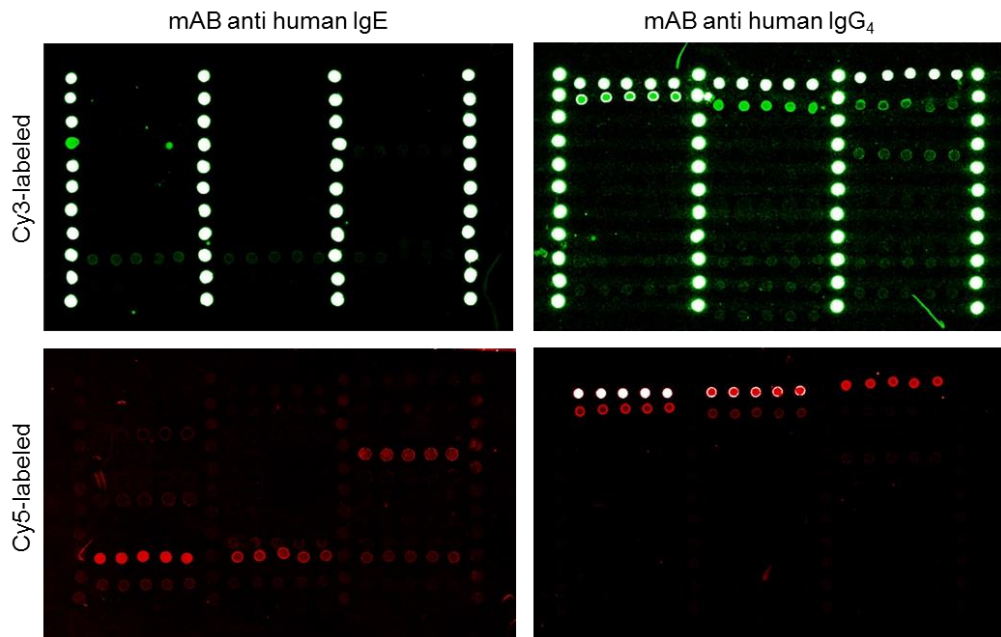
Figure 46: Cross-labeling of monoclonal antibodies to IgE and IgG$_4$. Differently labeled AB's were applied simultaneously. Scans were conducted at 80% laser power/PMT for both IgE and IgG$_4$-specific signals to maintain equal conditions.

Labeling of mAB anti human IgE to Cy5 led to an improved detection of spotted IgE to 3 µg/ml. On the other hand, unspecific fluorescence was observed for several allergens (αs2-, κ- and total casein). Labeling of mAB anti human IgG$_4$ to Cy3 resulted in saturated fluorescences for three IgG$_4$ concentrations and unspecific constant background for spotted IgE which was very likely due to the high content of HSA protein. Unspecific binding to native κ-casein occurred in all applications.

In parallel, a polyclonal AB (pAB) against IgE was also labeled with Cy3 and Cy5. Due to different concentrations of ABs and manufacturer's instructions, Cy3-labeld mABs were to be applied at 1:1000. All other labeled ABs were applied at 1:500. Several arrays were spotted and incubated with PBS, negative and atopic pool serum (Figure 47).
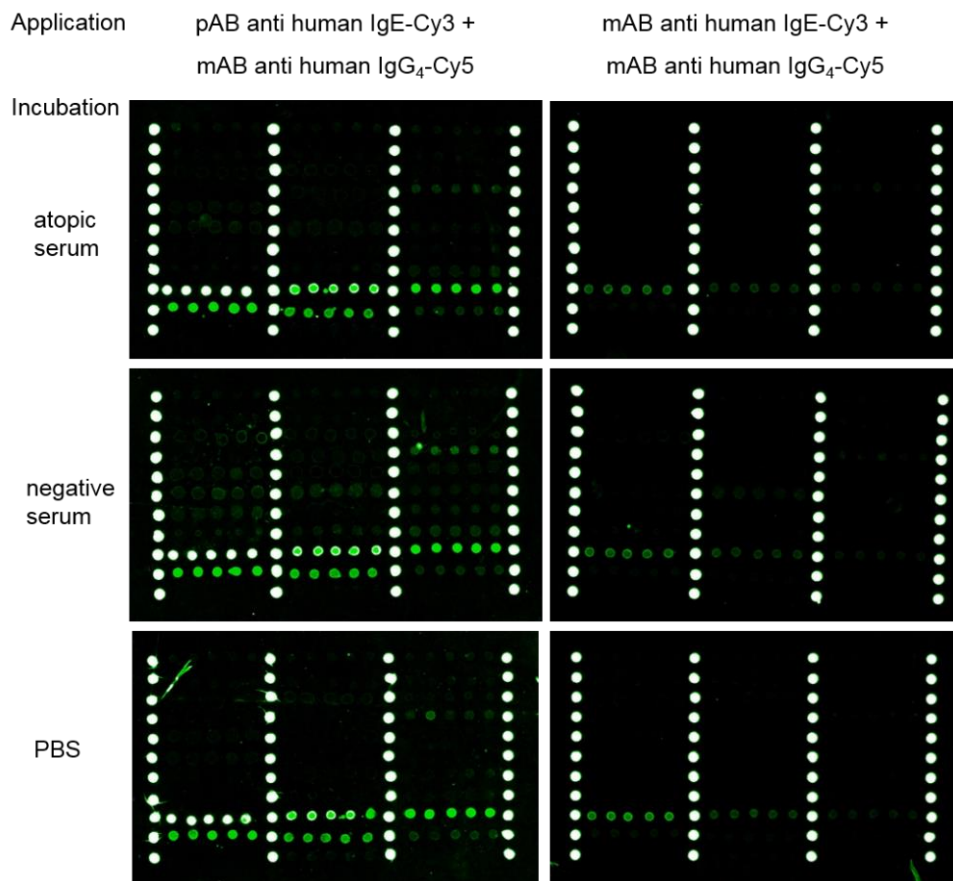
Figure 47: Comparison of a monoclonal with a polyclonal anti human IgE antibody. After incubation with atopic, negative pool serum or PBS, Cy3-labeled anti human IgE mAB and pAB were co-incubated with a Cy5-labeled anti human IgG$_4$ mAB. Only IgE-specific fluorescence is depicted. The polyclonal AB covers all spotted calibrators of IgE. 300 µg/ml calibrator is in saturation. On the other hand, unspecific fluorescence for some spotted allergens is visible, especially when negative pool serum was applied. The mAB hardly indicated the 35 µg/ml IgE calibrator (to be seen in PBS incubation).

The application of a polyclonal AB to IgE after incubation with PBS, negative and atopic pool sera revealed a drastically increased fluorescence compared to the mAB. Thereby, it was possible to detect small amounts of spotted IgE (3 µg/ml) without applying a higher laser power/PMT as it would have been necessary with the mAB. However, the increase of fluorescence also led to clear fluorescence from spotted allergens, especially in the negative pool serum. At this point, no explanation was found for this result. Unspecific binding to native κ-casein in all assays indicated limited value of this component in subsequent applications.

Subsequent assays were to be conducted with Cy5-labeled anti human IgG$_4$ mAB and Cy3-labeled pAB anti human IgE. Prior to assaying patient sera, positive pool sera were applied to assess crosstalk between ABs.

4.3.3.8 Secondary antibody specificity of mAB anti human IgG$_4$ and pAB anti human IgE

Sequential and parallel incubation assays with mAB anti human IgG$_4$-Cy5 and pAB anti human IgE-Cy3 were conducted with CM high, low and atopic pool sera. Negative pool serum was not used due to high unspecific background (Figure 48).
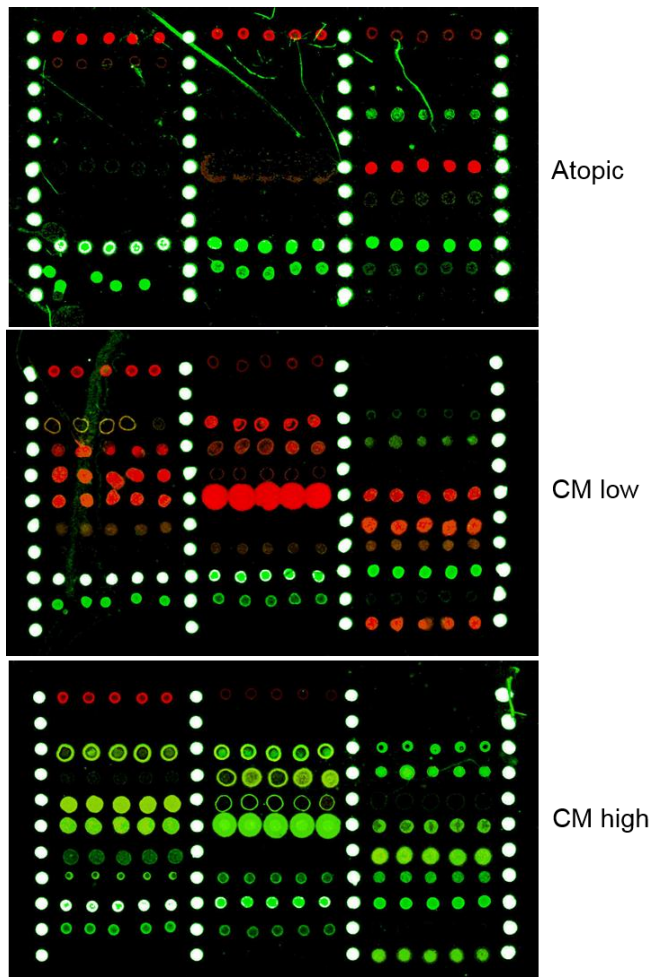
Figure 48: Comparison of IgE-and IgG$_4$-specific fluorescences in Atopic, CM low and high pool sera. Images are overlays and were auto adjusted.

IgE- and IgG$_4$-specific mean and median fluorescences were quantified from scans at 80% and 60% laser power/PMT. Of each microarray, fluorescences of IgE and IgG$_4$ calibration spots were compared. IgE-specific fluorescences of allergens were independently analyzed. IgG$_4$-specific fluorescences of allergens were not compared. Application of Kruskal-Wallis comparison test revealed significant differences which were assessed by Dunn's multiple comparison test. Results for mean fluorescences of IgE and IgG$_4$ spots are shown in Figure 49.
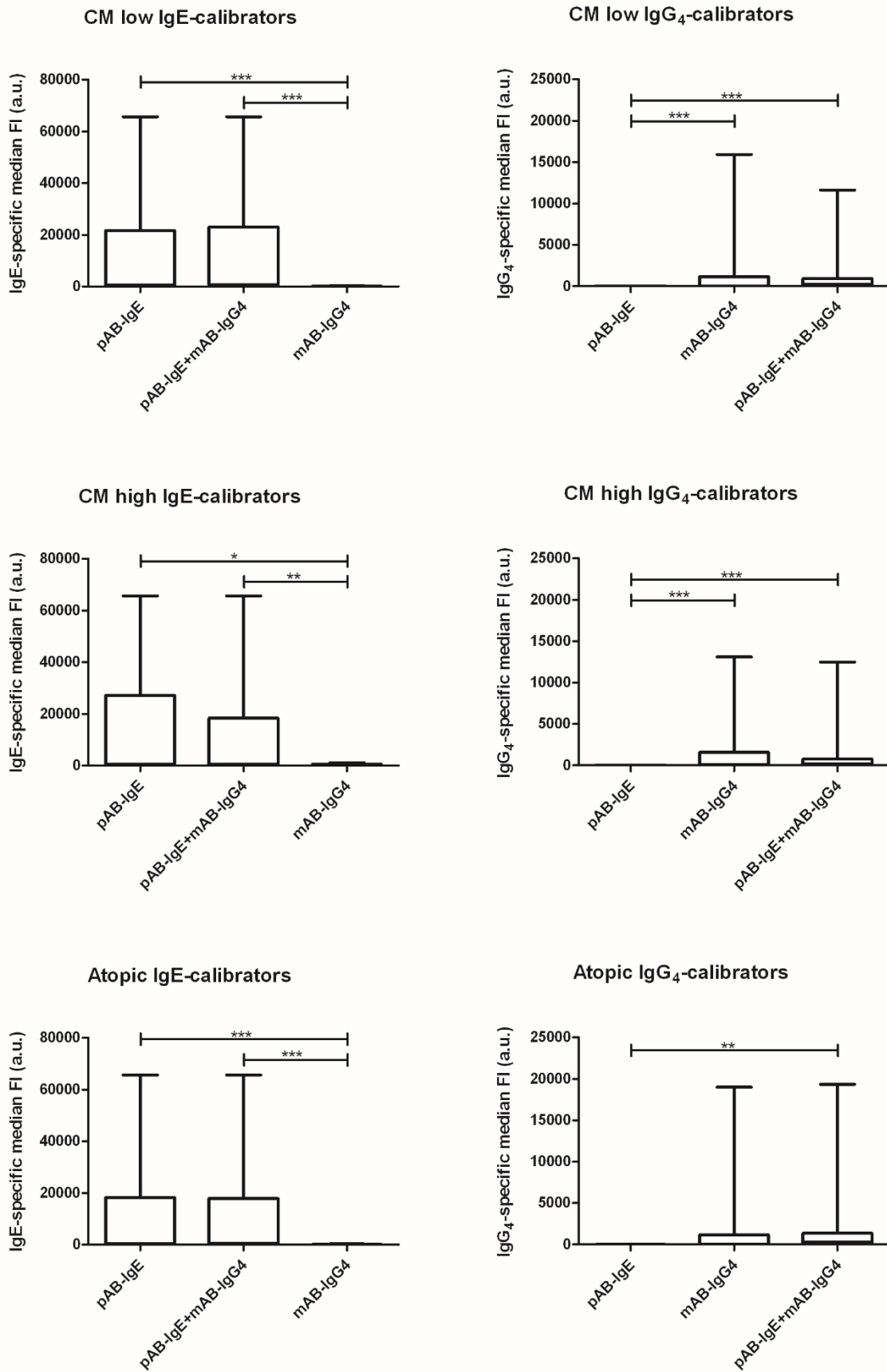
Figure 49: Comparison of IgE- and IgG$_4$-specific median fluorescences for spotted concentrations of IgE and IgG$_4$ between different sera and application of ABs.

No significant differences were calculated between single and parallel incubations of ABs in all three pool sera applied. Significant cross talk between ABs was not detected. Analysis of allergen-specific fluorescences confirmed these findings (Supplement Figure 2).

These assays were also used to address differentiality of atopic from CM low and high pool serum (Figure 50).
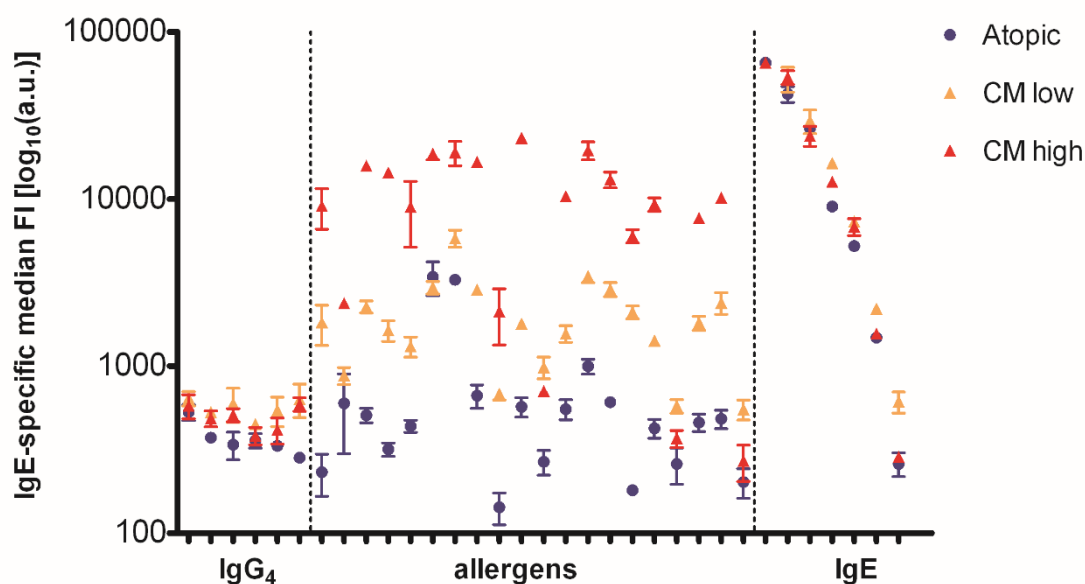
Figure 50: Comparison of IgE-specific median fluorescences of atopic and CM positive pool sera.

Atopic and CM low pool sera were different in IgE-specific fluorescence for the majority of components and extracts. Equal fluorescence to each native and recombinant κ-casein was attributed to unspecific binding as shown previously (4.3.2.3). Low binding and no concentration-dependent response was observed for recombinant β-lactoglobulin. Lack of function was confirmed for recombinant ovalbumin.

Comparison of total fluorescences, allergen-specific and IgE spot-specific fluorescences were submitted to test statistics (Table 23).

Table 23: Comparison of atopic, CM low and CM high pool sera in IgE-specific fluorescences.

Compared pair	total FI	allergen-specific FI	IgE spots
Atopic vs CM low	*	*	ns
Atopic vs CM high	***	***	ns
CM low vs CM high	ns	ns	ns

Atopic pool serum differed significantly from CM low and high pool serum. No difference between CM low and high pool sera was calculated for allergen-specific fluorescences.

4.3.4   Application of patient sera to assess the performance of the silicon microarray

The performance of copoly(DMA-NAS-MAPS) -coated silicon slides was assessed in large scale with 91 well-described sera (set B) of children with sensitization to hen's egg and cow's milk allergens. These sera had been determined quantitatively for several parameters: sIgE to CM and HE, sIgG$_4$ to casein, α-lactalbumin and β-lactoglobulin. Semi-quantitative results from ISAC were available for CM and HE components. These data were used in a study and published in Ahrens, Lopes de Oliveira *et al.* [129]. In parallel to patient sera, HE medium pool serum was used as control for later assessment of inter and intra slide variation.

4.3.4.1   Intra and inter slide variation

The coefficient of variation (CV in %) was determined within one batch (intra slide) and between batches (inter slide) with HE medium pool serum. For intra slide variation, 10 chips of one daily batch were used. Inter slide variation was calculated from the daily batch controls of all lots, including one randomly selected chip from the intra slide run (n=13). Median and mean fluorescence (cleared according to criteria described in 3.8.6) were considered (Supplement Table 19).

In general, CV`s for intra slide variation were lower compared to inter slide ones. No difference could be detected between median and mean fluorescence. The intra slide variation for cow's milk allergen IgE-specific mean fluorescences was 11.3% at average, and 26.9% for inter slide variation. For IgG$_4$-specific signals, 14.1% were calculated as mean intra slide variation and 33.1% as inter slide variation. Results for hen's egg allergens were very similar with 14.0% (intra) and 30.0% (inter) for IgE, and 16.6% (intra) and 32.2% (inter) for IgG$_4$.-specific fluorescences.

4.3.4.2   Correlations with quantitative and semi-quantitative results

For 91 sera assayed, quantitative results from ImmunoCAP (sIgE and sIgG$_4$) and semi-quantitative results from ImmunoCAP ISAC (ISU) were correlated with IgE- and IgG$_4$-specific fluorescences. These were submitted to further processing (4.3.4.3) and also correlated. A complete overview is provided in Supplement Table 17.

As a graphical example, the correlation of sIgE to HE and CM to IgE-specific median fluorescence of egg white and cow's milk is depicted in Figure 51.
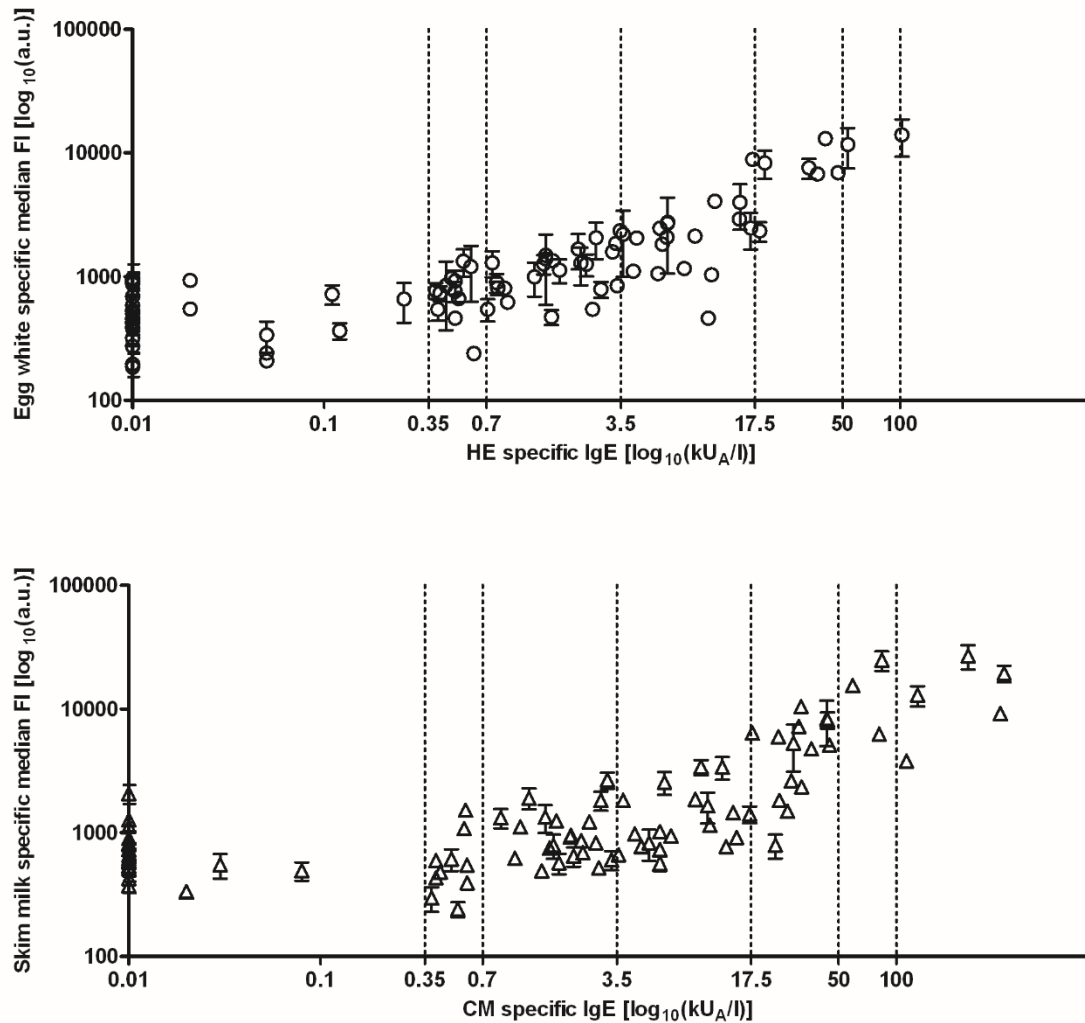
Figure 51: Correlation of sIgE to HE and CM with corresponding IgE-specific median fluorescence of egg white and skim milk on the silicon microarray.

Correlations of sIgE to HE and CM with egg white and skim milk specific fluorescences were highly significant with $r_{(s)}$=0.813 and 0.747. IgG$_4$-specific results also highly correlated with 0.924 for native α-lactalbumin to 0.849 for native β-lactoglobulin. Semi-quantitative results to CM components of ISAC correlated higher with native proteins than with recombinant variants and ranged from 0.719 to 0.758 and 0.536 to 0.729, respectively. Between native and recombinant ovomucoid equal correlations were calculated. No correlation was shown for recombinant ovalbumin.

Quantitative results for HE and CM from ImmunoCAP were also correlated with the sum of ISU and fluorescences of native or recombinant components (Supplement Table 18). In these correlations the silicon microarray showed higher results compared to ISAC. Native components correlated better compared to recombinant ones.

Two sets of each 15 sera (B1 and B2) were selected and sIgE to components was determined. Results were combined with fluorescences from silicon microarray and ISU from ISAC. 15 other sera (B3) with sIgG$_4$ between 0 and 0.72 mg$_A$/l were re-assayed side-by-side and correlated with available quantitative results in order to assess sensitivity in this range (Table 24).

Table 24: Correlations of sIgE to CM and HE components with ISAC and silicon microarray. Correlations for mean fluorescence are shown. 1) refers to the sum of Bos d5.0102 (β-lactoglobulin A) and Bos d5.0101 (β-lactoglobulin B); 2) refers to Bos d5.0102 (β-lactoglobulin A).

set	ImmunoCAP	ISAC	Silicon Microarray	spotted analyte
B.1	a-lactalbumin (IgE)	0.781**	0.957**	nat α-lactalbumin
			0.625*	rec α-lactalbumin
	b-lactoglobulin (IgE)	0.831**[1]	0.815**	nat β-lactoglobulin (A+B)
		0.704**[2]	0.667**	rec β-lactoglobulin (A)
B.2	ovomucoid (IgE)	0.932**	0.896**	nat ovomucoid
			0.928**	rec ovomucoid
	ovalbumin (IgE)	0.906**	0.872**	nat ovalbumin
			0.057	rec ovalbumin
B.3	a-lactalbumin (IgG$_4$)	n/a	0.857**	nat α-lactalbumin
		n/a	-0.242	rec α-lactalbumin
	b-lactoglobulin (IgG$_4$)	n/a	0.791**	nat β-lactoglobulin (A+B)
	casein (IgG$_4$)	n/a	0.530	casein
E	hen's egg (IgG$_4$)	n/a	0.691*	egg white

The correlation between quantitative α-lactalbumin and fluorescence for native α-lactalbumin was clearly positive compared to ISAC ($r_{(s)}$=0.957** to 0.781**) after exclusion of one serum due to implausible results. The quantitative results for β-lactoglobulin correlated equally well with ISU and the silicon microarray with slight differences depending on the variant of β-lactoglobulin.

The comparison of HE proteins showed an equally good correlation for native ovalbumin on both platforms. Recombinant ovomucoid correlated slightly better compared to the native variant and to the same level as ovomucoid on ISAC.

Repeated assays to IgG$_4$ showed similar correlations for α-lactalbumin and β-lactoglobulin compared to results from 91 sera. Casein correlated positively, but was not significant. This result was attributed to the fact that total casein contains κ-casein which caused unspecific background.

In general, more positive correlations with native cow's milk allergens compared to the recombinant variants were observed.

4.3.4.3   Internal and external calibration

Normalization to intra and inter slide variation was performed by calculating the average fluorescence of (intra: 10 arrays, inter: 13 arrays) each analyte. Values obtained were set to 100% and related to the values of the inter assay control that was run in every daily batch. Relative percentages obtained were then applied to in- or decrease fluorescences of chips of that batch incubated with patient serum. This procedure was repeated for the 13 inter assay slides.

Intra- and inter normalizations produced equal correlations (Supplement Table 17). Median and mean fluorescences results were very similar. One exception was recombinant α-lactalbumin with a clear higher correlation of mean compared to median fluorescence. Equal correlations reflected that fact of very similar average fluorescences of intra and inter slide measurements. For downstream analyses, intra normalized results were therefore not considered.

Spotted concentrations of IgE and IgG$_4$ were log-transformed and a curve calculated using a five-parameter-fit (log concentration against fluorescence). 1% HSA was regarded as 0 µg/ml calibrator and set to 0.001 to comply with log-transformation. This curve was used to transform the analyte specific fluorescence into CCU's (concentration curve units). Fluorescence values that were not captured by the curve, leading to no value for CCU, were set to "0" when below the minimum covered by the curve or to "4" when above the maximum covered by the curve. Details of these calculations are summarized in the supplement. The combination of normalization to intra and inter assay variation and expression in CCU was performed by first normalization analyte specific fluorescence as described above and then conversion into CCU. Figure 52 depicts the correlation of sIgE to HE and CM with calculated CCU.
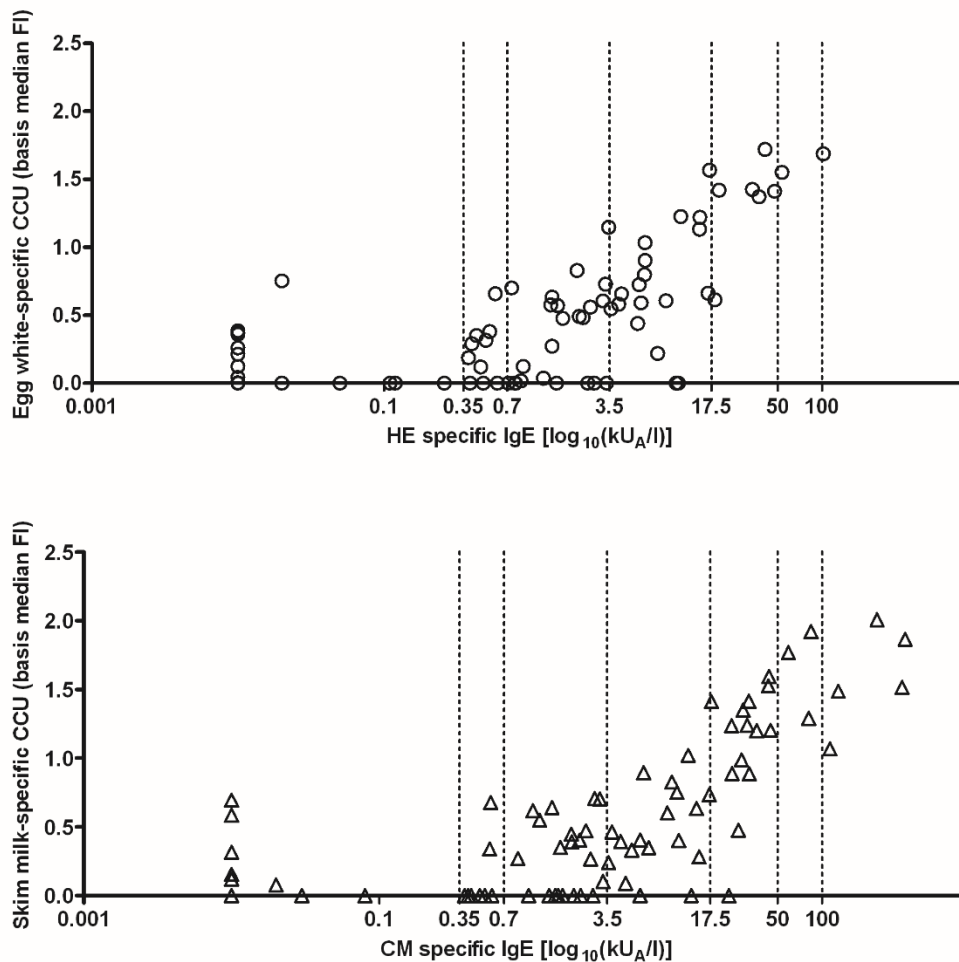
Figure 52: Correlation of sIgE to HE and CM with calculated CCU from median fluorescence. Correlation of sIgE and median fluorescence in **Figure 51**.

Significant positive correlations for sIgE to HE and CM with CCU were observed with $r_{(s)}$=0.692** and 0.749**, respectively. IgG$_4$-specific results showed similar significant correlations. However, a high degree of fluorescences not captured by IgE and IgG$_4$ calibration curves was observed. The IgE-calibration curve failed in the low range, not capturing low fluorescences for up to ~ 82% of values of rec β-lactoglobulin specific fluorescence. Similar observations were made for IgG$_4$-specific application where the calibration curve did not cover very low fluorescences and very high fluorescences. Missing values ranged from 14.3% (native α-lactalbumin) to 61.5% (recombinant αs2-casein) for median fluorescence (results for native k-casein and recombinant ovalbumin not considered). The application of normalizations prior to CCU transformation led to slightly correlations with quantitative and semi-quantitative results (Supplement Table 17).

In general, fluorescence and fluorescence normalized to inter slide variation gave higher positive correlations than other modes of processing.

4.3.4.4 Determination of working range and sensitivity

In order to assess linearity of signal and working range of the assay, two sera (set B) with sIgE >100 kU_A/l to HE and CM were diluted stepwise 1:2 with 1% BSA in PBS down to ~0.1 kU_A/l which is the detection limit of commercial quantitative ImmunoCAP.
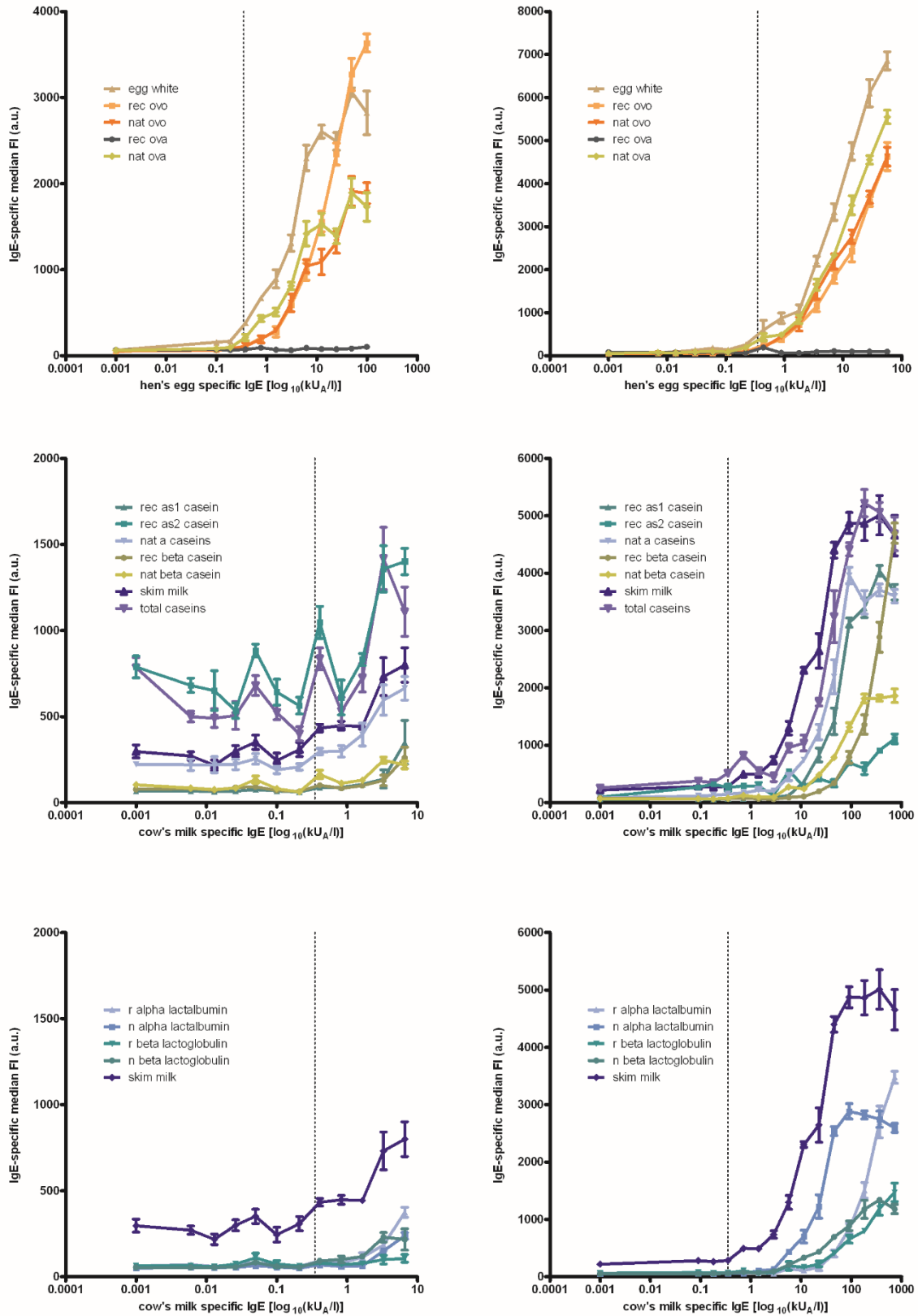
Figure 53: IgE-specific fluorescence in geometric dilutions of two sera. Specific median fluorescences of egg white, skim milk and components at 80% laser power/PMT from two sera with 98.4 (left) and 56.8 (right) kU_A/l sIgE to HE, and 6.6 (left) and 727 (right) kU_A/l sIgE to CM. The dashed line indicates the 0.35 kU_A/l cut-off on ImmunoCAP.

Very clear concentration-dependent fluorescence responses were observed for HE proteins starting between 0.1 and 1 kU_A/l for native ovalbumin, native and recombinant ovomucoid, and egg white. The upper limit for recombinant ovomucoid remained undetected because no saturation of fluorescence occurs. Other HE proteins slightly showed saturation near 100 kU_A/l. The working range for HE proteins in these assays covered all CAP classes from 0 to 6.

Concentration-dependent response for cow's milk proteins was within 0.1 and 1 kU_A/l, although differences between components were detected. Stable responses showed skim milk, native α-caseins, recombinant β-casein and recombinant αs1-casein. Native and recombinant α-lactalbumin and native β-lactoglobulin showed specific responses at approx.1 kU_A/l at the lower end of detection. The upper limit is near 100 kU_A/l (saturation for native α-lactalbumin). Component-specific IgE was not determined in these sera, whereas results for $sIgG_4$ to α-lactalbumin, β-lactoglobulin, casein, ovomucoid and ovalbumin were available. IgG_4-specific fluorescences were quantified from scans at 70% laser power/PMT due to a change of the Cy5-laser in the scanner (Figure 54, Figure 55).
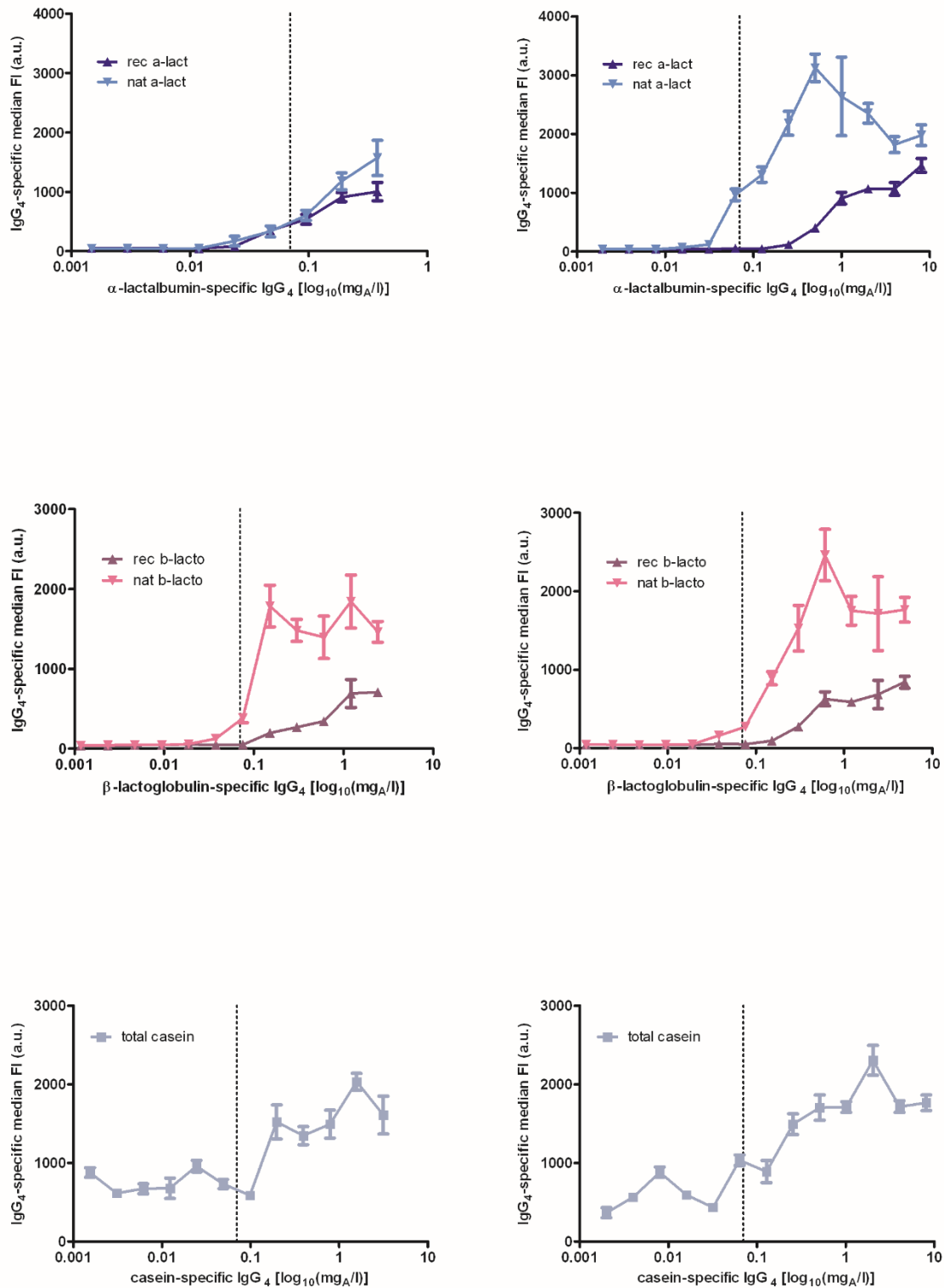
Figure 54: IgG₄-specific fluorescences at 70% laser power/ PMT for CM proteins from two sera. Specific IgG₄ titers for α-lactalbumin, β-lactoglobulin and casein were 0.38, 2.41 and 3.15 mg$_A$/l (left panel) and 7.99, 4.87 and 8.17 mg$_A$/l (right panel). The dashed line indicates LOD of 0.07 mg$_A$/l on ImmunoCAP.

The sensitivity for native and recombinant α-lactalbumin was between 0.01 and 0.1 mg_A/l and reached fast saturation near 1 mg_A/l. Native and recombinant β-lactoglobulin showed similar responses, although the upper limit of detection became not really clear (close to 0.1 mg_A/l or near 1 mg_A/l). Casein performed quite similarly to the proteins mentioned.
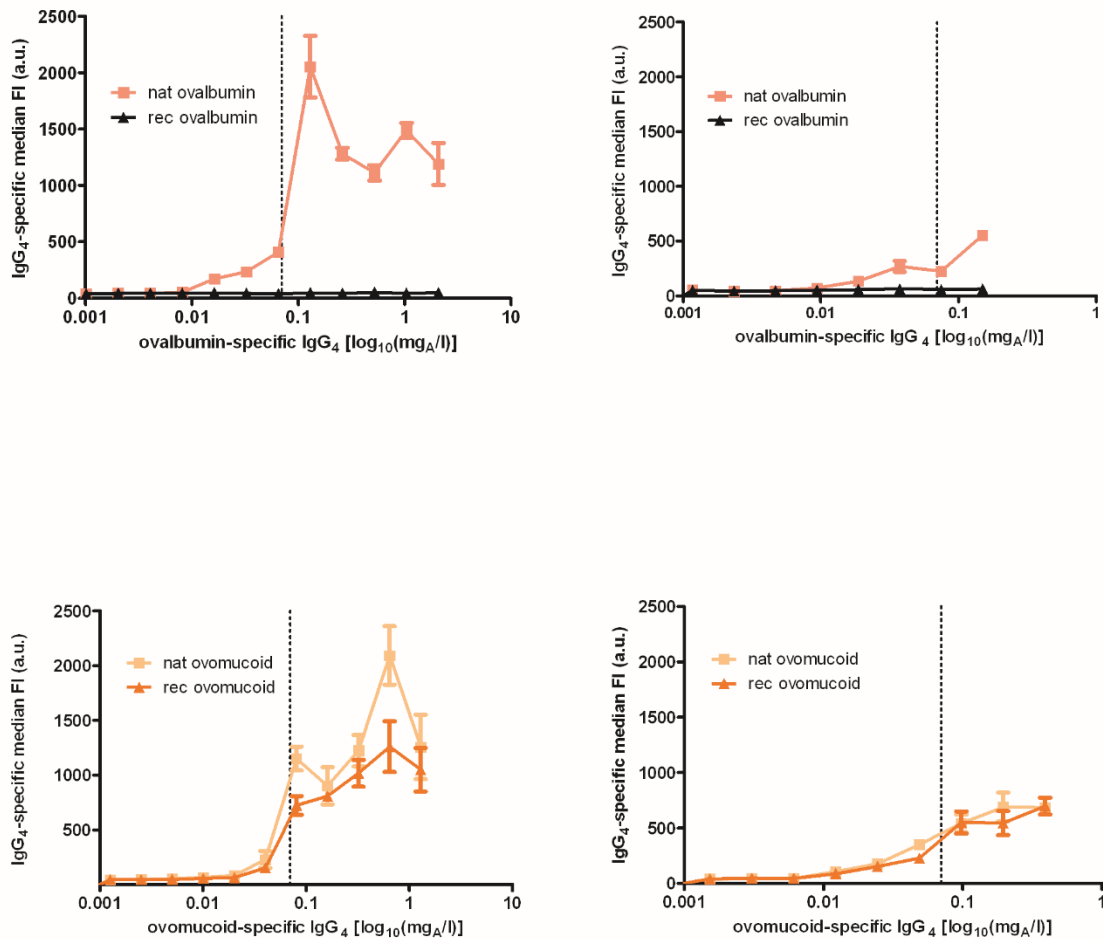
Figure 55: IgG$_4$-specific fluorescences at 70% laser power/PMT for HE proteins from two sera. Specific IgG$_4$ titers for ovalbumin and ovomucoid were 2.08 and 1.29 mg_A/l (left panel) and 0.15 and 0.39 mg_A/l (right panel). The dashed line indicates LOD of 0.07 mgA/l on ImmunoCAP.

HE protein native and recombinant ovomucoid showed concentration-dependent fluorescence near 0.01 mg_A/l IgG$_4$. Native ovalbumin was also clearly recognized at a concentration of 0.01 mg_A/l IgG$_4$. Its dynamic range could not be properly determined, but it was likely to be near 1 mg_A/l IgG$_4$ as well.

4.3.4.5   Interim conclusion

The conclusion from these detailed analyses was that fluorescence and inter slide normalized fluorescence were most suitable for downstream calculations. The silicon microarray was able to be comparably sensitive to ImmunoCAP for HE proteins, whereas for CM sensitivity can be assumed at concentrations >1 kU_A/l. Considering inter slide CVs, the microarray can only be regarded as semi-quantitative. However, there is evidence that it is more sensitive than ISAC. Parallel detection of IgE and IgG_4 is specific and sensitive.

4.3.5   Establishment of component-specific cut-offs

As shown in 4.3.4.2, fluorescence and inter-normalized fluorescence correlated best with quantitative and semi-quantitative results from ImmunoCAP and ImmunoCAP ISAC, respectively. Median and mean fluorescences did not clearly differ. Thus, only median fluorescences were considered for subsequent analyses and calculations.

For ISAC, MIA software converts measured fluorescences into ISU results based on inherent processing of an external calibration serum. In contrast, results from the silicon microarray exist only as metric arbitrary units or as normalized arbitrary units. Therefore, cut-offs needed to be established in order to distinguish unspecific noise from IgE-specific signals. Possible parameters for cut-off establishment were CM provocation result a/o sIgE to CM <0.35 kU_A/l (cut-off on ImmunoCAP). Table 25 gives an overview of provocation results and sIgE.

Table 25: Overview of clinical cut-off and provocation results.

sIgE to CM	Provo neg	Provo pos	total	%
<0.35 kUA/l	12	7	19	20.9
>0.35 kUA/l	19	53	72	79.1
total	31	60	91	100

As a negative provocation result does not exclude sensitization, and sIgE to CM <0.35 kU_A/l can coincide with a positive provocation result, none of these two criteria alone were reliable. In this population, 7 individuals showed symptoms despite a result <0.35 kU_A/l to CM. On the other hand, sIgE was detected in 19 patients with a negative provocation result. Thus, a combination of negative provocation result and sIgE to CM <0.35 kU_A/l was used. From the 91 sera in this study, 12 were identified as double-negative and IgE-specific fluorescences for components were plotted (Figure 56). Results for HE were not considered because provocation test results to this allergen were not available.
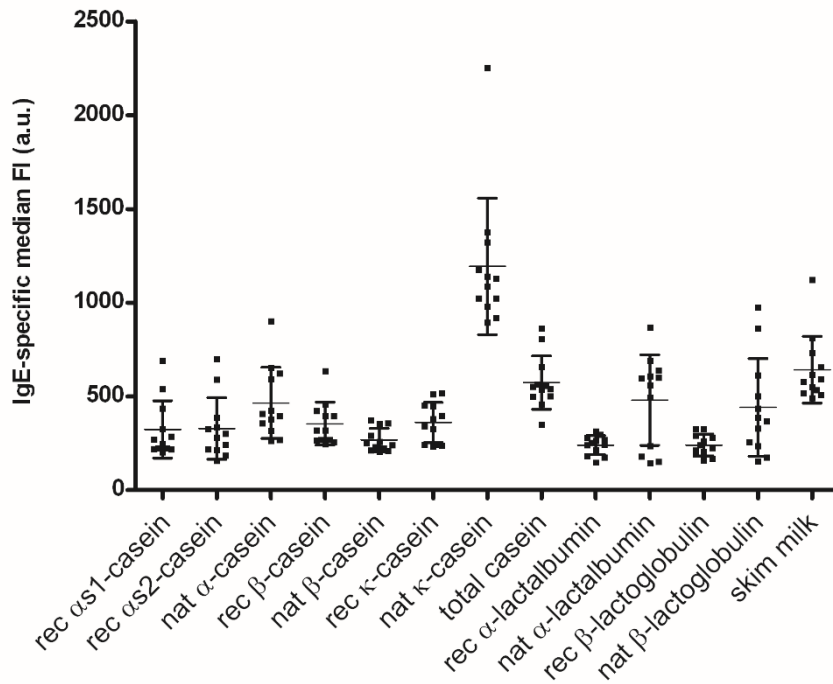
Figure 56: Median IgE-specific FI to CM allergens of 12 individuals with sIgE<0.35 kU$_A$/l and negative CM provocation result. Mean and SD are indicated.

For the 12 sera identified, component-specific fluorescences were averaged, and calculated means plus one, two and three SD were used as cut-offs (Figure 57). This calculation was also applied to the inter-normalized fluorescences.
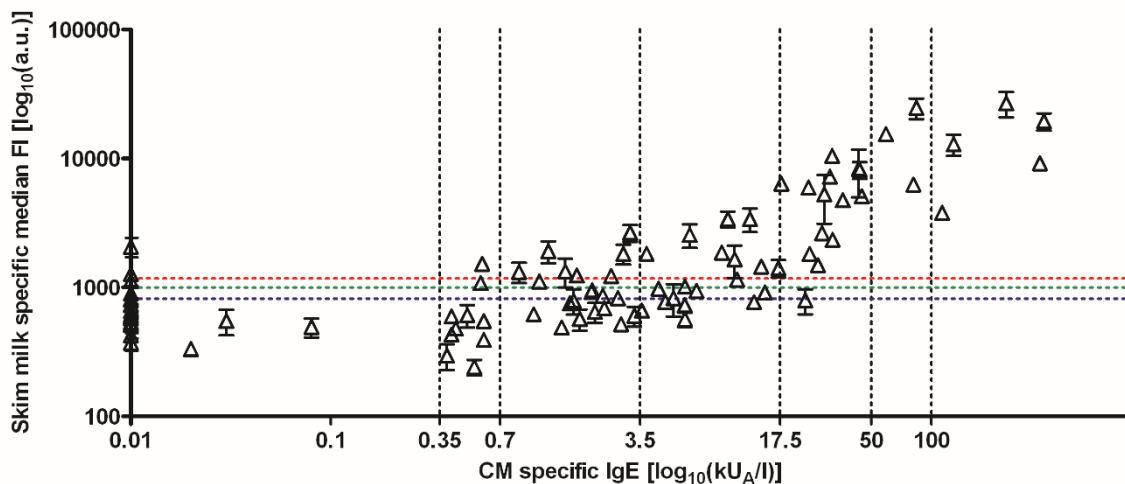
Figure 57: Suggested cut-offs on the basis of 12 individuals with sIgE to CM <0.35 kU$_A$/l and negative provocation resuls. Dashed horizontal lines indicate mean+1SD (blue), +2SD (green) and 3SD (red). Dashed vertical lines indicate CAP classes.

Absolute and relative frequencies of values above each of the three cut-offs were determined in order to select the most suitable cut-off and to identify significant components occurring with high fluorescence (Table 26).

Table 26: Application of different cut-offs on 91 sera. Calculated component-specific means plus 1. 2 or 3 SD's from 12 double-negative sera were used as cut-offs. Absolute and relative frequencies of values above the cut-offs were determined and are arranged with increasing frequency. Setting of cut-offs are discussed in the text and refer to skim milk which is depicted in bold.

component		Component-specific Fluorescence			Component-specific Inter-normalized Fluorescence		
	Mean	+1SD	+2SD	+3SD	+1SD	+2SD	+3SD
rec αs2-casein	abs.	36	26	19	37	21	13
	rel.	39.6	28.6	20.9	40.6	23.1	14.3
nat β-lactoglobulin A+B	abs.	40	31	27	34	30	27
	rel.	44.0	34.1	29.7	37.4	33.0	29.7
rect β-lactoglobulin A	abs.	40	32	27	35	22	17
	rel.	44.0	35.2	29.7	38.5	24.2	18.7
nat κ-casein	abs.	40	33	29	48	34	29
	rel.	44.0	36.3	31.9	52.7	37.4	31.9
rec α-lactalbumin	abs.	45	34	31	37	22	16
	rel.	49.5	37.4	34.1	40.6	24.2	17-6
rec β-casein	abs.	45	37	34	38	29	22
	rel.	49.5	40.7	37.4	41.7	31.9	24.2
rec κ-casein	abs.	46	38	35	40	33	27
	rel.	50.5	41.8	38.5	44.0	36.3	29.7
nat β-casein	abs.	50	38	35	56	48	39
	rel.	54.9	41.8	38.5	61.5	52.7	42.8
rec αs1-casein	abs.	51	45	39	52	41	39
	rel.	56.0	49.5	42.9	57.1	45.0	42.8
skim milk	**abs.**	**53**	**44**	**39**	**55**	**46**	**36**
	rel.	**58.2**	**48.4**	**42.9**	**60.4**	**50.5**	**39.6**
nat α-casein	abs.	54	43	39	47	42	39
	rel.	59.3	47.3	42.9	51.6	46.1	42.8
total casein	abs.	57	47	41	52	46	40
	rel.	62.6	51.6	45.1	57.1	50.5	43.9
nat α-lactalbumin	abs.	59	51	46	58	49	44
	rel.	64.8	56.0	50.5	63.7	53.8	48.3

With increasing cut-off, the frequency of positive absolute and relative values decreased. This grading offered the opportunity to consider a safety range to avoid false negative results. As depicted in Table 25, 79.1% of sera were above the 0.35 kU_A/l cut-off. This suggested a decision for mean+1SD as a cut-off, because 58.2% of the sera would be positive for skim milk on the microarray. On the other hand, the discrimination of false positive from true positive sera on the microarray was not reliable in the low range. A higher

cut-off needed to be applied to increase the probability of a true positive result. Therefore, mean+2SD was chosen and used for subsequent analyses. Mean+3SD was rejected due to loss of sensitivity, because only 42.9% of sera were positive for skim milk at this cut-off. This was in too strong contrast to the result from ImmunoCAP with in total 79.1% CM positive sera.

4.3.6   ROC-curves

To compare specificity of recombinant and native components, ROC curves were plotted. The 12 double negative patients were set to state 0 (negative), and the remaining patients to state 1 (positive). Median and inter-normalized median fluorescence were considered (Figure 58, Figure 59)
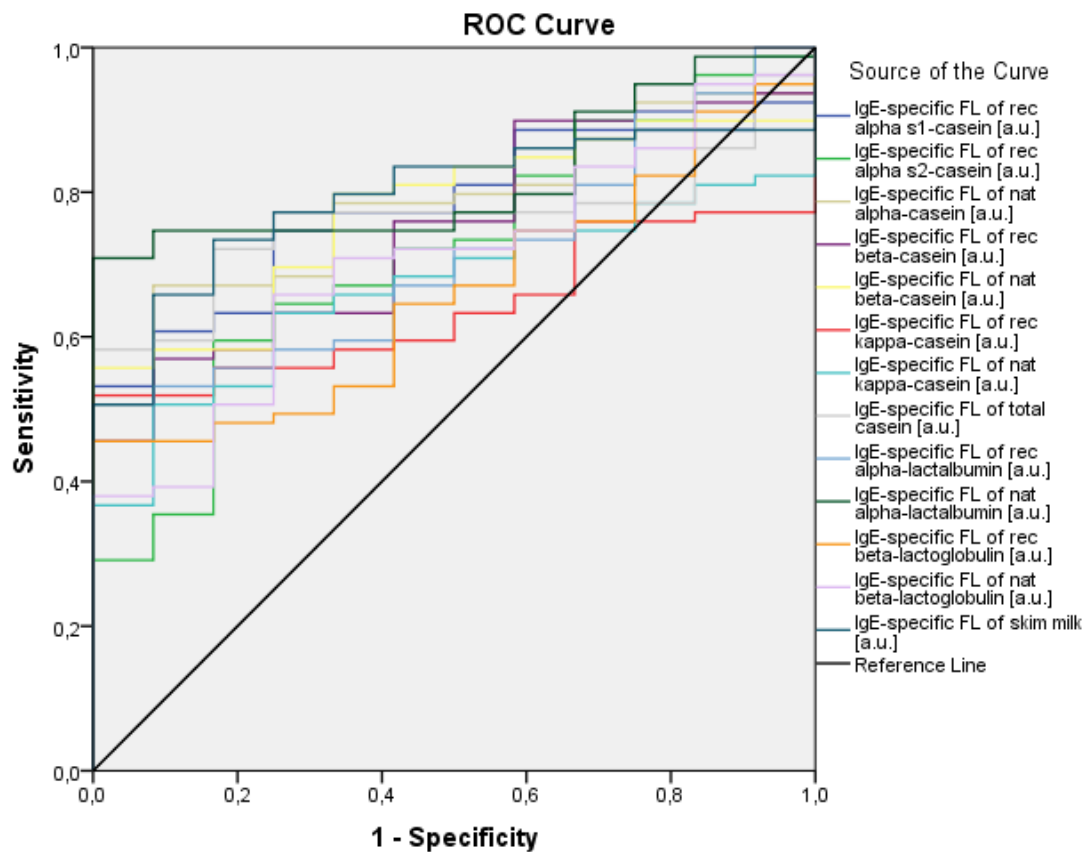
Figure 58: ROC-curves of native and recombinant components with median fluorescence.

Several curves calculated on the basis of median fluorescence slipped under the reference line in the upper right of the plot. This represented false negative patients who showed positive reactions but were measured with lower fluorescence for the respective component than defined by the 12 double negative patients.
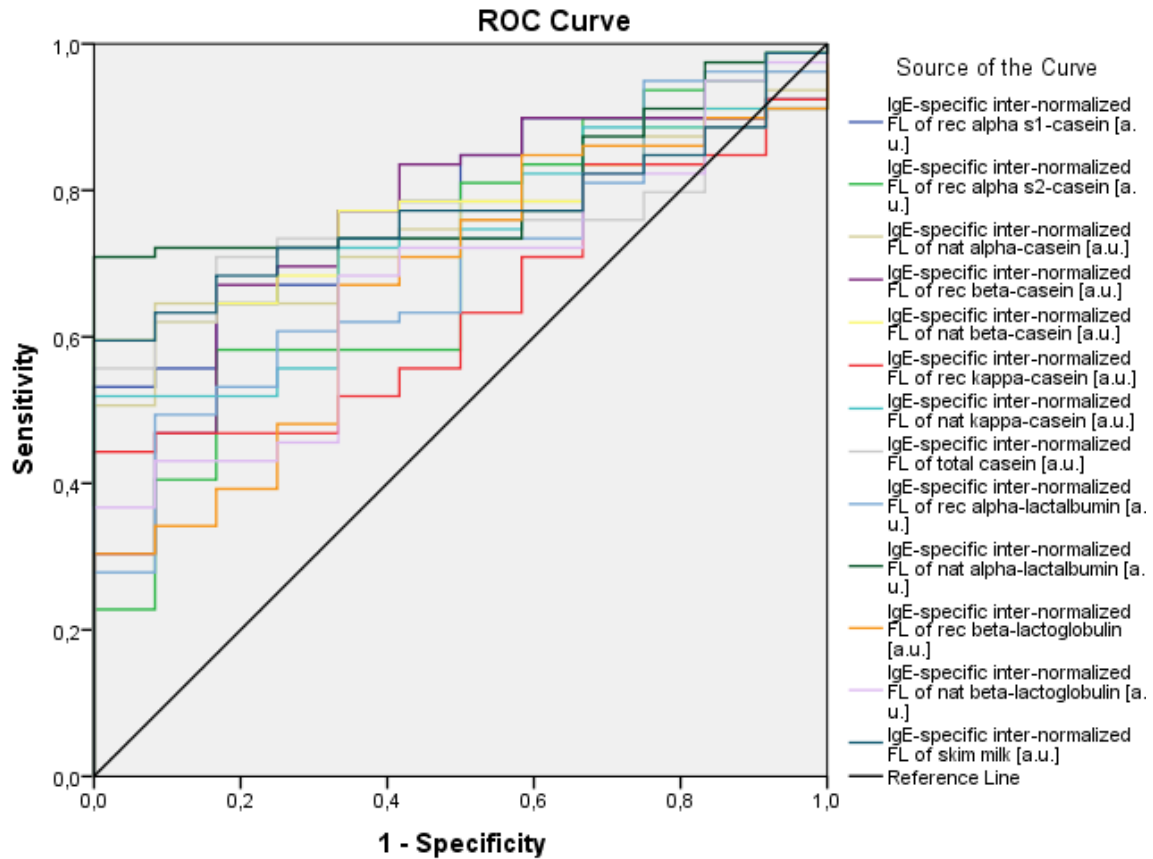
Figure 59: ROC-curves of native and recombinant components with median inter-normalized fluorescence.

Inter-normalization had a positive effect, fewer curves slipped under the reference line. The AUC of components and skim milk are summarized in Table 27.

Table 27: Comparison of computed AUC for median and inter-normalized median fluorescence of CM components and skim milk. Components are arranged with increasing AUC.

	Median FI		Inter-normalized median FI	
Component	AUC	Asymptotic Sig.	AUC	Asymptotic Sig.
rec κ-casein	0.640	0.119	0.642	0.113
rec β-lactoglobulin A	0.660	0.075	0.670	0.059
nat κ-casein	0.667	0.064	0.728	0.011
nat β-lactoglobulin A+B	0.704	0.024	0.675	0.052
rec α-lactalbumin	0.713	0.018	0.693	0.032
rec αs2-casein	0.715	0.017	0.698	0.027
rec β-casein	0.747	0.006	0.758	0.004
total casein	0.763	0.003	0.768	0.003
natβ-casein	0.773	0.002	0.751	0.005
rec αs1-casein	0.778	0.002	0.777	0.002
nat α-casein	0.782	0.002	0.750	0.005
skim milk	0.794	0.001	0.769	0.003
nat α-lactalbumin	0.821	0.000	0.800	0.001

AUC ranges of median and inter-normalized median fluorescence were very similar. Inter-normalization led to a slight increase in component-specific AUC. The largest AUC of components was calculated for native α-lactalbumin, followed by skim milk. Following significant components were all caseins. Native components, in general, were calculated with higher AUC.

4.3.7 Molecular analysis of native and recombinant proteins

4.3.7.1 Parallel determination of protein content relative to BSA

Protein solutions at working concentration used for spotting were assayed in parallel to determine protein content relative to BSA. Results of recombinant proteins were compared to those previously determined (Table 28).

Table 28: Protein content of native and recombinant protein solutions. Stock solutions as available in Milano microarray experiments, determined relative concentrations of the stocks and re-calculated relative concentrations actually existent at spotting are indicated with ~.

	proteins	stock conc. (rel.) [mg/ml]	stock conc. (abs.) [mg/ml]	rel. conc. [mg/ml]	applied rel. conc. on microarray [mg/ml]
recombinant	αs_1-casein	2.22	n/a	2.23	1.11
	αs_2-casein	0.85	n/a	0.56	~0.71
	ß-casein	3.51	n/a	3.51	1.75
	κ-casein	0.98	n/a	0.59	~0.39
	α-lactalbumin	1.33	n/a	1.13	~1.23
	ß-lactoglobulin A	1.06	n/a	1.08	~1.07
	ovomucoid	2.95	n/a	3.38	~1.58
	ovalbumin	1.21	n/a	1.33	~1.27
native	α-casein	n/a	1.06	0.52	0.52
	ß-casein	n/a	1.30	0.60	0.60
	κ-casein	n/a	1.08	0.64	0.32
	total casein	n/a	1.00	0.59	0.59
	α-lactalbumin	n/a	1.27	0.98	0.98
	ß-lactoglobulin A+B	n/a	1.25	0.79	0.79
	skim milk	n/a	2.00	0.55	0.55
	ovomucoid	n/a	1.38	0.98	0.98
	ovalbumin	n/a	1.20	0.99	0.99
	egg white	n/a	1.12	0.86	0.86

In general, previous results of protein content of recombinant proteins could be reproduced. Loss of protein was observed for $\alpha s2$- and κ-casein which was due to precipitation after thawing. Previous and recent results were averaged to calculate the protein content that was existent after thawing. Several native proteins were under-determined in BCA assay compared to the absolute amount that had been applied. In sum, native proteins were

applied at lower concentration compared to recombinant proteins. This result needed to be contextualized with purity of protein (4.3.7.2).

4.3.7.2 Identification with MALDI-TOF MS and determination of purity

Recombinant and native proteins were separated in SDS-PAGE, stained with colloidal Coomassie and scanned on GS-800 imager for later calculation of protein purity. Selected bands were excised and digested with trypsin or chymotrypsin (native β-, native and recombinant κ-casein, ovomucoids). Digestes were measured with MALDI-TOF MS and obtained *m/z* spectra (note CD) submitted to Swissprot "Other mammals" (CM proteins) or "Metazoa" (HE proteins) database (Figure 60).
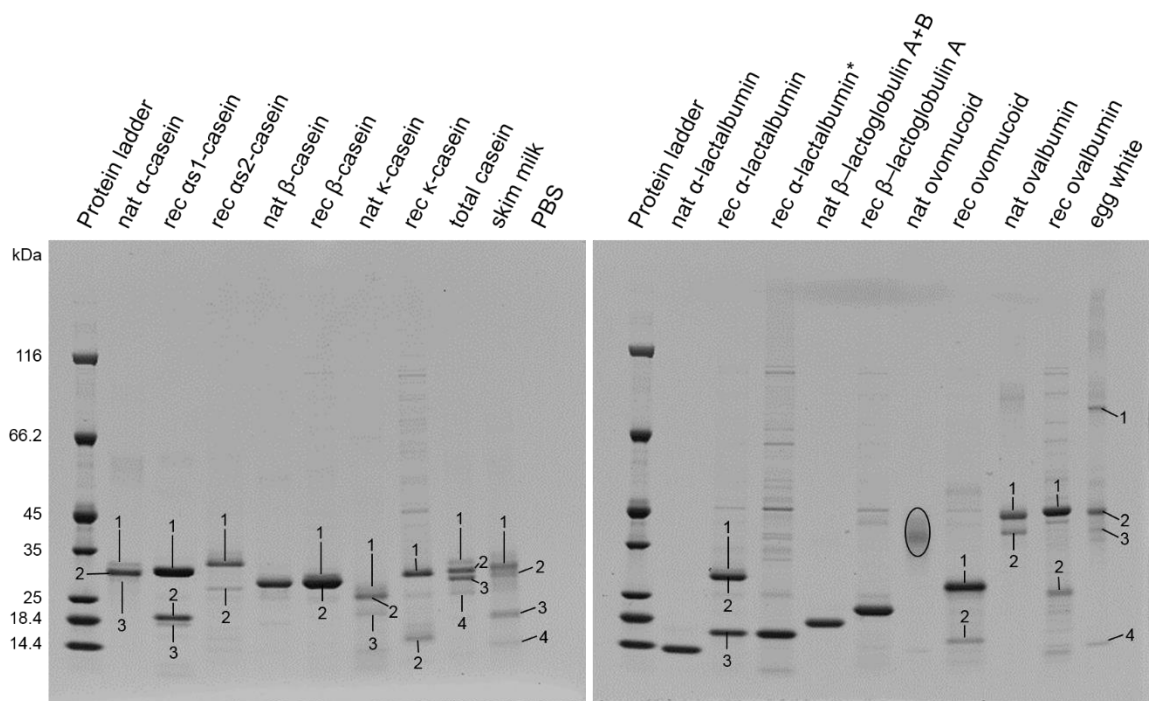
Figure 60: Determination of purity of recombinant and native allergens in SDS-PAGE. Of each component, 0.8 µg protein, determined relative to BSA, was applied. Amounts for skim milk and egg white were 1.6 and 1.7 µg, respectively. Natively purified recombinant α-lactalbumin is marked with an asterisk. Stain with colloidal Coomassie. Brightness and contrast were reduced to visualize faint bands. Several bands per protein were numbered and individually analyzed.

Purity of protein was calculated with consideration of identified bands in MALDI-TOF MS (Supplement Table 26) and from results in Western blot analyses (Figure 65, Figure 66).

Table 29: Determination of protein purity in CM and HE components and extracts.

protein	Band%	Lane%	provider
nat α-casein	86.2	83.2	≥70
rec αs1-casein	98.1	97.0	
rec αs2-casein	81.7	77.3	
nat β-casein	92.6	90.5	≥98
rec β-casein	95.7	95.1	
nat κ-casein	92.4	88.3	≥70
rec κ-casein	77.6	76.1	
total casein	94.6	89.6	technical grade
nat α-lactalbumin	98.6	96.6	≥85
rec α-lactalbumin (band 2)	52.8	52.4	
rec α-lactalbumin (band 3)	36.4	36.1	
rec α-lactalbumin (sum)	89.2	88.5	
rec α-lactalbumin (nat purified)	73.2	72.2	
nat β-lactoglobulin	94.3	92.4	≥90
rec β-lactoglobulin	86.4	85.9	
skim milk	95.5	90.2	for microbiology
nat ovomucoid	91.6	88.4	n/a
rec ovomucoid	88.5	87.7	
nat ovalbumin	89.2	85.5	≥98
rec ovalbumin	70.5	69.8	
egg white	68.3	65.5	≥90
ovotransferrin	12.5	12.0	
ovalbumin	52.1	50.0	
ovomucoid			
lysozym	3.7	3.5	

Information given on purity of purchased components was confirmed with exception of ovalbumin. Recombinant proteins were found to be of similar purity >70%. Components in egg white were identified and revealed existence of two other allergic components (ovotransferrin and lysozyme), not spotted on the silicon microarray. Allergic components in skim milk were only partially identified in MALDI-TOF MS, and purity was inferred from comparisons with purified components.

4.3.7.3   Interferometric quantification

The interferometric label free quantification [135] was performed on two IRIS-chips with four replicates of each analyte. This analysis was performed by courtesy of Paula Gagni of CNR lab personnel (Figure 61).
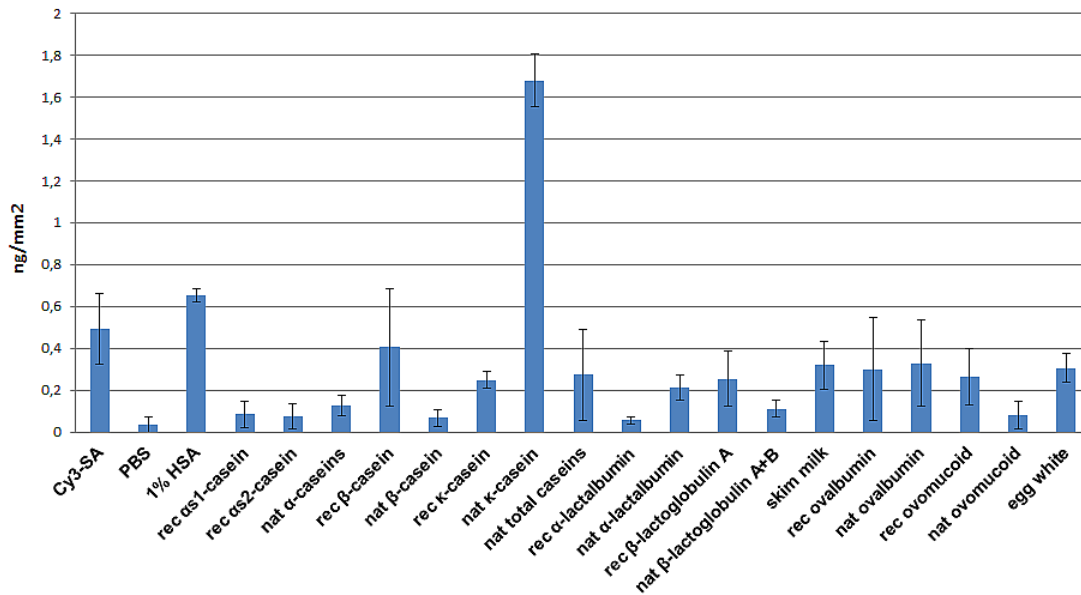
Figure 61: Analysis of binding capacity with the IRIS system. The amounts of bound protein [ng/mm²] were calculated relative to a calibration with IgG and do not represent absolute values. Cy3-Strepavidin, which is used for orientation spots, was also determined. Analysis results courtesy of Paula Gagni, CNR.

Interferometric results were calculated with high SD for all analytes. This may reflect irregular distribution of protein within a spot and between replicate spots. Differences in determined amounts of recombinant and native counterparts were considerable except for α-caseins and ovalbumins. Native κ-casein exceeded results from other spotted analytes 4fold. As these results are relative values to an IgG standard, they do not represent absolute values.

4.3.7.4 Glycosylation

In order to assign glycosylation especially in extracts, proteins were separated in SDS-PAGE under reducing conditions and blotted on nitrocellose (Figure 62, Figure 63).
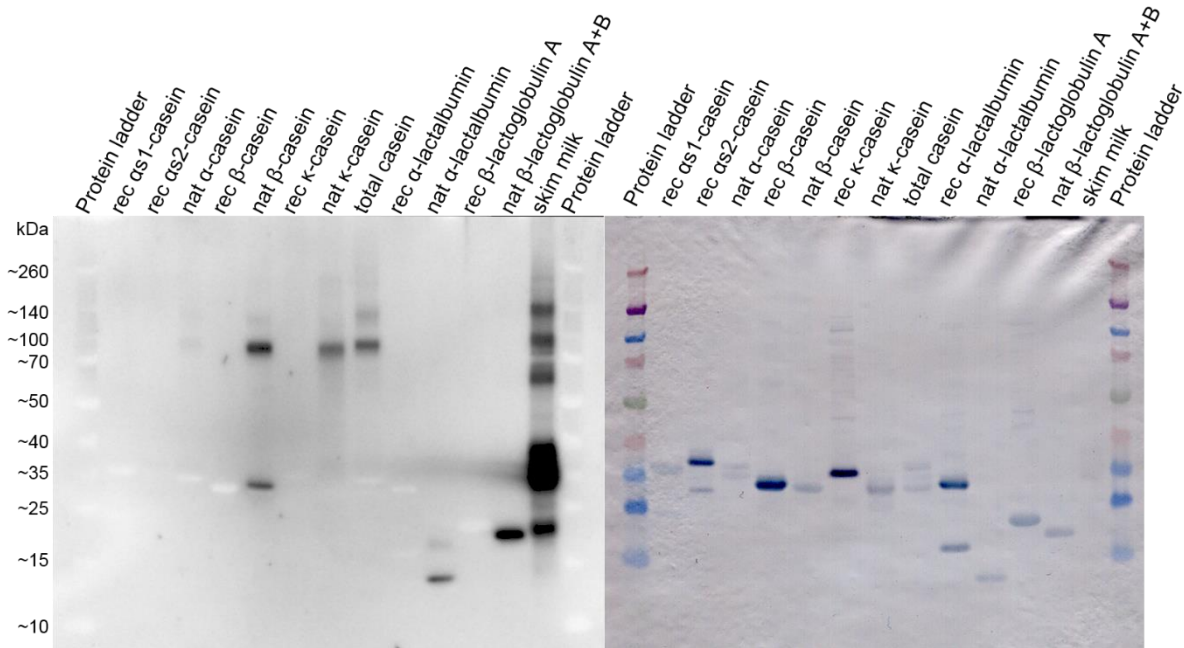
Figure 62: Detection of glycosylation in native and recombinant CM proteins. 1 µg of each protein were separated in SDS-PAGE (Bis-tris, 4-12%, MES buffer) and blotted on nitrocellulose. Glycosylation was detected with Ricin-HRP 1:10000 in PBS-T 0.05% (left). Time of exposure was 2 sec. The membrane was then stained with Amido-Black (right).

No glycosylation was observed in recombinant CM proteins. Native β-casein, α-lactalbumin and β-lactoglobulin showed clear signals at specific protein band positions, whereas specific signals between ~70 and ~100 kDa in β-, κ-, and total casein did not refer to bands visible in Coomassie or Amido-Black stains. In skim milk, the range between ~25 and ~40 kDa appeared strongly positive. According to the provider, skim milk contains ≥50% reducing sugars as lactose monohydrate which explained the strong specific signal in Western blot analysis and problems in identification of proteins in MALDI-TOF MS due to altered peptide masses.
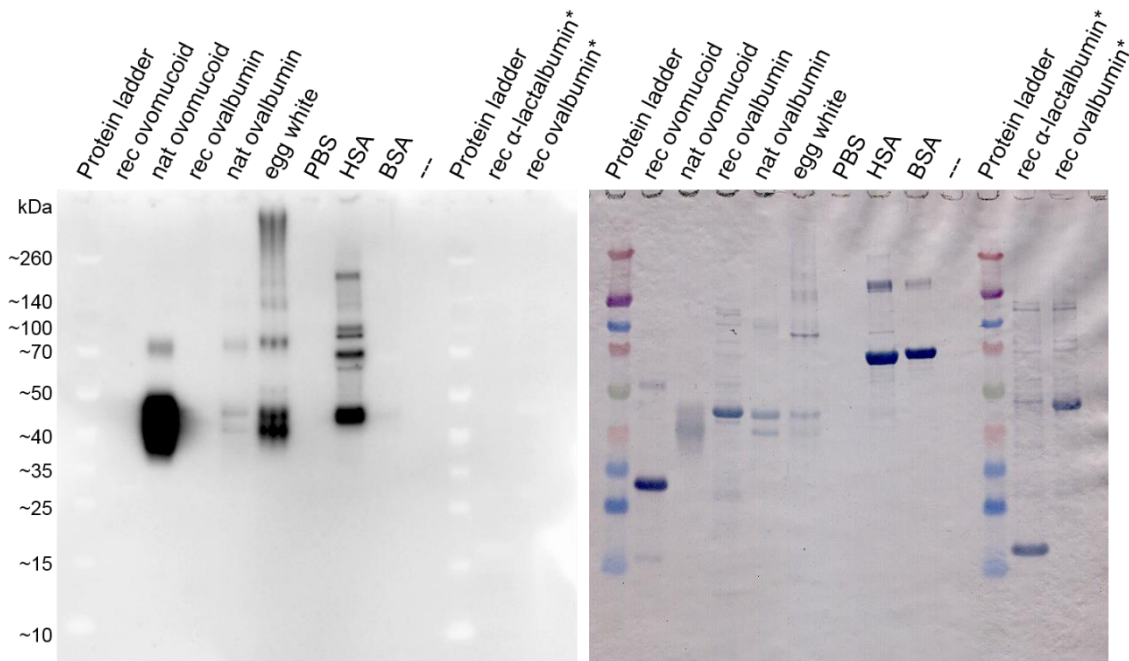
Figure 63: Detection of glycosylation in native and recombinant HE proteins. 1 µg of each protein were separated in SDS-PAGE (Bis-tris, 4-12%, MES buffer) and blotted on nitrocellulose. In addition to HE proteins, also recombinant α-lactalbumin and ovalbumin, purified under native conditions, were applied and are indicated with *. Glycosylation was detected with Ricin-HRP 1:10000 in PBS-T 0.05% (left). Time of exposure was 2 sec. The membrane was then stained with Amido-Black (right).

Also in recombinant HE proteins, no glycosylation was detected. Strong glycosylation occurred in ovomucoid and fitted findings from Amido-Back stain where ovomucoids did not appear with a sharp band but with a smear. In egg white, glycosylation at approximately the position of ovomucoid occured, giving further evidence to its content in the extract (Supplement Table 26). Of note, HSA is highly contaminated with glycosylated proteins in contrast to BSA, visible in Amido-Black stain.

4.3.7.5 Dot blots (native conditions)

Aliquots of recombinant and native proteins were dotted with 1 µl each on nitrocellulose membrane. The concentration of proteins was equal to applications on silicon microarray. In addition, recombinant α-lactalbumin, purified under native conditions, and human IgG were used (Table 30).

Table 30: Identification numbers in dot blot analysis.

No.	analyte	No.	analyte
1	rec αs1-casein	22	IgE 240 µg/ml
2	nat β-casein	23	IgE 120 µg/ml
3	nat κ-casein	24	IgE 60 µg/ml
4	rec αs2-casein	25	IgE 35 µg/ml
5	rec β-casein	26	IgE 15 µg/ml
6	rec κ-casein	27	IgE 3 µg/ml
7	nat α-casein	28	IgG$_4$ 300 µg/ml
8	rec α-lactalbumin	29	IgG$_4$ 120 µg/ml
9	nat β-lactoglobulin A+B	30	IgG$_4$ 60 µg/ml
10	total casein	31	IgG$_4$ 35 µg/ml
11	nat α-lactalbumin	32	IgG$_4$ 15 µg/ml
12	rec β-lactoglobulin A	33	IgG$_4$ 3 µg/ml
13	skim milk	34	IgG 0,5 µg/ml
14	nat ovomucoid	35	IgG 0,05 µg/ml
15	nat ovalbumin	36	IgG 0,005 µg/ml
16	egg white		
17	rec ovomucoid		
18	rec ovalbumin		
19	rec α-lactalbumin (native)		
20	HSA		
21	PBS		

CM pool sera were diluted 1:5 or 1:10 (CM high) in block solution, binding of IgE was detected with [125]iodine goat anti human IgE AB which is not identical to pAB goat anti human IgE used on the silicon microarray (Figure 64).
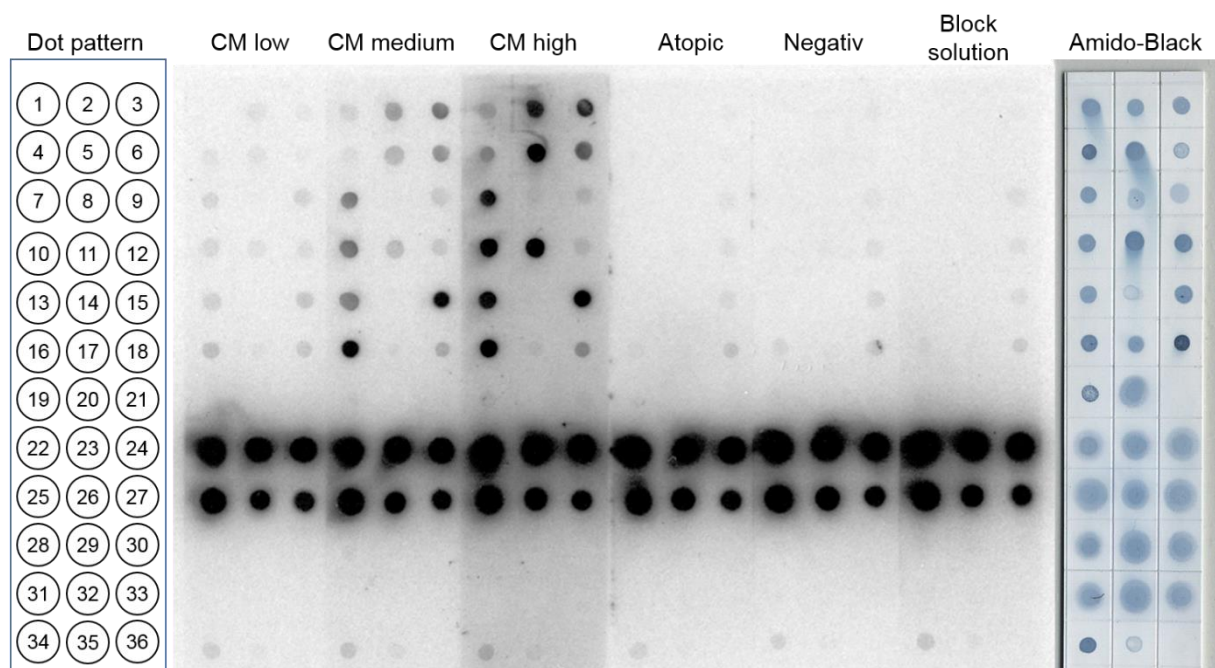
Figure 64: Detection of IgE-binding to recombinant and native proteins in dot blots. Exposition time was 14 days, scan on color scanner in gray mode (212-5-2.2) and auto adjustment with Irfanview software. The dot pattern is decoded in **Table 30**.

In dot blots, functionality of most recombinant and native proteins was confirmed. A considerable exception was native ovomucoid which remained undetectable in all dot blots incubated with positive serum. Similarly, recombinant ovomucoid appered only faintly. As both were dotted as shown in Amido-Black stain, interaction with the surface of the nitrocellulose membrane could account for prevented binding to IgE. Unspecific binding to native and recombinant β-lactoglobulins and ovalbumins occurred, visible in applications with negative controls (Negative and Atopic pool, block solution). Negative controls were unremarkable as concerns unspecific background from κ-caseins. Detection of IgG_4 was not conducted.

4.3.7.6 Western Blots (denaturing conditions)

Western blot analysis was used to assess linear epitopes which could have been masked by improper folding of recombinant proteins. Therefore, proteins were separated in SDS-APGE under reducing conditions which linearized proteins. HE and CM proteins were analyzed on different membranes which were incubated in sequence with CM pool serum, diluted 1:5 in blocking solution. Detection of IgE-specific signals was performed with [125]iodine goat anti human IgE AB, IgG_4 was detected with a mAB from mouse (at 1:10000 in blocking buffer), coupled with HRP.
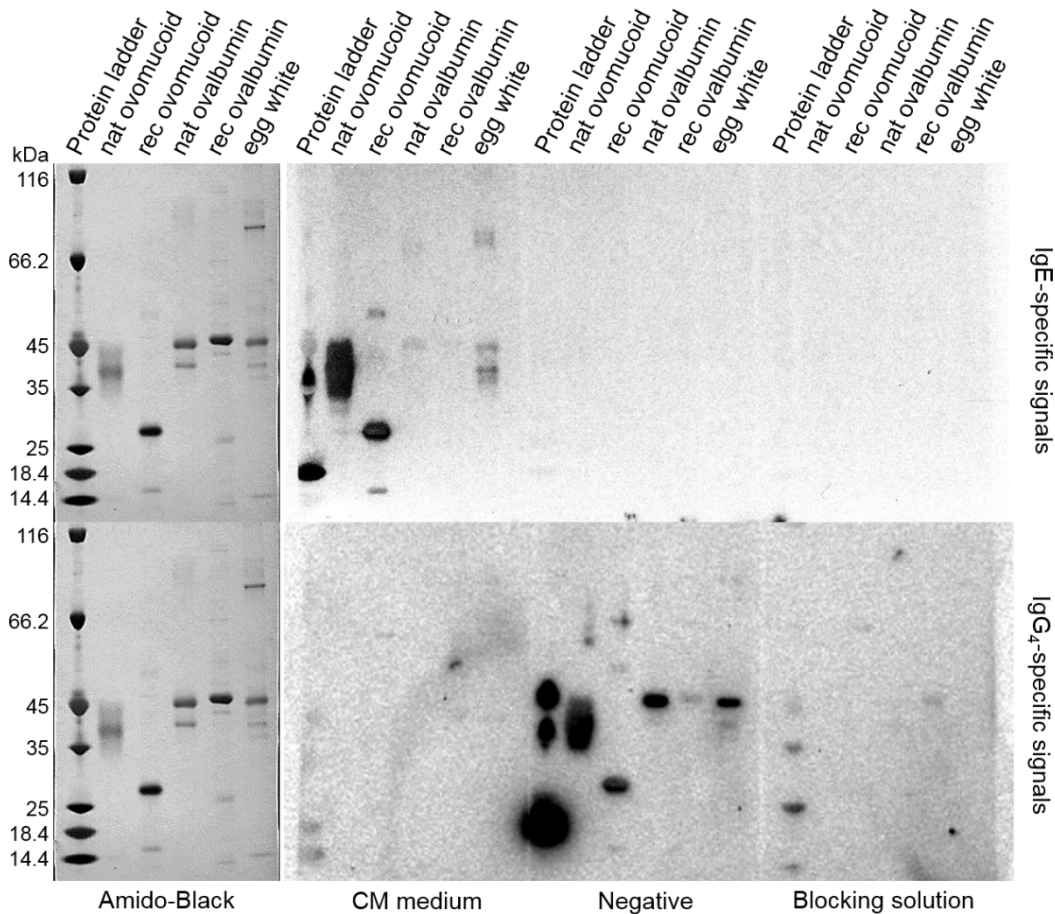
Figure 65: Detection of IgE- and IgG₄ binding to HE proteins in Western blot analysis. Proteins were separated in 4-12% Bis-tris gels using MES buffer. Films were exposed to membranes for two weeks. Scanned images were auto adjusted with Irfanview software.

Application of CM medium pool serum revealed strong binding of IgE to both native and recombinant ovomucoid. In recombinant ovomucoid, two additional faint bands occurred. In egg white, bands typical of ovotransferrin, ovalbumin and ovomucoid were detected (4.3.7.2). The protein ladder contained β-lactoglobulin (at 18.4 kDa) which was also highly positive. IgG₄-specific signals were clearly visible in application with negative pool serum. Recombinant ovalbumin was not recognized and agreed with findings in microarray analysis. With regard to dot blot analysis, this gave evidence that this recombinant protein was misconstructed and thus with no function.
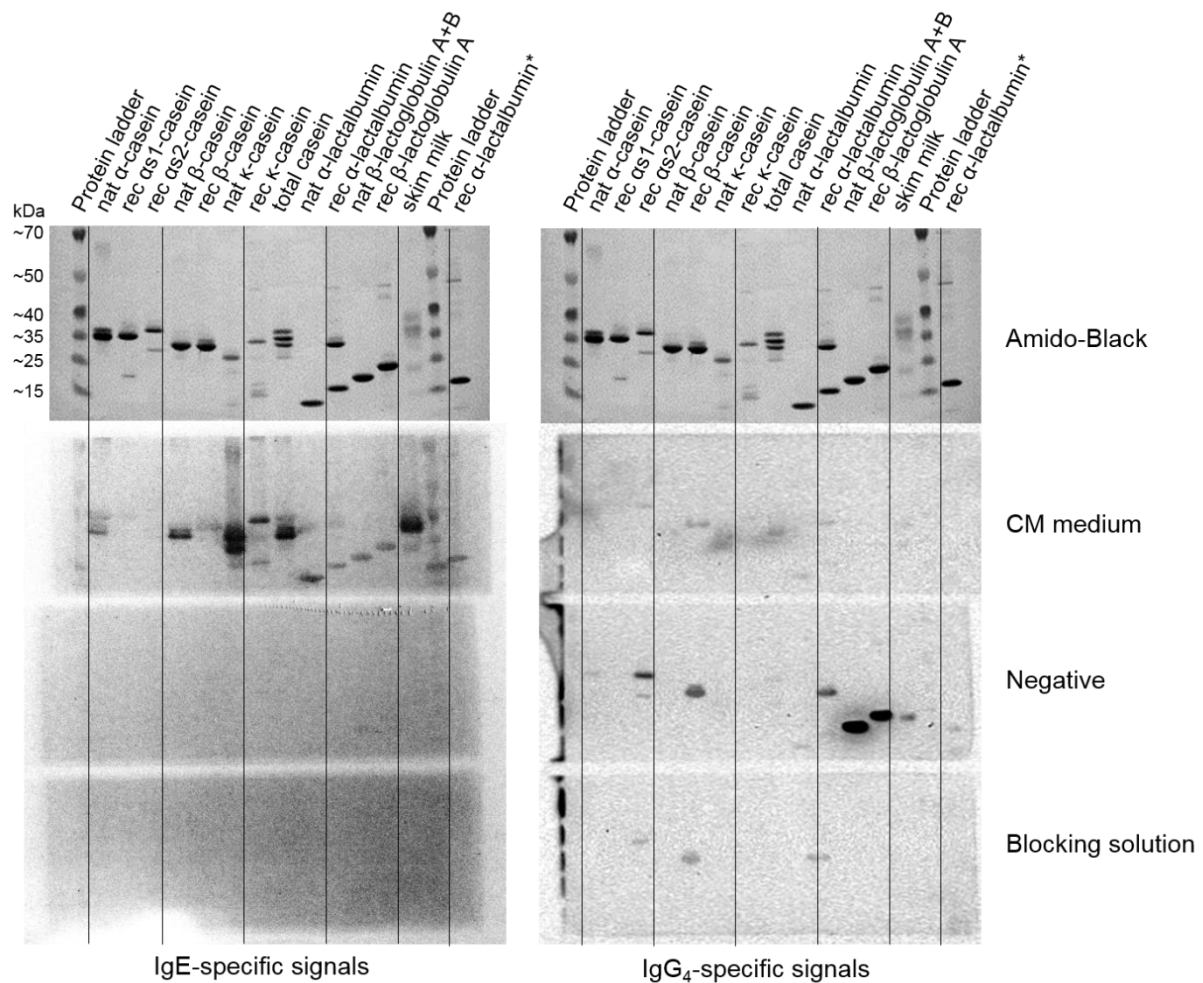
Figure 66: Detection of IgE- and IgG₄ binding to CM proteins in Western blot analysis. Proteins were separated in 4-12% Bis-tris gels using MES buffer. Films were exposed to membranes for two weeks. Scanned images were auto adjusted with Irfanview software.

In Western blot analysis to IgE, all native CM components and skim milk were positive. Recombinant caseins were weaker (β-, κ-casein) or not detected (αs1-, αs2-casein). Especially native κ-casein showed strong IgE-binding to more than the clear protein band visible in Amido-Black stain. Recombinant κ-casein, on the other hand, appeared clearly positive in only one band. This difference indicated modifications on the native, but not the recombinant variant of κ-casein. Recombinant and native β-lactoglobulin appeared with equal intensity. Native α-lactalbumin was clearly recognized, whereas recombinant α-lactalbumin only showed binding with the lower band at ~15 kDa.

IgG₄-specific signals were clearly detected to native and recombinant β-lactoglobulins in Negative pool serum application. In all applications, binding to recombinant αs2- and β-casein, and to the upper band of recombinant α-lactalbumin occurred. If this was due to non-specificity of the secondary AB to these proteins, or bound contents in HSA as part of the blocking solution, could not be determined.

4.3.7.7   Interim conclusion

Analysis of the nucleotide sequences showed good to very good agreements between clones and plasmid sequences. Data base queries identified related proteins with scores >96%. The majority of recombinant proteins were identified in MALDI-TOF MS with significant scores except for κ-casein, α-lactalbumin and ovomucoid. Sequence coverages ranged between 19.5% (κ-casein) and 66.2% (αs2-casein). Purity of paired native and recombinant proteins was very similar except for recombinant α-lactalbumin, whereas protein concentration was misbalanced in favour of recombinant proteins. Quantification from Flamingo fluorescence stain and interferometric assessment of spotted protein on the microarray surface suggested native κ-casein was applied at a concentration higher than intended. Several native proteins were shown to be highly glycosylated, whereas recombinant proteins were not. Functionality of recombinant proteins agreed with findings from microarray assays. Lack of function was confirmed for recombinant ovalbumin in dot blot and Western blot analysis. There is strong evidence that recombinant α-lactalbumin lacked sufficient purity due to contamination with a protein that could not be identified.

5 Discussion

5.1 Wheat study

5.1.1 Immunoblot analyses

The study populations consisted of 106 WT and WA individuals who were determined for sIgE to whole wheat. IgE-binding patterns to wheat water/salt soluble albumins and globulins, and to water insoluble gliadins/glutenins proteins were compared between WT and WA children by means of one-dimensional SDS-PAGE on 4-12% gels and immunoblotting. The hypothesis was that there may be differences in the IgE-recognition pattern between WA and WT patients which may be attributed to water-insoluble gliadins/glutenins.

The water and salt soluble albumin/globulin fraction showed a broad spectrum of IgE binding. In general, WA patients seem to recognize more water and salt soluble proteins between 25 and 35 kDa and below 10 kDa than WT patients, to a lesser extent, proteins from 35 to >50 kDa. No difference of frequency in both groups was first observed for proteins in the range of 10 to 25 kDa. By refined assessment of molecular weights and application of MALDI-TOF MS, proteins in this range were identified and could be related to more precisely counted bands. By trend, non-specific lipid transfer protein (NLTP1), α-amylase inhibitor 0.28 (IAA2) and α-amylase inhibitor 0.19 (IAA1) were recognized more often by WA than WT individuals. The α-amylase inhibitor 0.19 was recently suggested as relevant allergen to distinguish WA from WT individuals by Makela, Eriksson et al. [76]. These authors also tested for avenin-like protein and found that 47% of the examined WA individuals reacted positive. In the population discussed here, a band which could be related to avenin-like a1 or wheatwin-1 was found more often in the WA group, but this difference was not significant with regard to >0.35 kU$_A$/l sensitized WT and WA individuals. The percentage of the respective proteins on the total IgE recognition pattern is very low (<10%) and thus their diagnostic value is questionable.

In the molecular range >25 kDa, refined analysis of 13 WT and 25 WA individuals confirmed no statistical difference in band counts. In MALDI-TOF MS, serpins were identified as common ingestive allergens in both groups. Two other proteins which showed binding to IgE were glyceraldehyde-3-phosphate dehydrogenase, described as inhalational allergen, and aldose reductase, which is neither described as inhalational nor ingestive allergen in wheat. Especially the latter may be subject to future research. However, both proteins were only important to few individuals and are likely not suitable to generally distinguish WT from WA

patients. Still, there is a considerable amount of detected IgE-binding to proteins of the albumin/globulin fraction, which are not clearly identified or characterized as food allergens yet. Their involvement in clinical reactivity of food allergy is unknown and needs to be applied to a large study population.

IB results confirmed findings from several previous studies that gliadins and LMW glutenin subunits are frequently recognized in immunoblots by patients' IgE [93]. HMW glutenin subunits were only found in four patients, one tolerant and three allergic. As the number of WT and WA patients differs, this finding might be incidental, because HMW-glutenins were shown to be major allergens in several studies which investigated WDEIA in young adults [136] and wheat allergy in children [76]. On the other hand, as shown by Takahashi, Matsuo, Chinuki*et al.* [137] and Baar, Pahr, Constantin *et al.* [138], fragments of recombinant HMW-glutenin show higher specificity compared to full forms. In this study, SDS-PAGE and Western blotting were performed under denaturing and reducing conditions which unfolds and linearizes proteins, but this might be insufficient for proper IgE binding when applied on PVDF membrane.

Refined analysis to gliadins/glutenins was only possible for 7 WT and 25 WA individuals. Separation of proteins in SDS-PAGE was less clear, and MALDI-TOF MS could only identify HMW subunits. Some evidence was found that WT and WA individuals differ in two bands between ~35 and ~40 kDa. However, this result may be insignificant due to the limiting factors mentioned.

These data show that food allergy in infants can be triggered by all storage proteins. No difference could be found in the protein pattern of water insoluble proteins. This may be due to the fact that gliadins and glutenins feature a wide polymorphism, and a huge amount of highly repetitive sequences and non-repetitive domains as well as sequence homologies, resulting in cross-reactions and similar immunological recognition patterns [139-142].

In sum, these analyses showed that many proteins from both, the water and salt soluble albumin/globulin and the water insoluble gliadin/glutenin fraction, are recognized by WT and WA patients, implying a sensitization to several wheat proteins of both fractions. Unique bands that clearly distinguish by frequency WT from WA patients could not be detected. By means of MALDI-TOF MS, previous findings by Sotkovsky, Sklenar *et al.* [85] that the cultivar Akteur contains several important allergens in the albumin/globulin fraction could be replicated and taken as confirmation of own results. However, the ImmunoCAP's wheat panel probably consists of several wheat cultivars and thus covers more variants of allergenic proteins that might be absent in the cultivar Akteur. This could explain the deviations between measured IgE and non-detected bands. The value of component-resolved diagnostics with qualitative immunoblot-based assays is also limited for other

reasons. Band counts can only be regarded as semi-quantitative, because no internal standard was used for highly precise alignment of strips, especially when electrophoresis was extended and bands became irregular. One-dimensional SDS-PAGE is a suitable means for screening, but lacks high resolution. Instead, 2D gel electrophoresis could be employed, provided sufficient amounts of patient material. In connection with extraction protocols specific for each protein fraction, 2D gel electrophoresis would improve identification of IgE-reactive proteins. As shown in this study, despite intense washing during the protein purification process, some unspecific binding occured due to cross-contamination. This underlines the necessity of highly purified extracts but, on the other hand, the sensitivity of immunoblot analysis.

5.1.2   ω5-gliadin

The role of ω5-gliadin was of special interest as it is one of the most reported examples in favour of CRD [117]. There was no significant band in IB analysis at the proposed position at ~65 kDa. Low general abundance of ω5-gliadin may be taken into account for this result, and insufficient transfer onto the PVDF membrane cannot be excluded despite optimization of conditions. Coomassie and Amido Black stains in the proposed range only showed faint bands. Tryptic digestion of these bands in the range around 65 kDa and analysis with MALDI-TOF MS were also not successful. The probable reason for this finding is that trypsin cleaves ω5-gliadin in large peptides which are beyond the detection range. In linear mode, which covers mass ranges up to 220 m/z, some evidence for the existence and position of ω5-gliadin was found. However, these findings could not help specify ω5-gliadin-specific signals in immunoblots. Other methods for purification and application of ω5-gliadin to IB analyses should be considered, e.g. RP-HPLC as suggested by Matsuo, Kohno, Morita et al. [143].

Quantitative assessment of specific IgE to ω5-gliadin in 40 randomly chosen sera showed elevated levels of specific IgE to ω5-gliadin in the WA group, but there was no significant difference between WT and WA individuals. This is consistent with findings of Makela, Eriksson et al. [76], who showed that ω5-gliadin has a specificity of only 50% in WA children. The correlation of IgE to wheat and ω5-gliadin was significant in the WT group, suggesting that ω5-gliadin levels are a function of IgE to whole wheat rather than a specific sensitization of WA children. However, as recently shown by Nilsson, Sjolander et al. [106], quantitative component-resolved diagnostics has now reached a level to near 100% specificity as concerns the prediction of wheat challenges when considering all four components of gluten (ω5-gliadin, gliadin, HMW- and LMW-glutenins) quantitatively. The fact that ω5-gliadin-specific IgE was existent in in some sera, but was not detectable in IB analysis, may be evaluated in favour of CRD, because measurements on ImmunoCAP use a recombinant

fragment of only 37 kDa size and compensate for low abundance in crude native extracts. On the other hand, diagnostics with ImmunoCAP (or ImmunoCAP ISAC and the immunoblot technique applied) are highly artificial compared to conditions in daily diet where proteins of different sources might chemically interact by mixing, processing, fermenting, heating and being digested in the gastrointestinal tract [144]. In this regard, the impact of de-amidation on allergenicity has been discussed [145-148]. Diagnostic procedures might evoke epitopes which are irrelevant in normal life and overestimate the role of IgE reactivity patterns [149].

5.1.3   Comparison of cultivars

Using pooled serum of young patients sensitized to ingested wheat was to reveal the majority of possible IgE binding sites in the albumin/globulin and gliadin fractions of the cultivars and species applied. Pooled serum is not representative for single patient IgE recognition patterns, however, it provides an overview that is helpful in assessing the possible allergenic potency of a grain species and cultivar compared to others. In this respect, old cultivars cannot be generally considered an alternative to wheat, because they also show a broad IgE recognition pattern and thus could be as allergenic as wheat. On the other hand, individuals differ in their IgE recognition pattern, and some cultivars may be well tolerated compared to others. A broad screen of old cultivars is useful to assess differences in general immunoreactivity which might help identify e.g. less allergenic isoforms of proteins or different levels of expressed allergens. This information could direct breeding strategies. Whether old cultivars are really less allergenic (or more healthy compared to wheat) can only be proven by clinical tests (DBPCFC) and diagnostics (ImmunoCAP, immunoblots, microarray) of a large number of patients.

5.2   Microarray study

5.2.1   Performance of the silicon microarray

The polymer coated silicon microarray for simultaneous detection of food allergen-specific IgE and IgG$_4$ showed an overall good correlation to quantitative and semi-quantitative results from the ImmunoCAP and ImmunoCAP ISAC platforms. Key issues to be improved are inter slide variation and sensitivity for certain components and cow's milk extract.

In general, the IgE- and IgG$_4$-specific intra slide variations of the mean fluorescence were good with 12.6% and 15.7% at average. Results of inter slide variation were higher with 30.7% for IgE and 32.9% for IgG$_4$. However, the silicon microarray performed considerably better in comparison to available results for inhalative components on ISAC reported by Melioli, Bonifazi et al. [59]. Intra assay CV's on ISAC in the range 0.3 to 1 ISU were greater

100%, and CV's for ISU between 1 to 15 were determined with (intra assay) 17% and (inter assay) 33%. In this study, the HE medium pool serum was used as intra and inter assay control. It was determined on ISAC for α-lactalbumin and β-lactoglobulin with 0.68 and 0.54 ISU, respectively. Calculated intra assay CV's on the silicon microarray for IgE-specific median fluorescence were 7.4 and 16.5%, and 16.5 and 20.8%, respectively. In the range 1 to 15 ISU, ovomucoid and ovalbumin were determined on ISAC with 3.67 and 1.28. CV's for spotted ovomucoid and ovalbumin were 20.9 and 12% for intra assay, and 26.4 and 23.1% for inter assay variance. IgG_4-specific CV's are not directly comparable, but are still better in the respective ranges for the components mentioned. These results are a strong evidence of higher reproducibility of the silicon microarray and confirm previous findings by Cretich, Di Carlo *et al.* [61].

The relatively high inter slide variation can be ascribed to several factors: most steps in assaying the slides were manual (e.g. washing), secondary antibody dilutions were prepared daily, several batches and lots of slides were used, only one array per slide was spotted (ISAC has 4 arrays per slide), the slide was scanned being maybe not fully planar placed in the holder. Apart these factors that could quite easily be solved, a well-known problem is the spotting procedure itself with an estimated error of 20-25% in depositing protein onto the surface [150]. This would imply using 3 colors for an additional total protein control, which would provide further data on the quality of spotting, but would also increase costs or the introduction of an internal calibration method to normalize the allergen fluorescence response by its actual immobilization yield [151]. Indeed, scanning systems with three or four colours are available and have been applied to parallel detection of IgA, IgM, IgG and IgE [152,153]. In this study, instrumentation was limited to two colours.

Another well know problem that affects proper quantification and thus inter assay variability is sub-optimal spot morphology ("donut-effect"). The ring-shaped structure was especially observed for β-caseins. According to Wellhausen, Seitz *et al.* [154], this effect could be compensated by application of e.g. Triton or glycerol which lowers the surface tension of the protein solution, or mathematically by using median instead of mean fluorescence. In this study, both mean and median fluorescence were correlated with quantitative results and showed very similar coefficients.

Although recent guidelines do not recommend the determination of IgG_4 for the diagnostic of food allergy, IgG_4 has been suggested as a candidate involved in tolerance development [155,156]. Therefore, quantitative data are needed, preferably simultaneously from the same blood sample, [157,158]. One issue for parallel detection of IgE and IgG_4 is the difference in concentration which is usually compensated by diluting the serum when IgG_4 is targeted. However, this alters binding kinetics of IgE and IgG_4 towards shared epitopes. Parallel detection from undiluted serum would mimic physiological conditions in the patient with

implications for the role of IgG$_4$ as competing with IgE. But, as outlined by Yman [159] or Aalberse and Aalberse [22] the problem for microarrays is that the low amount of spotted allergen favours interference with immunoglobulins other than IgE. Shreffler [36], on the other hand, points out that assays like ImmunoCAP, which provide allergen in excess, may even detect low-affinity IgE with little or no clinical relevance. A possible solution was suggested by Cretich, Di Carlo, Giudici *et al.* [160] who showed that microfluidics coupled with microarray improves the accuracy of IgE vs IgG detection when low and high affinity antibodies compete for the binding with the same allergen.

In this study, IgE and IgG$_4$ were detected in parallel with a pair of Cy3 and Cy5 labelled polyclonal and monoclonal antibodies. Correlations with quantitative results from individual sera were very high and specific. This was confirmed by assays with geometric dilutions of two sera in 1% BSA in PBS, where no interference in the parallel measurement of IgE and IgG$_4$-specific fluorescence was observed. Thus, dye interaction ("quenching") of Cy3 and Cy5 is neglectable low, which is in addition to their brightness, an appreciated characteristic of these dyes [161]. The silicon microarray could be a suitable tool in co-detection of IgE and IgG$_4$, irrespective of clear inter assay variations. IgE/IgG$_4$ ratios can be calculated because deviations in e.g. protein deposition should apply equally to IgE and IgG$_4$ binding and detection. On the other hand, the working range of IgG$_4$ detection is limited, and very high levels of IgG$_4$ would probably not be captured. However, competing fluorescent dyes (Alexa) have been successfully applied in co-detection of IgE and IgG$_4$ to peptides of ovomucoid and CM components [157,162]. The ImmunoCAP ISAC is not available for co-detection. The most advanced version uses undiluted serum for IgE detection, for IgA 1:10 and 1:50 for IgG and IgG$_4$ [163].

Sensitivity was shown in assays with atopic and low positive pooled sera, and in geometric dilutions assays on microarrays from one batch. In dilution assays, hen's egg-specific IgE correlated well with the ImmunCAP system and showed sensitivity in the low range down to <0.35 kU$_A$/l. In contrast, for cow's milk-specific IgE only limited sensitivity and correlation could be found. The sensitivity for skim milk starts only near 1 kU$_A$/l with clear titer-dependent increase of fluorescence up to 100 kU$_A$/l. As shown by Gaudin *et al.* [164], large differences between cow's milk substrates exist. The high number of allergenic components (caseins, α-lactalbumin, β-lactoglobulin and other allergens not assessed in this assay) which differ in content in differently processed cow's milk and matrix-dependent binding affinities contribute to divergent performances on different platforms. Therefore, skim milk might be an inappropriate analyte in comparison to the cow's milk panel on the ImmunoCAP. The working range for IgG$_4$ seems to be limited from 0.01 to 1 mg$_A$/l, whereas the ImmunoCAP covers up to 30 mg$_A$/l. Attempts to assess sensitivity to different individual sera proved to be difficult. On

the ImmunoCAP platform, the LOD for IgG_4 currently is 0.07 mg_A/l. However, results down to 0 mg_A/l are reported. Therefore, correlations of IgG_4-specific fluorescence with values below the LOD are questionable, and the sensitivity of the silicon microarray is difficult to determine due to missing reliable quantitative references in this range and "real" negative controls.

Various kinds of normalizations and transformations were applied and showed that cleared fluorescence correlates best with quantitative and semiquantitative results. Normalization to intra/inter assay variation resulted in slightly reduced correlations. Transformation in internal calibration curve units and combinantions with normalization to intra/inter assay variation showed lowest correlations. The reasons identified were misbalanced concentrations of spotted IgE in the low range, leading to insufficient capture of low IgE-specific fluorescence from components. As reported by Noh, Ahn *et al.* [19], IgE- and IgG_4-specific internal calibrations curves are applicable. These authors used concentrations of purified IgE and IgG_4 at concentrations of 80 to 1 µg/ml to establish IBU (immunoglobulin binding unit) which were used for calculation the IgE/IgG_4 ratio. To compensate the lower concentration of antigen, an additional amplification step was introduced. This could also be applied to the silicon microarray. In this study, IgG_4 lacked sufficient coverages of both high and low concentrations of specific IgG_4 from components. This agrees with the finding that the silicon microarray has a limited working range for IgG_4. Application of external calibration by means of Egg medium pool serum, which served as inter and inter assay control, was not possible due to its composition of sIgE between 4.06 to 26.3 kU_A/l.

In sum, the silicon microarray assay is only semi-quantitative. This result matches findings frequently reviewed. Despite intense research on surface chemistry and detection techniques [161,165-168], accurate quantification of captured IgE in kU_A/ on microarrayed platforms for the diagnosis of food allergy is still not achieved, despite promising results as concerns reliability, sensitivity and correlations with established routine platforms [169,170].

5.2.2 Comparison of recombinant with native components

In general, native components showed higher correlations with quantitative and semiquantitative results, independent of data processing.

Recombinant ovalbumin showed absolutely no binding, despite it was retrieved in BLAST data base query and identified with significant score in MALDI-TOF MS. This is in contrast to microarray, Western and dot blot results. Whereas a positive result in MALDI-TOF MS can be attributed to the fact that digested peptides are measured and assigned to proteins by bio-informatical algorithms with a certain probability, the negative Western blot result cannot be explained. For ovalbumin, 5 binding sites for IgE were proposed by means of peptide arrays [171]. At least some of these 5 supposedly linear epitopes should have been recognized in Western blot analysis where the protein is linearized and epitopes are exposed. The existence of structural epitopes has been inferred from reduced allergenicity after heating [172]. Assumed the recombinant protein was misfolded, the lack of function was explainable. Purification of ovalbumin under mild native conditions was performed, but the protein rapidly precipitated, indicating improper folding. Further studies with e.g. digested peptides of this recombinant protein could help unravel its failure in the assays reported. A verification of the cDNA clone is also advisable.

Comparisons of functioning paired recombinant and native proteins are difficult. As shown in assessment of protein purity, β-caseins, α-caseins and ovomucoids were found to be quite similar. Clear differences were found for κ-caseins, β-lactoglobulins and ovalbumins. Rekombinant α-lactalbumin was determined with least purity after evaluation of Western blot and MALDI TOF MS results. The relative protein concentration applied on the silicon microarray was disbalanced in favour of recombinant proteins (Table 28). Results in Flamingo stain imply approximately equal amounts of caseins, α-lactalbumins and β-lactoglobulins. Clear differences were determined for β- and κ-caseins, ovomucoids and ovalbumins (Figure 45). The interferometric evaluation of immobilized protein on the surface showed differences for α-lactalbumins and β-lactogolublins, but not for ovalbumins (Figure 59). In sum, it is not clear if same amounts of paired recombinant and native protein were immobilized on the surface. Thereby, direct comparisons are of very limited value. This finding may explain why, as mentioned afore, the results for IgE in the dilution assays showed a very good performance for recombinant ovomucoid which exceeded its native variant in sensitivity and working range. However, besides an excess of protein, this result could also be ascribed to different spot morphology because native ovomucoid spots are much densed, which could be due to the fact that native ovomucoid is highly glycosylated, as shown in Western blot analysis. On the other hand, as only two sera were assayed in geometric dilutions, these assays must be repeated with several sera to be generalized.

Nonetheless, differences for applied β- and κ-caseins can be compared because in all methods applied, recombinant β-casein exceeds the native variant, and vice versa native κ-casein its recombinant counterpart. Despite of lower amount, native β-casein showed clearly higher correlations compared to recombinant β-casein. Both proteins were found to be of very similar purity. In dot blot and Western blot analysis, native β-casein showed slightly stronger signals. These results imply a certain role for glycosylation as shown in Western blot analysis. The difference between κ-caseins, on the other hand, is due to the fact of applying native κ-casein in large excess. Interestingly, a stronger fluorescence had already been observed in initial microarray assays when another preparation of native κ-casein was applied and detected with a monoclonal antibody to IgE.

Another issue was the function of recombinant α-lactalbumin. In SDS-PAGE, two bands had been observed and ascribed to be the monomer (lower band) and a dimer (upper band). As both were purified in affinity chromatography to HIS-tag, this was reasonable to assume. Observations during quantification of the microarrays, and finally poor correlations with quantitative and semi-quantitative results implied a repetition of purification, which was performed under mild native conditions. Applied in SDS-PAGE, only one band of typical size occurred. In Western blot analysis, IgE only showed binding to this band in both preparations of recombinant α-lactalbumin. In MALDI-TOF MS provided additional evidence. In sum, these results show that purification of recombinant proteins must be monitored very closely.

The specificity of CM components was also assessed with ROC analysis to identify significant components. As mentioned afore, the amount of immobilized protein is not equal. Another limiting factor of this analysis is that is considers all 91 sera equally, although some individuals contributed with several sera from different time points. This was accepted to keep constant conditions, although results of respective individuals should have been weighted. However, this analysis served as a means to set component- and extract-specific cut-offs. So far, no baseline had been established due to the fact that background was not subtracted and that internal calibration failed. The results, with all limitations mentioned, give some further evidence for superior performance of native CM components.

Despite issues on protein content and purity, heterogenity and genetic polymorphism with several isoforms, as mentioned in the introduction, may be the reasons for better performance of native components in these assays [118,119]. On the other hand, except for ovalbumin, all recombinant components do correlate to their native counterparts with at least 75% which is a strong indicator of function [173]. This is important especially for cDNA clones ovomucoid and β-casein, which were so far only predicted.

6 Conclusion and Outlook

Children with clinical relevant food allergy and tolerant ones had a similar spectrum of IgE-binding to individual wheat allergens. A broad spectrum of proteins in the water/salt soluble and the water insoluble protein fraction can trigger IgE-specific binding. Neither quantitatively determined sIgE to ω5-gliadin nor results from immunoblot analyses justify component-resolved diagnostics in the diagnostic workup of wheat food allergy. Quantitative determination of sIgE to components or panels of components need to be investigated along with extended application of highly purified components using microarrays or dot blots. Longitudinal studies with individuals should be conducted to closely monitor the development of IgE-recognition pattern to wheat proteins which would propel deducible causalities.

It was shown that the copoly(DMA-NAS-MAPS) Si/SiO_2 based microarray is a semiquantitative assay with a working range comparable to the ImmunoCAP ISAC. Correlations between quantitative results from the ImmunoCAP correlated better with IgE-specific fluorescence from silicon based microarray than with ISU from ISAC. If this is due to data processing with the MIA software remains unclear as no raw fluorescence of ISAC was accessible. There is evidence that copoly(DMA-NAS-MAPS) Si/SiO_2 array is more sensitive in IgE detection than the ImmunoCAP ISAC as shown in geometric dilution assays. Parallel measurements of IgE and IgG_4 are possible, but only with limited working range for IgG_4. Slides with a four-field-format, together with the known relatively cheap and easy-to-perform surface chemistry, might make the copoly(DMA-NAS-MAPS) silicon chip an alternative to the ImmunoCAP ISAC. Further analyses of data with clinical symptoms are necessary to determine IgE-specific component-specific cut-offs. Calibration and normalization, close monitoring of spot quality and protein content, and clearly defined quality criteria and data processing are indispensable. In order to clearly compare different platforms, repeated measurements with standard materials (serum and allergens) under defined conditions need to be performed side-by-side [159,174].

Component-resolved diagnostics to food allergens is challenging, and as suggested by the EFSA, other factors than specific IgE are likely to contributionally mediate and/or modulate allergic reactions [175]. In this respect, microarrays are the platform of choice. The copoly(DMA-NAS-MAPS) silicon chip could be of great benefit.

7 Summary

Food allergy is a severe disease with increasing prevalence. Until now, no single laboratory parameter was identified that could prove the diagnosis of food allergy nor predict its course. This may be due to insufficient test systems with natural extracts. Component-resolved diagnostics tests for single allergenic components and tries to relate them to the disease. Recombinant proteins have become alternatives to native components which may be impure. On the other hand, recombinant proteins may be misfolded and lack functionality. Therefore, both need to be compared in parallel. In pediatric populations, where patient material is limited, the application of microarrayed disgnostics is the matter of choice. The most common microarray, the ImmunoCAP ISAC, provides several food allergens that can be tested for. On the other hand, this microarray is only semiquantitative, lacks sensitivity as well as reproducibility, and only detects one class of immunoglobulins.

In this study, an alternative to ISAC was used, the copoly(DMA-NAS-MAPS) silicon chip which was reported to be more sensitive and robust. The performance of this microarray was assessed by assaying a large number of well-described sera to native and recombinant components of cow's milk and hen's egg. IgE and IgG_4, which may be involved in tolerance development, were detected in parallel from one drop of blood. The silicon microarray was sensitive and showed a broad working range for hen's egg comparable to ImmunoCAP. For cow's milk it was less sensitive, and the working range for IgG_4 may be limited at higher concentrations. In the lower range, its intra and inter assay variance was better compared to ISAC. Native components were found to correlate better with quantitative and semiquantitative results than recombinant variants. The exact reason for this result could not be determined. However, the silicon microarray is a semiquantitative assay and a good alternative to ISAC.

Another important disease in childhood is food allergy to ingested wheat. The diagnosis of wheat allergy is still difficult, because wheat-specific IgE, determined in blood, does not correlate well with clinical symptoms. Component-resolved diagnostics by means of qualitative immunoblots was applied to compare wheat allergic and tolerant individuals in their IgE-recognition pattern to water and salt soluble proteins and to water-insoluble proteins which may be underrepresented in commercial test systems. A broad spectrum of protein bands in both protein fractions was detected, and some were identified, but no differences in the pattern between WA and WT individuals was found. The significance of ω5-gliadin, which is reported to be a specific marker of wheat allergy, could not be confirmed. The benefit of component-resolved diagnostics in the workup of wheat allergy is therefore questionable.

Zusammenfassung

Die Nahrungsmittelallergie ist eine ernsthafte Erkrankung mit zunehmender Prävalenz. Bis dato wurde kein einzelner Laborparameter identifiziert, der die Diagnose belegen und den Verlauf vorhersagen könnte. Ein Grund dafür könnten unzulängliche Testsysteme mit natürlichen Extrakten sein. Komponenten-basierte Diagnostik testet auf einzelne allergene Bestandteile (Komponenten) und versucht, diese mit der Erkrankung in Beziehung zu setzen. Rekombinante Proteine sind dabei Alternativen im Vergleich zu nativen Proteinen, die verunreinigt sein können. Auf der anderen Seite können rekombinante Proteine fehlgefaltet und somit funktionslos sein. Daher müssen beide parallel verglichen werden. In pädiatrischen Populationen ist Patientenmaterial begrenzt und die Anwendung von miniaturisierter Diagnostik auf Microarrays die Methode der Wahl. Am weitesten verbreitet ist der ImmunoCAP ISAC, der mehrere Nahrungsmittelallergene zur Testung bereitstellt. Andererseits ist dieser Microarray nur semiquantitativ, es fehlt ihm an Sensitivität und Reproduzierbarkeit und er detektiert nur je eine Klasse von Immunglobulinen.

In dieser Arbeit wurde daher alternativ der copoly(DMA-NAS-MAPS) Siliziumchip verwendet, der zuvor als robuster und sensitiver beschrieben wurde. Die Leistungsfähigkeit dieses Microarrays wurde ermittelt, indem eine Vielzahl gut beschriebener Seren an nativen und rekombinanten Komponenten aus Kuhmilch und Hühnerei erprobt wurden. IgE und IgG$_4$, welches in der Toleranzentwicklung involviert sein könnte, wurden parallel in einem Tropfen Blutflüssigkeit bestimmt. Der Silizium-Microarray war sensitiv und zeigte einen breiten Arbeitsbereich für Hühnerei vergleichbar mit dem ImmunoCAP, der quantitativen Routineplatform. Für Kuhmilch war er weniger sensitiv. Der Arbeitsbereich für IgG$_4$ dürfte bei höheren Konzentrationen begrenzt sein. Im unteren Bereich waren intra- und inter-Assay-Varianz besser als beim ImmunoCAP ISAC. Native Komponenten korrelierten besser mit quantitativen und semiquantitativen Ergebnissen als die rekombinanten Varianten. Der genaue Grund für diesen Befund konnte nicht bestimmt werden. Nichtsdestotrotz ist der Silizium-Microarray ein semiquantitativer Assay und eine gute Alternative zum ISAC.

Eine andere wichtige Nahrungsmittelallergie im Kindesalter ist die gegen Weizen. Die Diagnose Weizenallergie ist immer noch schwierig zu stellen, da das im Blut bestimmte Weizen-spezifische IgE nicht gut mit der klinischen Symptomatik korreliert. Komponenten-basierte Diagnostik auf Basis von qualitativen Immunoblots wurde angewendet, um Weizen-allergische und Weizen-tolerante Patienten in ihren IgE-Erkennungsmustern auf wasser- und salzlösliche sowie wasser-unlösliche Proteine zu vergleichen, zudem letztere in kommerziellen Testsystemen unterrepräsentiert sein könnten. Es wurde ein breites Spektrum an Proteinbanden in beiden Fraktionen ermittelt. Einige Proteine wurden

identifiziert, jedoch wurden keine Unterschiede im Erkennungsmuster zwischen Weizen-allergischen und Weizen-toleranten Patienten gefunden. Die Bedeutung von ω5-gliadin, das ein spezifischer Marker der Weizenallergie sein soll, konnte nicht bestätigt werden. Der Nutzen Komponenten-basierter Diagnostik in der Abklärung der Weizenallergie ist somit fraglich.

8 Reference List

1.   Sicherer SH, Sampson HA. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *Journal of Allergy and Clinical Immunology*, 133(2), 291-307.e295 (2014).

2.   Lack G. Update on risk factors for food allergy. *J.Allergy Clin.Immunol.*, 129(5), 1187-1197 (2012).

3.   Platts-Mills TA, Commins SP. Emerging antigens involved in allergic responses. *Curr.Opin.Immunol.*, 25(6), 769-774 (2013).

4.   Nwaru BI, Hickstein L, Panesar SS *et al.* The epidemiology of food allergy in Europe: a systematic review and meta-analysis. *Allergy*, 69(1), 62-75 (2014).

5.   Grabenhenrich LB, Dolle S, Moneret-Vautrin A *et al.* Anaphylaxis in children and adolescents: The European Anaphylaxis Registry. *J Allergy Clin Immunol*, (2016).

6.   Ballmer-Weber BK. Allergic reactions to food proteins. *Int.J.Vitam.Nutr.Res.*, 81(2-3), 173-180 (2011).

7.   Meyer R, De KC, Dziubak R *et al.* Malnutrition in children with food allergies in the UK. *J.Hum.Nutr.Diet.*, 27(3), 227-235 (2014).

8.   Versluis A, Knulst AC, Kruizinga AG *et al.* Frequency, severity and causes of unexpected allergic reactions to food: a systematic literature review. *Clin.Exp.Allergy*, (2014).

9.   Bocking C, Renz H, Pfefferle PI. [Prevalence and socio-economic relevance of allergies in Germany]. *Bundesgesundheitsblatt.Gesundheitsforschung.Gesundheitsschutz.*, 55(3), 303-307 (2012).

10.   Muraro A, Werfel T, Hoffmann-Sommergruber K *et al.* EAACI food allergy and anaphylaxis guidelines: diagnosis and management of food allergy. *Allergy*, 69(8), 1008-1025 (2014).

11.   Nermes M, Niinivirta K, Nylund L *et al.* Perinatal pet exposure, faecal microbiota, and wheezy bronchitis: is there a connection? *ISRN allergy*, 2013, 827934 (2013).

12.   Nermes M, Salminen S, Isolauri E. Is there a role for probiotics in the prevention or treatment of food allergy? *Curr Allergy Asthma Rep*, 13(6), 622-630 (2013).

13.   Lau S. What is new in the prevention of atopy and asthma? *Curr.Opin.Allergy Clin.Immunol.*, 13(2), 181-186 (2013).

14.   Esparza-Gordillo J, Matanovic A, Marenholz I *et al.* Maternal filaggrin mutations increase the risk of atopic dermatitis in children: an effect independent of mutation inheritance. *PLoS genetics*, 11(3), e1005076 (2015).

15.   Martino D, Joo JE, Sexton-Oates A *et al.* Epigenome-wide association study reveals longitudinally stable DNA methylation differences in CD4+ T cells from children with IgE-mediated food allergy. *Epigenetics*, 9(7), 998-1006 (2014).

16.   WHO. Prevention of allergy and allergic asthma. 14 (2003).

17.   James LK, Till SJ. Potential Mechanisms for IgG4 Inhibition of Immediate Hypersensitivity Reactions. *Curr Allergy Asthma Rep*, 16(3), 23 (2016).

18.   Okamoto S, Taniuchi S, Sudo K *et al.* Predictive value of IgE/IgG4 antibody ratio in children with egg allergy. *Allergy, Asthma & Clinical Immunology*, 8(1), 1-7 (2012).

19.   Noh G, Ahn HS, Cho NY, Lee S, Oh JW. The clinical significance of food specific IgE/IgG4 in food specific atopic dermatitis. *Pediatr.Allergy Immunol.*, 18(1), 63-70 (2007).

20.   Lee JH, Kim WS, Kim H, Hahn YS. Increased cow's milk protein-specific IgG4 levels after oral desensitization in 7- to 12-month-old infants. *Ann Allergy Asthma Immunol*, 111(6), 523-528 (2013).

21.   Ahrens B, Lopes de Oliveira LC, Schulz G *et al.* The role of hen's egg-specific IgE, IgG and IgG4 in the diagnostic procedure of hen's egg allergy. *Allergy*, 65(12), 1554-1557 (2010).

22.   Aalberse RC, Aalberse JA. Molecular Allergen-Specific IgE Assays as a Complement to Allergen Extract–Based Sensitization Assessment. *The Journal of Allergy and Clinical Immunology: In Practice*, 3(6), 863-869 (2015).

23.   Lieberman JA, Sicherer SH. Diagnosis of food allergy: epicutaneous skin tests, in vitro tests, and oral food challenge. *Curr Allergy Asthma Rep*, 11(1), 58-64 (2011).

24.   Bellini F, Ricci G, Dondi A, Piccinno V, Angelini F, Pession A. End point prick test: could this new test be used to predict the outcome of oral food challenge in children with cow's milk allergy? *Ital.J.Pediatr.*, 37, 52 (2011).

25.   Bellini F, Ricci G, Remondini D, Pession A. Cow's milk allergy (CMA) in children: identification of allergologic tests predictive of food allergy. *Eur.Ann.Allergy Clin.Immunol.*, 46(3), 100-105 (2014).

26.   Hill DJ, Heine RG, Hosking CS. The diagnostic value of skin prick testing in children with food allergy. *Pediatr Allergy Immunol*, 15(5), 435-441 (2004).

27.   Peters RL, Allen KJ, Dharmage SC *et al.* Skin prick test responses and allergen-specific IgE levels as predictors of peanut, egg, and sesame allergy in infants. *J Allergy Clin Immunol*, 132(4), 874-880 (2013).

28.   Peters RL, Gurrin LC, Allen KJ. The predictive value of skin prick testing for challenge-proven food allergy: a systematic review. *Pediatr Allergy Immunol*, 23(4), 347-352 (2012).

29.   Sicherer SH, Wood RA. Allergy testing in childhood: using allergen-specific IgE tests. *Pediatrics*, 129(1), 193-197 (2012).

30.   Hamilton RG, Williams PB. Human IgE antibody serology: a primer for the practicing North American allergist/immunologist. *J.Allergy Clin.Immunol.*, 126(1), 33-38 (2010).

31.   Asero R, Fernandez-Rivas M, Knulst AC, Bruijnzeel-Koomen CA. Double-blind, placebo-controlled food challenge in adults in everyday clinical practice: a reappraisal of their limitations and real indications. *Curr Opin Allergy Clin Immunol*, 9(4), 379-385 (2009).

32.   Ahrens B, Niggemann B, Wahn U, Beyer K. Positive reactions to placebo in children undergoing double-blind, placebo-controlled food challenge. *Clin.Exp.Allergy*, 44(4), 572-578 (2014).

33.   Jarvinen KM, Sicherer SH. Diagnostic oral food challenges: procedures and biomarkers. *J Immunol Methods*, 383(1-2), 30-38 (2012).

34.   Rolinck-Werninghaus C, Niggemann B, Grabenhenrich L, Wahn U, Beyer K. Outcome of oral food challenges in children in relation to symptom-eliciting allergen dose and allergen-specific IgE. *Allergy*, 67(7), 951-957 (2012).

35.   Soares-Weiser K, Takwoingi Y, Panesar SS *et al.* The diagnosis of food allergy: a systematic review and meta-analysis. *Allergy*, 69(1), 76-86 (2014).

36.   Shreffler WG. Microarrayed recombinant allergens for diagnostic testing. *J.Allergy Clin.Immunol.*, 127(4), 843-849 (2011).

37. Melioli G, Compalati E, Bonini S, Canonica GW. The added value of allergen microarray technique to the management of poly-sensitized allergic patients. *Curr.Opin.Allergy Clin.Immunol.*, 12(4), 434-439 (2012).

38. Breiteneder H, Wagner B. Expression of allergens in E. coli and plants--benefits and drawbacks. *Arb.Paul Ehrlich Inst.Bundesamt Sera Impfstoffe Frankf A M.*, (94), 178-187 (2003).

39. Incorvaia C, Rapetti A, Aliani M *et al.* Food allergy as defined by component resolved diagnosis. *Recent Pat Inflamm.Allergy Drug Discov.*, 8(1), 59-73 (2014).

40. Valenta R, Hochwallner H, Linhart B, Pahr S. Food allergies: the basics. *Gastroenterology*, 148(6), 1120-1131.e1124 (2015).

41. Hompes S, Kohli A, Nemat K *et al.* Provoking allergens and treatment of anaphylaxis in children and adolescents - data from the anaphylaxis registry of German-speaking countries. *Pediatr.Allergy Immunol.*, (2011).

42. Sicherer SH. Epidemiology of food allergy. *J Allergy Clin Immunol*, 127(3), 594-602 (2011).

43. Nicolaou N, Murray C, Belgrave D, Poorafshar M, Simpson A, Custovic A. Quantification of specific IgE to whole peanut extract and peanut components in prediction of peanut allergy. *J.Allergy Clin.Immunol.*, 127(3), 684-685 (2011).

44. Beyer K, Grabenhenrich L, Hartl M *et al.* Predictive values of component-specific IgE for the outcome of peanut and hazelnut food challenges in children. *Allergy*, 70(1), 90-98 (2015).

45. Eller E, Bindslev-Jensen C. Clinical value of component-resolved diagnostics in peanut-allergic patients. *Allergy*, 68(2), 190-194 (2013).

46. Lieberman JA, Glaumann S, Batelson S, Borres MP, Sampson HA, Nilsson C. The utility of peanut components in the diagnosis of IgE-mediated peanut allergy among distinct populations. *J.Allergy Clin.Immunol.Pract.*, 1(1), 75-82 (2013).

47. Flinterman AE, van Hoffen E, den Hartog Jager CF *et al.* Children with peanut allergy recognize predominantly Ara h2 and Ara h6, which remains stable over time. *Clin Exp Allergy*, 37(8), 1221-1228 (2007).

48. Dang TD, Tang M, Choo S *et al.* Increasing the accuracy of peanut allergy diagnosis by using Ara h 2. *J Allergy Clin Immunol*, 129(4), 1056-1063 (2012).

49. Amin MR, Khoury JC, Assa'ad AH. Food-specific serum immunoglobulin E measurements in children presenting with food allergy. *Ann.Allergy Asthma Immunol.*, 112(2), 121-125 (2014).

50. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science*, 289(5485), 1760-1763 (2000).

51. Zhu H, Bilgin M, Bangham R *et al.* Global analysis of protein activities using proteome chips. *Science*, 293(5537), 2101-2105 (2001).

52. Ekins RP. Multi-analyte immunoassay. *Journal of pharmaceutical and biomedical analysis*, 7(2), 155-168 (1989).

53. Mari A, Scala E, Alessandri C. The IgE-microarray testing in atopic dermatitis: a suitable modern tool for the immunological and clinical phenotyping of the disease. *Curr.Opin.Allergy Clin.Immunol.*, 11(5), 438-444 (2011).

54. Tu S, Jiang HW, Liu CX, Zhou SM, Tao SC. Protein microarrays for studies of drug mechanisms and biomarker discovery in the era of systems biology. *Current pharmaceutical design*, 20(1), 49-55 (2014).

55.   Ayoglu B, Haggmark A, Neiman M *et al.* Systematic antibody and antigen-based proteomic profiling with microarrays. *Expert.Rev.Mol.Diagn.*, 11(2), 219-234 (2011).

56.   Hu B, Niu X, Cheng L *et al.* Discovering cancer biomarkers from clinical samples by protein microarrays. *Proteomics Clin Appl*, 9(1-2), 98-110 (2015).

57.   Goikoetxea MJ, Sanz ML, Garcia BE *et al.* Recommendations for the use of in vitro methods to detect specific immunoglobulin E: are they comparable? *J.Investig.Allergol.Clin.Immunol.*, 23(7), 448-454 (2013).

58.   Cabrera-Freitag P, Goikoetxea MJ, Beorlegui C *et al.* Can component-based microarray replace fluorescent enzimoimmunoassay in the diagnosis of grass and cypress pollen allergy? *Clin.Exp.Allergy*, 41(10), 1440-1446 (2011).

59.   Melioli G, Bonifazi F, Bonini S *et al.* The ImmunoCAP ISAC molecular allergology approach in adult multi-sensitized Italian patients with respiratory symptoms. *Clin.Biochem.*, 44(12), 1005-1011 (2011).

60.   Canonica GW, Ansotegui IJ, Pawankar R *et al.* A WAO - ARIA - GA(2)LEN consensus document on molecular-based allergy diagnostics. *The World Allergy Organization journal*, 6(1), 17 (2013).

61.   Cretich M, Di Carlo G, Longhi R *et al.* High sensitivity protein assays on microarray silicon slides. *Analytical Chemistry*, 81(13), 5197-5203 (2009).

62.   Pirri G, Damin F, Chiari M, Bontempi E, Depero LE. Characterization of a polymeric adsorbed coating for DNA microarray glass slides. *Anal Chem*, 76(5), 1352-1358 (2004).

63.   Cretich M, Breda D, Damin F *et al.* Allergen microarrays on high-sensitivity silicon slides. *Anal.Bioanal.Chem.*, 398(4), 1723-1733 (2010).

64.   Cretich M, Monroe MR, Reddington A *et al.* Interferometric silicon biochips for label and label-free DNA and protein microarrays. *Proteomics.*, 12(19-20), 2963-2977 (2012).

65.   Palosuo K. Update on wheat hypersensitivity. *Curr.Opin.Allergy Clin.Immunol.*, 3(3), 205-209 (2003).

66.   Pourpak Z, Mansouri M, Mesdaghi M, Kazemnejad A, Farhoudi A. Wheat allergy: clinical and laboratory findings. *Int.Arch.Allergy Immunol.*, 133(2), 168-173 (2004).

67.   Tatham AS, Shewry PR. Allergens to wheat and related cereals. *Clin.Exp.Allergy*, 38(11), 1712-1726 (2008).

68.   Zuidmeer L, Goldhahn K, Rona RJ *et al.* The prevalence of plant food allergies: a systematic review. *J Allergy Clin Immunol*, 121(5), 1210-1218.e1214 (2008).

69.   Venter C, Pereira B, Grundy J *et al.* Incidence of parentally reported and clinically diagnosed food hypersensitivity in the first year of life. *J.Allergy Clin.Immunol.*, 117(5), 1118-1124 (2006).

70.   Poole JA, Barriga K, Leung DY *et al.* Timing of initial exposure to cereal grains and the risk of wheat allergy. *Pediatrics*, 117(6), 2175-2182 (2006).

71.   Inomata N. Wheat allergy. *Curr Opin Allergy Clin Immunol*, 9(3), 238-243 (2009).

72.   Keet CA, Matsui EC, Dhillon G, Lenehan P, Paterakis M, Wood RA. The natural history of wheat allergy. *Ann.Allergy Asthma Immunol.*, 102(5), 410-415 (2009).

73.   Kotaniemi-Syrjanen A, Palosuo K, Jartti T, Kuitunen M, Pelkonen AS, Makela MJ. The prognosis of wheat hypersensitivity in children. *Pediatr.Allergy Immunol.*, 21(2 Pt 2), e421-e428 (2010).

74.   DuPont FM, Vensel WH, Tanaka CK, Hurkman WJ, Altenbach SB. Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional

electrophoresis, three proteases and tandem mass spectrometry. *Proteome.Sci.*, 9, 10 (2011).

75.   Pahr S, Constantin C, Mari A *et al.* Molecular characterization of wheat allergens specifically recognized by patients suffering from wheat-induced respiratory allergy. *Clin.Exp.Allergy*, 42(4), 597-609 (2012).

76.   Makela MJ, Eriksson C, Kotaniemi-Syrjanen A *et al.* Wheat allergy in children - new tools for diagnostics. *Clin Exp Allergy*, 44(11), 1420-1430 (2014).

77.   Armentia A, Rodriguez R, Callejo A *et al.* Allergy after ingestion or inhalation of cereals involves similar allergens in different ages. *Clin.Exp.Allergy*, 32(8), 1216-1222 (2002).

78.   Constantin C, Touraev A, Heberle-Bors E, Quirce S, Huber WD, Valenta R. Detection of antigens reactive to IgE and IgA during wheat seed maturation and in different wheat cultivars. *Int Arch.Allergy Immunol*, 149(3), 181-187 (2009).

79.   Larre C, Lupi R, Gombaud G *et al.* Assessment of allergenicity of diploid and hexaploid wheat genotypes: identification of allergens in the albumin/globulin fraction. *J.Proteomics.*, 74(8), 1279-1289 (2011).

80.   Sampson HA, Ho DG. Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J.Allergy Clin.Immunol.*, 100(4), 444-451 (1997).

81.   Sampson HA. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J.Allergy Clin.Immunol.*, 107(5), 891-896 (2001).

82.   Perry TT, Matsui EC, Kay Conover-Walker M, Wood RA. The relationship of allergen-specific IgE levels and oral food challenge outcome. *J.Allergy Clin.Immunol.*, 114(1), 144-149 (2004).

83.   Celik-Bilgili S, Mehl A, Verstege A *et al.* The predictive value of specific immunoglobulin E levels in serum for the outcome of oral food challenges. *Clin Exp Allergy*, 35(3), 268-273 (2005).

84.   Simonato B, De LF, Pasini G *et al.* IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion. *Clin.Exp.Allergy*, 31(11), 1771-1778 (2001).

85.   Sotkovsky P, Sklenar J, Halada P *et al.* A new approach to the isolation and characterization of wheat flour allergens. *Clin.Exp.Allergy*, 41(7), 1031-1043 (2011).

86.   Wan Y, Gritsch CS, Hawkesford MJ, Shewry PR. Effects of nitrogen nutrition on the synthesis and deposition of the omega-gliadins of wheat. *Annals of botany*, 113(4), 607-615 (2014).

87.   Jones SM, Magnolfi CF, Cooke SK, Sampson HA. Immunologic cross-reactivity among cereal grains and grasses in children with food hypersensitivity. *J Allergy Clin Immunol*, 96(3), 341-351 (1995).

88.   Matricardi PM, Bockelbrink A, Beyer K *et al.* Primary versus secondary immunoglobulin E sensitization to soy and wheat in the Multi-Centre Allergy Study cohort. *Clin.Exp.Allergy*, 38(3), 493-500 (2008).

89.   Sutton R, Hill DJ, Baldo BA, Wrigley CW. Immunoglobulin E antibodies to ingested cereal flour components: studies with sera from subjects with asthma and eczema. *Clin.Allergy*, 12(1), 63-74 (1982).

90.   Walsh BJ, Sutton R, Wrigley CW, Baldo BA. Allergen discs prepared from nitrocellulose: detection of IgE binding to soluble and insoluble allergens. *J.Immunol.Methods*, 73(1), 139-145 (1984).

91.   Constantin C, Huber WD, Granditsch G, Weghofer M, Valenta R. Different profiles of wheat antigens are recognised by patients suffering from coeliac disease and IgE-mediated food allergy. *Int Arch.Allergy Immunol*, 138(3), 257-266 (2005).

92.   Battais F, Courcoux P, Popineau Y, Kanny GI, Moneret-Vautrin DA, Denery-Papini S. Food allergy to wheat: differences in immunoglobulin E-binding proteins as a function of age or symptoms. *Journal of Cereal Science*, 42(1), 109-117 (2005).

93.   Battais F, Pineau F, Popineau Y *et al.* Food allergy to wheat: identification of immunogloglin E and immunoglobulin G-binding proteins with sequential extracts and purified proteins from wheat flour. *Clin.Exp.Allergy*, 33(7), 962-970 (2003).

94.   Shewry PR, Tatham AS, Forde J, Kreis M, Miflin BJ. The classification and nomenclature of wheat gluten proteins: A reassessment. *Journal of Cereal Science*, 4(2), 97-106 (1986).

95.   Tatham AS, Field JM, Smith SJ, Shewry PR. The conformations of wheat gluten proteins, II, aggregated gliadins and low molecular weight subunits of glutenin. *Journal of Cereal Science*, 5(3), 203-214 (1987).

96.   Shewry PR, Tatham AS. Disulphide Bonds in Wheat Gluten Proteins. *Journal of Cereal Science*, 25(3), 207-227 (1997).

97.   Shewry PR, Halford NG. Cereal seed storage proteins: structures, properties and role in grain utilization. *J.Exp.Bot.*, 53(370), 947-958 (2002).

98.   Shewry PR, Halford NG, Belton PS, Tatham AS. The structure and properties of gluten: an elastic protein from wheat grain. *Philos.Trans.R.Soc.Lond B Biol.Sci.*, 357(1418), 133-142 (2002).

99.   Tatham AS, Shewry PR. The S-poor prolamins of wheat, barley and rye: Revisited. *Journal of Cereal Science*, 55(2), 79-99 (2012).

100.   Sotkovsky P, Hubalek M, Hernychova L *et al.* Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. *Proteomics.*, 8(8), 1677-1691 (2008).

101.   Palosuo K, Varjonen E, Kekki OM *et al.* Wheat omega-5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *J.Allergy Clin.Immunol.*, 108(4), 634-638 (2001).

102.   Shibata R, Nishima S, Tanaka A, Borres MP, Morita E. Usefulness of specific IgE antibodies to omega-5 gliadin in the diagnosis and follow-up of Japanese children with wheat allergy. *Ann Allergy Asthma Immunol*, 107(4), 337-343 (2011).

103.   Nilsson N, Nilsson C, Hedlin G, Johansson SG, Borres MP, Nopp A. Combining analyses of basophil allergen threshold sensitivity, CD-sens, and IgE antibodies to hydrolyzed wheat, omega-5 gliadin and timothy grass enhances the prediction of wheat challenge outcome. *Int Arch Allergy Immunol*, 162(1), 50-57 (2013).

104.   Ebisawa M, Shibata R, Sato S, Borres MP, Ito K. Clinical utility of IgE antibodies to omega-5 gliadin in the diagnosis of wheat allergy: a pediatric multicenter challenge study. *Int Arch Allergy Immunol*, 158(1), 71-76 (2012).

105.   Beyer K, Chung D, Schulz G *et al.* The role of wheat omega-5 gliadin IgE antibodies as a diagnostic tool for wheat allergy in childhood. *J Allergy Clin Immunol*, 122(2), 419-421 (2008).

106.   Nilsson N, Sjolander S, Baar A *et al.* Wheat allergy in children evaluated with challenge and IgE antibodies to wheat components. *Pediatr Allergy Immunol*, 26(2), 119-125 (2015).

107. Ito K, Futamura M, Borres MP *et al.* IgE antibodies to omega-5 gliadin associate with immediate symptoms on oral wheat challenge in Japanese children. *Allergy*, 63(11), 1536-1542 (2008).

108. Sievers S, Rawel HM, Ringel KP, Niggemann B, Beyer K. Wheat protein recognition pattern in tolerant and allergic children. *Pediatr Allergy Immunol*, (2015).

109. Hofmann SC, Fischer J, Eriksson C, Bengtsson Gref O, Biedermann T, Jakob T. IgE detection to alpha/beta/gamma-gliadin and its clinical relevance in wheat-dependent exercise-induced anaphylaxis. *Allergy*, 67(11), 1457-1460 (2012).

110. Daengsuwan T, Palosuo K, Phankingthongkum S *et al.* IgE antibodies to omega-5 gliadin in children with wheat-induced anaphylaxis. *Allergy*, 60(4), 506-509 (2005).

111. Rona RJ, Keil T, Summers C *et al.* The prevalence of food allergy: a meta-analysis. *J.Allergy Clin.Immunol.*, 120(3), 638-646 (2007).

112. Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. *J.Allergy Clin.Immunol.*, 120(5), 1172-1177 (2007).

113. Wal JM. Bovine milk allergenicity. *Ann Allergy Asthma Immunol*, 93(5 Suppl 3), S2-11 (2004).

114. Chatchatee P, Jarvinen KM, Bardina L, Beyer K, Sampson HA. Identification of IgE- and IgG-binding epitopes on alpha(s1)-casein: differences in patients with persistent and transient cow's milk allergy. *J.Allergy Clin.Immunol.*, 107(2), 379-383 (2001).

115. Chatchatee P, Jarvinen KM, Bardina L, Vila L, Beyer K, Sampson HA. Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clin.Exp.Allergy*, 31(8), 1256-1262 (2001).

116. Jarvinen KM, Chatchatee P, Bardina L, Beyer K, Sampson HA. IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int Arch.Allergy Immunol*, 126(2), 111-118 (2001).

117. Borres MP, Ebisawa M, Eigenmann PA. Use of allergen components begins a new era in pediatric allergology. *Pediatr.Allergy Immunol.*, 22(5), 454-461 (2011).

118. Tsabouri S, Douros K, Priftis KN. Cow's milk allergenicity. *Endocrine, metabolic & immune disorders drug targets*, 14(1), 16-26 (2014).

119. Lisson M, Novak N, Erhardt G. Immunoglobulin E epitope mapping by microarray immunoassay reveals differences in immune response to genetic variants of caseins from different ruminant species. *J.Dairy Sci.*, 97(4), 1939-1954 (2014).

120. Hochwallner H, Schulmeister U, Swoboda I *et al.* Microarray and allergenic activity assessment of milk allergens. *Clin Exp Allergy*, 40(12), 1809-1818 (2010).

121. D'Urbano LE, Pellegrino K, Artesani MC *et al.* Performance of a component-based allergen-microarray in the diagnosis of cow's milk and hen's egg allergy. *Clin.Exp.Allergy*, 40(10), 1561-1570 (2010).

122. Ito K, Futamura M, Moverare R *et al.* The usefulness of casein-specific IgE and IgG4 antibodies in cow's milk allergic children. *Clin Mol Allergy*, 10(1), 1 (2012).

123. Garcia-Ara C, Pedrosa M, Belver MT, Martin-Munoz MF, Quirce S, Boyano-Martinez T. Efficacy and safety of oral desensitization in children with cow's milk allergy according to their serum specific IgE level. *Ann.Allergy Asthma Immunol.*, 110(4), 290-294 (2013).

124. Ahrens B, de Oliveira LC Lopes, Schulz G *et al.* The role of hen's egg-specific IgE, IgG and IgG4 in the diagnostic procedure of hen's egg allergy. *Allergy*, 65 (2010).

125. Chokshi NY, Sicherer SH. Molecular diagnosis of egg allergy: an update. *Expert review of molecular diagnostics*, 1-12 (2015).

126. Ott H, Baron JM, Heise R *et al.* Clinical usefulness of microarray-based IgE detection in children with suspected food allergy. *Allergy*, 63(11), 1521-1528 (2008).

127. Jarvinen KM, Beyer K, Vila L, Bardina L, Mishoe M, Sampson HA. Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy*, 62(7), 758-765 (2007).

128. Alessandri C, Zennaro D, Scala E *et al.* Ovomucoid (Gal d 1) specific IgE detected by microarray system predict tolerability to boiled hen's egg and an increased risk to progress to multiple environmental allergen sensitisation. *Clin Exp Allergy*, 42(3), 441-450 (2012).

129. Ahrens B, Lopes de Oliveira LC, Grabenhenrich L *et al.* Individual cow's milk allergens as prognostic markers for tolerance development? *Clin Exp Allergy*, 42(11), 1630-1637 (2012).

130. Sapan CV, Lundblad RL, Price NC. Colorimetric protein assay techniques. *Biotechnol Appl Biochem*, 29 (Pt 2), 99-108 (1999).

131. DuPont FM, Chan R, Lopez R, Vensel WH. Sequential extraction and quantitative recovery of gliadins, glutenins, and other proteins from small samples of wheat flour. *J Agric.Food Chem.*, 53(5), 1575-1584 (2005).

132. Larre C, Lupi R, Gombaud G *et al.* Assessment of allergenicity of diploid and hexaploid wheat genotypes: identification of allergens in the albumin/globulin fraction. *J Proteomics*, 74(8), 1279-1289 (2011).

133. Vlieg-Boerstra BJ, van de Weg WE, van der Heide S *et al.* Additional indications for the low allergenic properties of the apple cultivars Santana and Elise. *Plant foods for human nutrition (Dordrecht, Netherlands)*, 68(4), 391-395 (2013).

134. Riascos JJ, Weissinger AK, Weissinger SM, Burks AW. Hypoallergenic legume crops and food allergy: factors affecting feasibility and risk. *Journal of agricultural and food chemistry*, 58(1), 20-27 (2010).

135. Cretich M, Reddington A, Monroe M *et al.* Silicon biochips for dual label-free and fluorescence detection: Application to protein microarray development. *Biosens.Bioelectron.*, (2011).

136. Morita E, Matsuo H, Chinuki Y, Takahashi H, Dahlstrom J, Tanaka A. Food-dependent exercise-induced anaphylaxis -importance of omega-5 gliadin and HMW-glutenin as causative antigens for wheat-dependent exercise-induced anaphylaxis. *Allergol Int*, 58(4), 493-498 (2009).

137. Takahashi H, Matsuo H, Chinuki Y *et al.* Recombinant high molecular weight-glutenin subunit-specific IgE detection is useful in identifying wheat-dependent exercise-induced anaphylaxis complementary to recombinant omega-5 gliadin-specific IgE test. *Clin Exp Allergy*, 42(8), 1293-1298 (2012).

138. Baar A, Pahr S, Constantin C *et al.* The high molecular weight glutenin subunit Bx7 allergen from wheat contains repetitive IgE epitopes. *Allergy*, 69(10), 1316-1323 (2014).

139. Salcedo G, Quirce S, Diaz-Perales A. Wheat allergens associated with Baker's asthma. *J.Investig.Allergol.Clin.Immunol.*, 21(2), 81-92 (2011).

140. Sandiford CP, Tatham AS, Fido R *et al.* Identification of the major water/salt insoluble wheat proteins involved in cereal hypersensitivity. *Clin.Exp.Allergy*, 27(10), 1120-1129 (1997).

141. Baur X, Posch A. Characterized allergens causing bakers' asthma. *Allergy*, 53(6), 562-566 (1998).

142. Sander I, Flagge A, Merget R, Halder TM, Meyer HE, Baur X. Identification of wheat flour allergens by means of 2-dimensional immunoblotting. *J.Allergy Clin.Immunol.*, 107(5), 907-913 (2001).

143. Matsuo H, Kohno K, Morita E. Molecular cloning, recombinant expression and IgE-binding epitope of omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. *FEBS J.*, 272(17), 4431-4438 (2005).

144. Simonato B, Pasini G, Giannattasio M, Peruffo AD, De LF, Curioni A. Food allergy to wheat products: the effect of bread baking and in vitro digestion on wheat allergenic proteins. A study with bread dough, crumb, and crust. *J.Agric.Food Chem.*, 49(11), 5668-5673 (2001).

145. Malandain H. Transglutaminases: a meeting point for wheat allergy, celiac disease, and food safety. *Eur.Ann.Allergy Clin.Immunol.*, 37(10), 397-403 (2005).

146. Kumagai H, Suda A, Sakurai H *et al.* Improvement of digestibility, reduction in allergenicity, and induction of oral tolerance of wheat gliadin by deamidation. *Biosci.Biotechnol.Biochem.*, 71(4), 977-985 (2007).

147. Denery-Papini S, Bodinier M, Larre C *et al.* Allergy to deamidated gluten in patients tolerant to wheat: specific epitopes linked to deamidation. *Allergy*, 67(8), 1023-1032 (2012).

148. Turner PJ, Wong M, Varese N, Rolland JM, O'Hehir RE, Campbell DE. Tolerance to wheat in whole-grain cereal biscuit in wheat-allergic children. *J.Allergy Clin.Immunol.*, 131(3), 920-923 (2013).

149. Mittag D, Niggemann B, Sander I *et al.* Immunoglobulin E-reactivity of wheat-allergic subjects (baker's asthma, food allergy, wheat-dependent, exercise-induced anaphylaxis) to wheat protein fractions with different solubility and digestibility. *Mol Nutr Food Res*, 48(5), 380-389 (2004).

150. Jahn-Schmid B, Harwanegg C, Hiller R *et al.* Allergen microarray: comparison of microarray using recombinant allergens with conventional diagnostic methods to detect allergen-specific serum immunoglobulin E. *Clin Exp.Allergy*, 33(10), 1443-1449 (2003).

151. Monroe MR, Reddington AP, Collins AD *et al.* Multiplexed method to calibrate and quantitate fluorescence signal for allergen-specific IgE. *Anal Chem*, 83(24), 9485-9491 (2011).

152. Renault NK, Gaddipati SR, Wulfert F *et al.* Multiple protein extract microarray for profiling human food-specific immunoglobulins A, M, G and E. *J Immunol Methods*, 364(1-2), 21-32 (2011).

153. Wulfert F, Sanyasi G, Tongen L *et al.* Prediction of tolerance in children with IgE mediated cow's milk allergy by microarray profiling and chemometric approach. *J.Immunol.Methods*, 382(1-2), 48-57 (2012).

154. Wellhausen R, Seitz H. Facing current quantification challenges in protein microarrays. *J.Biomed.Biotechnol.*, 2012, 831347 (2012).

155. Hofmaier S, Hatzler L, Rohrbach A *et al.* "Default" versus "pre-atopic" IgG responses to foodborne and airborne pathogenesis-related group 10 protein molecules in birch-sensitized and nonatopic children. *J Allergy Clin Immunol*, (2014).

156. Vickery BP, Lin J, Kulis M *et al.* Peanut oral immunotherapy modifies IgE and IgG4 responses to major peanut allergens. *J.Allergy Clin.Immunol.*, 131(1), 128-134 (2013).

157. Martinez-Botas J, Cerecedo I, Zamora J *et al.* Mapping of the IgE and IgG4 sequential epitopes of ovomucoid with a peptide microarray immunoassay. *Int Arch Allergy Immunol*, 161(1), 11-20 (2013).

158. Cerecedo I, Zamora J, Shreffler WG *et al.* Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *J Allergy Clin Immunol*, 122(3), 589-594 (2008).

159. Yman L. Standardization of in vitro methods. *Allergy*, 56, 70-74 (2001).

160. Cretich M, Di Carlo G, Giudici C *et al.* Detection of allergen specific immunoglobulins by microarrays coupled to microfluidics. *Proteomics.*, 9(8), 2098-2107 (2009).

161. Chandra H, Reddy PJ, Srivastava S. Protein microarrays and novel detection platforms. *Expert.Rev.Proteomics.*, 8(1), 61-79 (2011).

162. Matsumoto N, Okochi M, Matsushima M *et al.* Peptide array-based analysis of the specific IgE and IgG4 in cow's milk allergens and its use in allergy evaluation. *Peptides*, 30(10), 1840-1847 (2009).

163. Lupinek C, Wollmann E, Baar A *et al.* Advances in allergen-microarray technology for diagnosis and monitoring of allergy: the MeDALL allergen-chip. 66(1), 106-119 (2014).

164. Gaudin JC, Rabesona H, Choiset Y *et al.* Assessment of the immunoglobulin E-mediated immune response to milk-specific proteins in allergic patients using microarrays. *Clin Exp.Allergy*, 38(4), 686-693 (2008).

165. Grainger DW, Greef CH, Gong P, Lochhead MJ. Current microarray surface chemistries. *Methods Mol.Biol.*, 381, 37-57 (2007).

166. Wu P, Castner DG, Grainger DW. Diagnostic devices as biomaterials: a review of nucleic acid and protein microarray surface performance issues. *Journal of biomaterials science. Polymer edition*, 19(6), 725-753 (2008).

167. Sun H, Chen GY, Yao SQ. Recent advances in microarray technologies for proteomics. *Chemistry & biology*, 20(5), 685-699 (2013).

168. Sutandy FX, Qian J, Chen CS, Zhu H. Overview of protein microarrays. *Curr.Protoc.Protein Sci.*, Chapter 27, Unit (2013).

169. Ito Y, Moritsugu N, Matsue T *et al.* An automated multiplex specific IgE assay system using a photoimmobilized microarray. *J.Biotechnol.*, 161(4), 414-421 (2012).

170. Sanz ML, Blazquez AB, Garcia BE. Microarray of allergenic component-based diagnosis in food allergy. *Curr.Opin.Allergy Clin.Immunol.*, 11(3), 204-209 (2011).

171. Mine Y, Rupa P. Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin. *Protein Eng*, 16(10), 747-752 (2003).

172. Martos G, Lopez-Exposito I, Bencharitiwong R, Berin MC, Nowak-Wegrzyn A. Mechanisms underlying differential food allergy response to heated egg. *J Allergy Clin Immunol*, 127(4), 990-997 e991-992 (2011).

173. Aalberse JA, Meijer Y, Derksen N, Palen-Merkus T, Knol E, Aalberse RC. Moving from peanut extract to peanut components: towards validation of component-resolved IgE tests. *Allergy*, 68(6), 748-756 (2013).

174. Kricka LJ, Master SR. Validation and quality control of protein microarray-based analytical methods. *Mol.Biotechnol.*, 38(1), 19-31 (2008).

175. EFSA. Outcome of a public consultation on the draft Scientific Opinion of the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) on the evaluation of allergenic foods and food ingredients for labelling purposes. In: *EFSA supporting publication 2014.* (Ed.^(Eds) (European Food Safety Authority, 2014) 202

9 Supplementary

9.1 Tables

Supplement Table 1: Wheat allergens as listed in the Allergome database and referred to by the World Health Organization and International Union of Immunological Societies (WHO/IUIS).

Tri a *Triticum aestivum* [access February 27, 2016]

Allergome				Uniprot		WHO/IUIS			
Name Allergome	Common Name	Tissue	Routes of exposure	No. of sequences	MW (kDa)	Name	Food allergen	Isoforms	MW in SDS-PAGE (kDa)
Tri a aA_SI	alpha-Amylase/Subtilisin Inhibitor	seed	ingestion	1	19.6	n/a	n/a	n/a	
Tri a alpha_Gliadin	alpha-Gliadin	seed	ingestion	25	30.0 - 36.0	n/a	n/a	n/a	
Tri a bA	beta-Amylase	seed	ingestion	1	56.6	n/a	n/a	n/a	
Tri a Bd36K	Peroxidase	seed	inhalation	1	8.4	n/a	n/a	n/a	
Tri a beta_Gliadin	beta-Gliadin	seed	ingestion	n/a	n/a	n/a	n/a	n/a	
Tri a Chitinase	Class 1 Chitinase	seed	ingestion	1	27.1	n/a	n/a	n/a	
Tri a CMX	alpha-Amylase Inhibitor, Trypsin Inhibitor	seed	ingestion	3	3.1, 13.8 - 13.9	n/a	n/a	n/a	
Tri a DH	Deyhdrin	seed	inhalation	1	21.8	n/a	n/a	n/a	
Tri a Endochitinase	Endochitinase	seed	ingestion	3	33.4 - 33.6	n/a	n/a	n/a	
Tri a GBSS_I	Starch Synthase	seed	ingestion	13	58.9 - 67.7	n/a	n/a	n/a	
Tri a Germin	Germin-like protein	seed	inhalation	n/a	n/a	n/a	n/a	n/a	
Tri a Gliadin	gamma/omega-Gliadin	seed	ingestion	1	43.9	n/a	n/a	n/a	
Tri a GST	Glutathione-S-transferase	seed	inhalation	1	25.0	n/a	n/a	n/a	
Tri a LMW-GS B16	Glutenin Subunit	seed	ingestion	n/a	n/a	n/a	n/a	n/a	
Tri a LMW-GS P42	Glutenin Subunit	seed	ingestion	n/a	n/a	n/a	n/a	n/a	
Tri a LMW-GS P73	Glutenin Subunit	seed	ingestion	n/a	n/a	n/a	n/a	n/a	
Tri a omega2_Gliadin	Omega2-Gliadin	seed	ingestion	n/a	n/a	n/a	n/a	n/a	
Tri a PAP	Purple Acid Phosphatase	seed	contact	1	37.9	n/a	n/a	n/a	
Tri a Peroxidase	Peroxidase	seed	ingestion	29	30.1 - 33.6	n/a	n/a	n/a	
Tri a Peroxidase 1	Peroxidase	seed	ingestion	1	38.8	n/a	n/a	n/a	
Tri a TLP	Thaumatin-like Protein	seed	ingestion	3	17.6	n/a	n/a	n/a	
Tri a Tritin	Ribosomal Inactivation Protein	seed	ingestion	1	29.6	n/a	n/a	n/a	
Tri a Trx	Thioredoxin	seed	inhalation	1	12.7	n/a	n/a	n/a	
Tri a XI	Xylanase Inhibitor	seed	ingestion	3	33.3	n/a	n/a	n/a	
Tri a 1	Expansin	pollen	inhalation	5	29.8 - 29.9	n/a	n/a	n/a	
Tri a 2	Group 2	pollen	inhalation	1	13.4	n/a	n/a	n/a	
Tri a 3	Group 3	pollen	inhalation	1	13.3	n/a	n/a	n/a	
Tri a 4	Group 4	pollen	inhalation	2	57.4	n/a	n/a	n/a	
Tri a 5	Group 5	pollen	inhalation	3	11.9, 18.6, 23.9	n/a	n/a	n/a	
Tri a 7	Polcalcin	pollen	inhalation	n/a	n/a	n/a	n/a	n/a	
Tri a 7k-LTP	Lipid Transfer Protein 2	seed	ingestion, inhalation	5	7.0 - 10.1	n/a	n/a	n/a	
Tri a 10kd	not given	seed	ingestion	1	10.4	n/a	n/a	n/a	
Tri a 12	Profilin	pollen, seed	ingestion, inhalation	6	14.2	Tri a 12	no	4	
Tri a 13	Polygalacturonase	pollen	inhalation	n/a	n/a	n/a	n/a	n/a	
Tri a 14	Lipid Transfer Proteins	seed	ingstion, inhalation	11	9.5 - 12.3	Tri a 14	yes	1	
Tri a 15	alpha-Amylase Inhibitor, wmai-0.28	seed	inhalation	8	13.1 - 16.8	Tri a 15	no	1	
Tri a 18	Agglutinin isolectin 1, Hevein-like, Lectin	seed	ingestion	3	18.7 - 21.3	Tri a 18	yes	1	
Tri a 19	Omega-5 gliadin	seed	ingestion	10	32.5 - 32.7, 38.4, 43.9, 53.0	Tri a 19	yes	1	65

Tri a 20	gamma-Gliadin	seed	ingestion	48	20.0, 25.8 - 37.2	Tri a 20	yes	1	35 to 38
Tri a 21	alpha/beta-Gliadin	seed	inhalation	1	32.6	Tri a 21	no	1	
Tri a 23kd	Leucine-rich Repeat Protein	seed	ingestion	1	15.2	n/a		n/a	
Tri a 25	Thioredoxin	seed	ingestion	1	13.3	Tri a 25	yes	1	
Tri a 26	HMW Glutenin	seed	ingestion	68	49.0, 54.0, 66 - 108	Tri a 26	yes	2	88
Tri a 27	Thiol Reductase	seed	inhalation	3	22.7 - 23.9	Tri a 27	no	1	27
Tri a 28	Dimeric alpha-Amylase Inhibitor 0.19	seed	inhalation	27	12.8 - 15.3	Tri a 28	no	2	13
Tri a 29	Tetrameric alpha-Amylase Inhibitor CM1/CM2	seed	inhalation	4	13.1 - 15.5	Tri a 29	no	2	13
Tri a 30	Trypsin Inhibitor CM3	seed	ingestion, inhalation	2	18.2	Tri a 30	no	1	16
Tri a 31	Triosephosphate Isomerase	seed	inhalation	1	26.8	Tri a 31	no	1	
Tri a 32	Peroxiredoxine	seed	inhalation	2	23.9	Tri a 32	no	1	
Tri a 33	Serpin	seed	ingestion, inhalation	7	42.9 - 43.3	Tri a 33	no	1	
Tri a 34	GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	seed	inhalation	1	36.5	Tri a 34	no	1	
Tri a 35	Dehydrin	seed	inhalation	2	11.5	Tri a 35	no	1	
Tri a 36	LMW Glutenin GluB3-23	seed	ingestion	156	31.7 - 45.1	Tri a 36	yes	1	40
Tri a 37	Alpha purothionin	seed	ingestion	9	9.5 - 14.7	Tri a 37	yes	1	12
Tri a 39	Serine Protease Inhibitor	seed	inhalation	3	9.3 - 9.5	Tri a 39	no	1	
Tri a 40	alpha-Amylase Inhibitor, Trypsin Inhibitor (CM17)	seed	ingestion, inhalation	4	15.6 - 15.9	Tri a 40	yes	1	
Tri a 41	Mitochondrial Ubiquitin Ligase Activator of NFKB 1-like Protein	seed	ingestion	3	38.4 - 41.6, 6.9	Tri a 41	yes	1	
Tri a 42	Group 42	seed	ingestion	3	17.4 - 17.9, 8.3	Tri a 42	yes	1	
Tri a 43	Group 43	seed	ingestion	2	11.7, 16.3	Tri a 43	yes	1	
Tri a 44	Endosperm Transfer Cell Specific PR60	seed	ingestion	23	7.5 - 11.9	Tri a 44	yes	1	
Tri a 45	Elongation Factor	seed	ingestion	6	7.8 - 11.8	Tri a 45	yes	2	

Supplement Table 2: Characteristics of WT children. The dashed line indicates non-sensitized (<0.35 kU$_A$/l) and sensitized patients (>0.35 kU$_A$/l).

Pat.	Sex	Wheat-specific IgE (kU/l)	Age at blood donation (months)	AD	Asthma	Reaction to albumins and globulins	Reaction to glutenins and gliadins
1	female	<0.35	38	yes	no	-	-
2	female	<0.35	13	yes	no	-	-
3	male	<0.35	14	yes	no	-	-
4	male	<0.35	12	yes	no	-	-
5	male	<0.35	8	yes	no	-	-
6	male	<0.35	71	yes	no	-	-
7	male	<0.35	15	yes	no	-	-
8	male	<0.35	6	no	no	-	-
9	male	<0.35	15	no	no	-	-
10	male	<0.35	25	yes	no	-	-
11	female	<0.35	6	no	no	-	-
12	male	<0.35	51	no	no	-	-
13	male	<0.35	24	no	no	-	-
14	female	<0.35	13	yes	no	-	-
15	male	<0.35	42	yes	no	+	-
16	female	<0.35	31	yes	no	-	-
17	male	<0.35	173	yes	yes	-	-
18	male	<0.35	27	no	no	-	-
19	female	<0.35	53	yes	yes	-	-
20	male	<0.35	42	yes	no	-	-
21	female	<0.35	27	yes	no	-	-
22	female	<0.35	26	yes	no	-	-
23	male	<0.35	28	yes	no	-	-
24	male	0.41	9	yes	no	-	-
25	male	0.42	11	yes	no	+	+
26	male	0.45	57	yes	yes	+	-
27	female	0.50	9	yes	no	-	-
28	male	0.53	13	yes	no	-	-
29	female	0.61	12	yes	no	+	+
30	male	0.64	7	yes	no	+	-
31	male	0.66	9	yes	no	+	+
32	female	0.72	130	yes	yes	-	-
33	male	0.76	17	yes	no	+	-
34	male	0.79	50	yes	no	-	-
35	female	0.95	12	yes	no	-	-
36	male	0.97	11	yes	no	-	-
37	male	1.01	103	yes	yes	-	-
38	female	1.37	117	no	yes	+	-
39	female	1.53	10	yes	no	+	+
40	male	1.54	188	yes	no	-	-
41	male	1.92	13	yes	no	+	+
42	male	1.98	9	yes	no	+	-
43	female	2.04	54	yes	yes	-	-
44	male	2.05	5	yes	no	+	-
45	male	2.06	8	yes	no	+	+
46	male	2.06	55	yes	no	+	-
47	female	2.52	6	yes	no	+	+
48	female	2.66	53	yes	no	+	-
49	male	3.99	13	yes	no	-	-
50	male	5.31	8	yes	no	+	+
51	male	5.57	10	yes	no	+	+
52	female	6.32	7	yes	no	+	+
53	male	6.33	15	yes	no	+	-
54	male	6.62	5	yes	no	-	-
55	male	6.72	8	yes	no	+	-
56	male	7.41	95	yes	yes	+	-
57	male	8.20	86	yes	yes	+	+
58	female	8.74	13	yes	no	+	+
59	female	10.60	37	yes	yes	+	-
60	female	12.00	114	yes	yes	+	+
61	male	17.00	20	yes	no	-	-
62	male	90.80	60	yes	no	+	+

Supplement Table 3: Characteristics of WA children. The dashed line indicates non-sensitized (<0.35 kU$_A$/l) and sensitized patients (>0.35 kU$_A$/l).

Pat.	Sex	Wheat-specific IgE (kU/l)	Age at blood donation (months)	AD	Asthma	Reaction to albumins and globulins	Reaction to glutenins and gliadins	Clinical reaction
63	female	<0.35	16	yes	no	-	-	generalized erythema, scratching
64	female	<0.35	3	yes	no	-	-	vomiting, diarrhea
65	female	<0.35	14	yes	no	-	-	worsening of eczema
66	male	<0.35	102	no	no	-	-	diarrhea
67	male	<0.35	4	yes	no	-	-	urticaria
68	female	<0.35	37	yes	no	-	-	worsening of eczema
69	female	0.47	6	yes	no	-	-	urticaria, erythema
70	female	0.53	22	yes	no	-	-	generalized erythema, scratching
71	male	0.65	6	yes	no	-	+	worsening of eczema
72	male	0.97	8	yes	no	+	+	urticaria
73	male	1.17	6	yes	no	+	+	worsening of eczema
74	female	1.38	53	yes	no	-	-	urticaria, worsening of eczema
75	male	1.39	7	yes	no	+	+	urticaria, worsening of eczema
76	female	2.15	192	no	no	+	+	vomiting, diarrhea
77	male	2.36	11	yes	no	-	+	worsening of eczema
78	female	2.64	9	yes	no	+	+	erythema, rhinitis, worsening of eczema
79	male	3.10	6	yes	no	+	+	urticaria
80	female	4.26	119	yes	no	+	+	diarrhea, worsening of eczema
81	female	4.27	9	yes	no	+	+	urticaria, rhinitis
82	female	5.63	7	yes	no	+	+	urticaria
83	male	5.91	35	yes	yes	+	-	worsening of eczema
84	female	6.47	102	yes	yes	+	-	rhinitis
85	male	6.94	5	yes	no	+	+	urticaria, vomiting
86	male	7.39	4	yes	no	+	+	urticaria, wheezing
87	male	7.83	8	yes	no	+	+	urticaria, worsening of eczema
88	male	9.63	46	yes	no	+	+	worsening of eczema
89	male	9.70	9	yes	no	+	+	urticaria
90	male	10.5	8	yes	no	-	-	urticaria
91	female	11.20	9	yes	no	+	+	urticaria, angioedema
92	male	14.90	9	yes	no	+	+	urticaria
93	female	16.00	5	yes	no	+	-	generalized erythema, scratching
94	male	16.00	6	yes	no	+	+	generalized erythema, scratching
95	male	18.60	7	yes	no	+	+	urticaria
96	male	35.30	6	yes	no	+	+	urticaria
97	male	35.60	8	yes	no	+	+	urticaria, rhinitis
98	female	37.30	8	yes	no	+	+	vomiting
99	male	37.60	5	yes	no	+	+	urticaria, vomiting
100	female	43.10	5	yes	yes	+	+	vomiting
101	male	61.40	10	yes	no	+	+	generalized erythema, scratching
102	male	>100	11	yes	no	+	+	urticaria, worsening of eczema
103	male	>100	18	yes	no	+	+	vomiting
104	male	229.00	15	yes	no	+	+	vomiting
105	male	394.00	43	yes	no	+	+	urticaria, vomiting
106	male	539.00	37	yes	no	+	+	urticaria

Supplement Table 4: Absolute and relative positive reactions of WT children's IgE with water/salt soluble and insoluble proteins in immunoblot assays according to rel. molecular weight ranges. The total number patients was n=62, patients with sIgE>0.35 kU$_A$/l were n=39, positive in IB for water/salt soluble albumins/globulins were n= 27, for water insoluble gliadins/glutenins n= 14. The dashed line indicates non-sensitized (<0.35 kU$_A$/l) and sensitized patients (>0.35 kU$_A$/l).

Pat.	≤10	10-15	15-25	25-35	35-50	>50		<37	~37-65	~65-100	>100	kDa
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15						x						
16												
17												
18												
19												
20												
21												
22												
23												
---	---	---	---	---	---	---	---	---	---	---	---	---
24												
25					x				x			
26						x						
27												
28												
29					x				x			
30					x							
31					x				x			
32												
33			x									
34												
35												
36												
37												
38			x									
39					x				x			
40												
41					x	x			x	x		
42												
43												
44			x									
45			x		x	x			x	x	x	
46		x			x	x		x				
47					x	x			x	x		
48		x			x							
49												
50					x				x			
51					x				x	x		
52					x	x			x	x		
53	x											
54												
55		x		x	x							
56		x				x						
57												
58					x				x			
59		x			x	x						
60		x			x	x		x		x		
61					x							
62					x				x	x		
SUM abs.	1	6	4	1	19	10		2	12	7	1	**n**
% of total	1.6	9.7	6.5	1.6	30.6	16.1		3.2	19.4	11.3	2.0	**62**
% of >0.35	0.0	7.7	25.6	2.6	48.7	20.5		5.1	30.8	17.9	2.6	**39**
% of pos	3.7	22.2	14.8	3.7	70.4	37.0		14.3	85.7	50.0	7.1	**27 | 14**

Supplement Table 5: Absolute and relative positive reactions of WA children's IgE with water/salt soluble and insoluble proteins in immunoblot assays according to rel. molecular weight ranges. The total number of patients were n=44, patients with sIgE>0.35 kU$_A$/l were n=38, positive in the IB for water and salt soluble albumins/globulins were n= 32, for water insoluble gliadins/globulins n= 31. The dashed line indicates non-sensitized (<0.35 kU$_A$/l) and sensitized patients (>0.35 kU$_A$/l).

Pat.	Water and salt soluble proteins						Water insoluble proteins				kDa
	<10	10-15	15-25	25-35	35-50	>50	<37	~37-65	~65-100	>100	
63											
64											
65											
66											
67											
68											
- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -
69											
70											
71								x			
72				x	x	x		x			
73					x			x			
74											
75				x	x	x		x		x	
76		x			x	x	x				
77										x	
78				x	x	x		x	x		
79				x	x	x		x	x		
80								x			
81					x			x			
82				x	x	x		x	x		
83			x	x	x	x					
84		x			x	x					
85					x			x	x		
86								x			
87	x		x	x	x			x	x		
88		x						x			
89					x	x		x	x		
90											
91					x	x		x	x		
92					x	x		x	x		
93			x		x						
94					x	x		x	x		
95					x	x		x	x		
96		x			x			x	x		
97					x	x		x	x		
98			x		x		x	x	x		
99				x	x	x		x	x		
100		x		x	x	x		x	x		
101				x	x	x		x	x		
102				x	x	x		x	x		
103	x				x	x		x	x		
104					x	x		x	x		
105	x	x	x	x	x			x		x	
106	x	x	x	x	x	x	x	x	x		
SUM abs.	4	7	6	13	29	21	3	29	20	3	**n**
% of total	9.1	15.9	13.6	29.5	65.9	47.7	6.8	65.9	45.5	6.8	**44**
% of >0.35	2.6	18.4	23.7	5.3	73.7	52.6	7.9	76.3	52.6	7.9	**38**
% of pos	12.5	21.9	18.8	40.6	90.6	65.6	9.7	93.5	64.5	9.7	**32 | 31**

Supplement Table 6: Absolute and relative positive reactions of WT children's IgE with selected and identified water / salt soluble protein bands <25 kDa in immunoblot assays. IB, immunoblot

Pat ID	35	34	33	32	31	30	29	
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								
26								
27								
28								
29								
30								
31								
32								
33						x		
34								
35								
36								
37								
38						x		
39								
40								
41								
42								
43								
44								
45						x		
46					x			
47								
48					x			
49								
50								
51								
52								
53		x						
54								
55					x			
56				x	x			
57								
58								
59					x			
60					x			
61								
62								
SUM abs.	0	1	0	1	6	3	0	**n**
% of total WT	0.0	1.6	0.0	1.6	9.7	4.8	0.0	**62**
% of pos in IB	0.0	2.6	0.0	2.6	15.4	7.7	0.0	**39**
% of pos<25kDa	0.0	10.0	0.0	10.0	60.0	30.0	0.0	**10**

Supplement Table 7: Absolute and relative positive reactions of WA children's IgE with selected and identified water / salt soluble protein bands <25 kDa in immunoblot assays. IB, immunoblot

Pat ID	35	34	33	32	31	30	29	
63								
64								
65								
66								
67								
68								
69								
70								
71								
72								
73								
74								
75								
76					x			
77								
78								
79								
80					x			
81								
82								
83						x		
84					x			
85								
86								
87		x					x	
88			x	x				
89								
90								
91								
92								
93							x	
94								
95								
96			x	x				
97								
98							x	
99								
100								
101								
102								
103		x						
104								
105		x	x	x				
106	x	x			x		x	
SUM abs.	1	4	3	3	4	1	4	n
% of total WA	2.3	9.1	6.8	6.8	9.1	2.3	9.1	44
% of pos in IB	2.6	10.5	7.9	7.9	10.5	2.6	10.5	38
% of pos<25kDa (n=12)	8.3	33.3	25.0	25.0	33.3	8.3	33.3	12

Supplement Table 8: Absolute and relative positive reactions of WT patients' IgE with selected and identified water / salt soluble proteins >25 kDa in immunoblot assays. Calculated percentages refer to WT patients sera that we re-assessed in the second screen (n=13), to the number of positively reacting sera (n=12) and to the total of WT sera (n=62).

Pat ID	28	26/27	25	22	19/20/21	18	17	16	14/15	13	11/12	10	8/9	7	6	3	2	1	
15															x				
26																		x	
41																			
46					x	x	x	x			x					x			
47		x																	
48											x							x	
50											x					x			
51																			
52											x								
56			x		x		x		x		x				x		x		
59		x	x		x		x	x			x					x	x	x	
60		x	x		x		x	x			x					x	x	x	
62								x											
SUM abs.	0	3	3	0	4	1	4	4	1	0	7	0	0	0	2	4	3	4	**n**
% of total in 2nd IB	0.0	23.1	23.1	0.0	30.8	7.7	30.8	30.8	7.7	0.0	53.8	0.0	0.0	0.0	15.4	30.8	23.1	30.8	**13**
% of pos in 2nd IB	0.0	25.0	25.0	0.0	33.3	8.3	33.3	33.3	8.3	0.0	58.3	0.0	0.0	0.0	16.7	33.3	25.0	33.3	**12**
% of total WT	0.0	4.8	4.8	0.0	6.5	1.6	6.5	6.5	1.6	0.0	11.3	0.0	0.0	0.0	3.2	6.5	4.8	6.5	**62**

Supplement Table 9: Absolute and relative positive reactions of WA patients' IgE with selected and identified water / salt soluble proteins >25 kDa in immunoblot assays. Calculated percentages refer to WA patients sera that we re-assessed in the second screen (n=25), to the number of positively reacting sera (n=22) and to the total of WT sera (n=44).

Pat ID	28	26/27	25	22	19/20/21	18	17	16	14/15	13	11/12	10	8/9	7	6	3	2	1	
73																			
75																			
76			x		x	x	x	x			x					x	x	x	
79											x								
82																			
83			x				x		x		x				x		x	x	
84					x	x	x	x			x					x	x	x	
85							x	x			x		x	x					
87	x	x																	
89							x	x			x		x						
90																			
91											x		x						
92											x		x						
94							x		x		x	x		x					
95							x			x		x		x					
96																x			
97											x								
98				x															
99							x	x			x		x						
100																			
101							x	x	x		x		x		x	x			
102							x	x			x		x						
103																			
104	x	x							x	x	x								
106			x							x	x						x	x	
SUM abs.	2	2	3	1	2	2	8	9	4	3	15	2	7	3	2	4	4	4	**n**
% of total in 2nd IB	8.0	8.0	12.0	4.0	8.0	8.0	32.0	36.0	16.0	12.0	60.0	8.0	28.0	12.0	8.0	16.0	16.0	16.0	**25**
% of pos in 2nd IB	9.1	9.1	13.6	4.5	9.1	9.1	36.4	40.9	18.2	13.6	68.2	9.1	31.8	13.6	9.1	18.2	18.2	18.2	**22**
% of total WA	4.5	4.5	6.8	2.3	4.5	4.5	18.2	20.5	9.1	6.8	34.1	4.5	15.9	6.8	4.5	9.1	9.1	9.1	**44**

Supplement Table 10: Absolute and relative positive reactions of WT patients' IgE with water insoluble proteins >35 kDa in immunoblot assays. Calculated percentages refer to WT patients sera that we re-assessed in the second screen (n=7), to the number of positively reacting sera (n=7) and to the total of WT sera (n=62). x?, not clear and neither counted positive or negative

Pat ID	1	2	3	4	5	6	7	8	9/10	11/12	13	14	15	16	17	18	19	20	21	22	
41											x	x	x	x	x		x?			x	
47													x	x	x	x	x?		x	x	
50											x		x	x	x	x			x	x	
51													x		x	x	x?		x	x	
52							x				x		x	x	x	x	x	x?	x	x	
58																	x		x	x	
62																	x		x	x	
SUM abs.	0	0	0	0	0	0	1	0	0	0	3	1	5	4	5	4	3	0	6	7	n
% of total in 2nd IB	0.0	0.0	0.0	0.0	0.0	0.0	14.3	0.0	0.0	0.0	42.9	14.3	71.4	57.1	71.4	57.1	42.9	0.0	85.7	100.0	7
% of pos in 2nd IB	0.0	0.0	0.0	0.0	0.0	0.0	14.3	0.0	0.0	0.0	42.9	14.3	71.4	57.1	71.4	57.1	42.9	0.0	85.7	100.0	7
% of total >0.35 kU$_A$/l	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	0.0	7.7	2.6	12.8	10.3	12.8	10.3	7.7	0.0	15.4	17.9	39
% of total WT	0.0	0.0	0.0	0.0	0.0	0.0	1.6	0.0	0.0	0.0	4.8	1.6	8.1	6.5	8.1	6.5	4.8	0.0	9.7	11.3	62

Supplement Table 11: Absolute and relative positive reactions of WA patients' IgE with water insoluble proteins >35 kDa in immunoblot assays. Calculated percentages refer to WA patients sera that we re-assessed in the second screen (n=25), to the number of positively reacting sera (n=25) and to the total of WT sera (n=44). x?, not clear and neither counted positive or negative

Pat ID	1	2	3	4	5	6	7	8	9/10	11/12	13	14	15	16	17	18	19	20	21	22	
73																					
75	x	x	x								x	x	x	x	x	x	x		x	x	
78											x	x	x	x		x					
79													x	x	x	x	x	x	x	x	
80	x	x	x	x	x						x	x					x	x	x	x	
82													x	x	x	x	x		x	x	
85										x	x	x	x		x	x	x		x	x	
86	x	x	x								x	x	x								
87											x	x	x		x	x	x		x	x	
88												x	x		x	x				x	
89						x	x			x			x	x	x	x	x	x	x	x	
91										x			x	x	x	x	x	x	x	x	
92										x	x		x	x	x	x	x	x	x	x	
94									x	x	x	x	x	x	x	x	x	x	x	x	
95										x	x		x	x	x	x	x	x	x	x	
96																					
97							x			x	x		x		x	x	x	x	x	x	
98											x	x	x	x	x	x			x	x	
99							x		x	x			x	x	x	x	x?	x	x	x	
100												x	x	x	x	x	x		x	x	
101							x		x	x			x	x	x	x	x		x	x	
102										x	x		x	x	x	x	x		x	x	
103																x	x	x			
104													x		x	x	x	x		x	
106							x				x						x	x			
SUM abs.	3	3	3	1	1	1	5	0	3	10	13	10	20	15	18	20	19	12	17	19	**n**
% of total in 2nd IB	12.0	12.0	12.0	4.0	4.0	4.0	20.0	0.0	12.0	40.0	52.0	40.0	80.0	60.0	72.0	80.0	76.0	48.0	68.0	76.0	**25**
% of pos in 2nd IB	12.0	12.0	12.0	4.0	4.0	4.0	20.0	0.0	12.0	40.0	52.0	40.0	80.0	60.0	72.0	80.0	76.0	48.0	68.0	76.0	**25**
% of total >0.35 kU$_A$/l	7.9	7.9	7.9	2.6	2.6	2.6	13.2	0.0	7.9	26.3	34.2	26.3	52.6	39.5	47.4	52.6	50.0	31.6	44.7	50.0	**38**
% of total WA	6.8	6.8	6.8	2.3	2.3	2.3	11.4	0.0	6.8	22.7	29.5	22.7	45.5	34.1	40.9	45.5	43.2	27.3	38.6	43.2	**44**

Supplement Table 12: Identified and calculated proteins of the albumin/globulin wheat fraction. *m/z* spectra of digested protein bands, shown in **Figure 14** and **Figure 17**, were submitted to Swissprot "Other green plants" database via Mascot interface. Hits are reported with score and sequence coverage (SC). Significant scores are marked red. Retrieved proteins with high but no significant score were assessed by *in silico* digestion from sequences of the Uniprot database, referred to by Allergome database entries. Probable proteins, calculated by *in silico* digestion, do not have a Mascot score. For identified and probable proteins, relative molecular weights with signal peptide (w SP) and without (mature form) are shown. par, partials; WHO/IUIS, World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee.)* ß-Amylase (Hor v 17) is listed as food allergen, but no Uniprot sequence is referred to. Band IDs without any result are not listed.

Band ID	Uniprot entry	Mascot Score	SC (%)	ppm	par	w SP MW (kDa)	mature MW (kDa)	Remarks	Name	Allergome entry	routes of exposure	WHO/IUIS food allergen	confirmed
3	AMYB_HORVU	66	14.6	50	0	59.6	n/a		ß-Amylase	Hor v 17	ingestion	(yes)*	yes
4	AMYB_HORVU	139	29.0	50	0	59.6	n/a		ß-Amylase	Hor v 17	ingestion	(yes)*	yes
	AMYB_HORVS	119	24.9	50	0	59.6	55.2		ß-Amylase	Hor v 17	ingestion	(yes)*	
	AMYB_WHEAT	31	9.9	50	0	56.6	n/a	100% peptide	ß-Amylase	Tri a bA	ingestion		
5	AMYB_HORVU	50	14.8	50	0	59.6	n/a		ß-Amylase	Hor v 17	ingestion	(yes)*	yes
		-	14.8	50	0								
	AMYB_HORVS	51	14.8	50	0	59.6	55.2		ß-Amylase	Hor v 17	ingestion	(yes)*	
		-	16.2	50	0								
6	AMYB_HORVU	52	17.9	50	0	59.6	n/a		ß-Amylase	Hor v 17	ingestion	(yes)*	yes
		-	20.9	100	2								
7	SPZ2B_WHEAT	84	38.9	100	0	43.0	n/a		Serpin-Z2B	Tri a 33	ingestion, inhalataion	no	yes
	SPZ1B_WHEAT	81	34.8	100	0	43.0	n/a		Serpin-Z1B	Tri a 34	ingestion, inhalataion		yes
	SPZ2A_WHEAT	66	30.9	100	0	43.3	n/a		Serpin-Z2A	Tri a 35	ingestion, inhalataion		yes
9													
10	G3PC1_HORVU	89	29.1	50	0	36.5	n/a		Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic				
	C7C4X1_WHEAT	-	37.1	50	1	36.5	n/a		Glyceraldehyde-3-phosphate dehydrogenase	Tri a 34	inhalation	no	
13	ALDR_HORVU	43	38.1	50	1	35.8	n/a		Aldose reductase				
		-	38.1	50	1								
14	GAO1A_WHEAT	69	39.7	100	1	40.7	n/a		Gibberellin 20 oxidase 1-A				
	GAO1D_WHEAT	57	33.8	100	1	40.3	n/a		Gibberellin 20 oxidase 1-D				
15	GAO1A_WHEAT	60	38.1	100	1	40.7	n/a		Gibberellin 20 oxidase 1-A				
18	CO410_WHEAT	44	42.0	100	2	28.2	n/a		Dehydrin COR410				
		-	34.4	50	1								
20	CHIA_SECCE	51	29.3	50	1	33.6	31.8		Basic endochitinase A				
		-	31.1	50	1								
	CHI1_HORVU	48	26.1	50	1	33.4	31.4		26 kDa endochitinase 1				yes
		-	27.8	50	1								
21	XIP1_WHEAT	61	22.0	50	0	33.3	30.3		Xylanase inhibitor protein 1 [Class III chitinase homolog]	Tri a XI	ingestion	n/a	yes
	CHIA_SECCE	55	26.8	50	0	33.6	31.8	100% peptide	Basic endochitinase A				
		-	24.7	50	1			100% peptide					
	CHI1_HORVU	50	23.6	50	0	33.4	31.4	100% peptide	26 kDa endochitinase 1				yes
		-	39.1	50	1			100% peptide					
22	XIP1_WHEAT	146	43.1	50	1	33.3	30.3	100% peptide	Xylanase inhibitor protein 1	Tri a XI	ingestion	n/a	yes

No	Protein								Description	Allergen	Route		
									[Class III chitinase homolog]				
24	TPIS_HORVU	68	33.6	50	0	26.7	26.6		Triosephosphate isomerase, cytosolic				
	TPIS_SECCE	68	33.6	50	0	26.9	26.8		Triosephosphate isomerase, cytosolic				
	Q9FS79_WHEAT	-	33.6	50	0	26.8	n/a		Triosephosphate isomerase	Tri a 31	inhalation	no	
25	L0GED8_WHEAT	-	27.6	100	2	29.3	n/a	100% peptide	14-3-3 protein				
26	CHIC_SECCE	58	37.2	50	0	28.3	26.1	100% peptide	Basic endochitinase C [Rye seed chitinase-c]		ingestion		
	CHI2_HORVU	101	42.1	50	1	28.2	25.9	100% peptide	26 kDa endochitinase 2 [CHI-26]		ingestion		
27	1433B_HORVU	41	49.2	100	3	29.7	n/a		14-3-3-like protein B				
		-	34.4	100	2								
28	Q8S4P7_WHEAT	-	32.9	100	0	23.6	23.6	100% peptide	Thaumatin-like protein				yes
29	AVLA1_WHEAT	61	48.2	50	0	18.4	16.4		Avenin-like a1				
	WHW1_WHEAT	-	32.0	50	0	15.6	13.7	100% peptide	Wheatwin-1 [Pathogenesis-related protein 4a]				yes
30	LEA1_HORVU	53	25.8	100	2	21.8	-		ABA-inducible protein PHV A1				
31	IAAC3_WHEAT	162	88.1	50	0	18.2	15.8		α-Amylase/trypsin inhibitor CM3	Tri a 30	ingestion, inhalataion	no	yes
32	IAA1_WHEAT	105	58.9	50	0	13.3	-		α-Amylase inhibitor 0.19 / 0.53	Tri a 28	inhalation	no	yes
33	IAA2_WHEAT	113	56.9	50	1	16.8	13.3		α-Amylase inhibitor 0.28 [WMAI-0.28]	Tri a 15	inhalation	no	yes
34	NLTP1_WHEAT	69	63.7	50	1	11.9	9.5		Non-specific lipid-transfer protein (9k-LTP)	Tri a 14	ingestion, inhalataion	yes	yes
35	NLT2P_WHEAT	72	92.5	50	0	7.0	-		Non-specific lipid-transfer protein 2 (nsLTP2)	Tri a 7k-LTP	ingestion, inhalataion	n/a	

Supplement Table 13: Identified and calculated proteins of the gliadin/glutenin wheat fraction. *m/z* spectra of digested protein bands, shown in **Figure 14** and **Figure 18**, were submitted to Swissprot "Other green plants" database via Mascot interface. Hits are reported with score and sequence coverage (SC). Significant scores are marked red. Retrieved proteins with high but no significant score were assessed by *in silico* digestion from sequences of the Uniprot database, referred to by Allergome database entries. Probable proteins, calculated by *in silico* digestion, do not have a Mascot score. For identified and probable proteins, relative molecular weights with signal peptide (w SP) and without (mature form) are shown. par, partials; WHO/IUIS, World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. Band IDs without any result are not listed.

Band ID	Uniprot entry	Score	Mascot SC (%)	ppm	par	w SP MW (kDa)	mature MW (kDa)	Remarks	Name	Allergome entry	routes of exposure	WHO/IUIS food allergen
1	GLT5_WHEAT	55	9.1	30	0	90.2	88.1	100% peptide	Glutenin, high molecular weight subunit DX5	Tri a 26	ingestion	yes
		-	11.1	50	1			100% peptide	Glutenin, high molecular weight subunit DX5			
	GLT4_WHEAT	37	6	30	0	89.1	86.9	100% peptide	Glutenin, high molecular weight subunit PW212	Tri a 26	ingestion	yes
		-	8	50	1			100% peptide	Glutenin, high molecular weight subunit PW212			
2	D1MJA1_9POAL	-	27.7	50	0	91.4	89.3	100% peptide	High molecular weight glutenin subunit Ax-dp			
3	Q45R38_WHEAT	-	22.9	50	0	85.2	83.1		HMW glutenin x-type subunit Bx7	Tri a 26	ingestion	yes
	Q18MZ6_WHEAT		20.2	50	0	80	77.9		High-molecular-weight glutenin subunit Bx17	Tri a 26	ingestion	yes
4	GLT0_WHEAT	62	19.1	30	0	69.6	67.4		Glutenin, high molecular weight subunit DY10	Tri a 26	ingestion	yes
	Q03871_WHEAT	-	23.1	50	0	75.6	73.5	100% peptide	HMW glutenin subunit 1By9	Tri a 26	ingestion	yes
5	GLT0_WHEAT	113	24.5	100	0	69.6	67.4		Glutenin, high molecular weight subunit DY10	Tri a 26	ingestion	yes
	GLT3_WHEAT	61	11.8	100	0	70.8	68.7		Glutenin, high molecular weight subunit 12	Tri a 26	ingestion	yes
		-	13.8	100	0	70.8	68.7		Glutenin, high molecular weight subunit 12	Tri a 26	ingestion	yes
13	GLTA_WHEAT	36	11.8	30	0	41	39.1	100% peptide	Glutenin, low molecular weight subunit	Tri a 26	ingestion	yes
		-	13.9	30	1			100% peptide	Glutenin, low molecular weight subunit			
16	GLTA_WHEAT	32	13.2	100	1	41	39.1		Glutenin, low molecular weight subunit	Tri a 26	ingestion	yes
		-	13.9	100	1	41	39.1		Glutenin, low molecular weight subunit	Tri a 26	ingestion	yes
	GLTB_WHEAT	30	16.3	50	0	34.9	32.5		Glutenin, low molecular weight subunit 1D1	Tri a 26	ingestion	yes
18	GDA7_WHEAT	22	15.7	200	0	36.1	34		Alpha/beta-gliadin clone PW8142			
19	GDB3_WHEAT	44	23.4	40	0	27.7			Gamma-gliadin (Fragment)			
	GLTB_WHEAT	41	18.6	40	0	34.9	32.5		Glutenin, low molecular weight subunit 1D1	Tri a 26	ingestion	yes

Supplement Table 14: Selected patients for quantitative determination of omega5-gliadin-specific IgE. 21 WT and 19 WA individuals were randomly selected and omega5-gliadin-specific IgE (f416) was determined. f4, wheat-specific IgE

	Pat.	f4 (kU$_A$/l)	f416 (kU$_A$/l)	Sex	age (months)	AD	Asthma	Symptoms after provocation
tolerant	2	<0.35	0.10	female	13	yes	no	
	7	<0.35	0.02	male	15	yes	no	
	10	<0.35	0.01	male	25	yes	no	
	18	<0.35	0.00	male	27	no	no	
	19	0.05	0.00	female	53	yes	yes	
	31	0.66	0.37	male	9	yes	no	
	32	0.72	0.14	female	130	yes	yes	
	34	0.79	0.00	male	50	yes	no	
	38	1.37	0.25	female	117	no	yes	
	40	1.54	0.96	male	188	yes	no	
	42	1.98	0.15	male	9	yes	no	
	43	2.04	0.22	female	54	yes	yes	
	47	2.52	0.87	female	6	yes	no	
	51	5.57	2.08	male	10	yes	no	
	54	6.62	0.02	male	5	yes	no	
	55	6.72	1.09	male	8	yes	no	
	56	7.41	0.56	male	95	yes	yes	
	59	10.6	0.37	female	37	yes	yes	
	60	12.0	0.41	female	114	yes	yes	
	61	17.0	0.38	male	20	yes	no	
	62	90.8	2.59	male	60	yes	no	
allergic	63	<0.35	0.03	female	16	yes	no	redn early, itch early
	64	<0.35	0.00	female	3	yes	no	vomit early+late; diarr early+late
	65	<0.35	0.00	female	14	yes	no	AD early+late
	66	<0.35	0.00	male	102	no	no	diarr early
	68	<0.35	0.06	female	37	yes	no	AD late
	70	0.53	0.07	female	22	yes	no	redn early+late, itch early+late, AD early
	74	1.38	0.44	female	53	yes	no	urtic early, AD early
	77	2.36	0.85	male	11	yes	no	AD early
	78	2.64	0.14	female	9	yes	no	redn early, AD early, rhino early
	79	3.10	2.78	male	6	yes	no	urtic early
	85	6.94	2.03	male	5	yes	no	urtic early, redn early, vomit early
	88	9.63	0.05	male	46	yes	no	AD early+late
	89	9.70	13.4	male	9	yes	no	urtic early
	91	11.2	3.25	female	9	yes	no	urtic early, angi early+late
	92	14.9	7.93	male	9	yes	no	urtic early, itch early
	93	16.0	1.09	female	5	yes	no	redn early, itch early
	96	35.3	0.21	male	6	yes	no	urtic early
	100	43.1	2.18	female	5	yes	yes	vomit early
	102	>100	22.9	male	11	yes	no	urtic early, redn early, AD early

Supplement Table 15: Comparison of total fluorescences obtained from microarrays incubated with PBS, negative, atopic, HE low, medium, high and CM low, medium, high pool sera at 70%, 80% and 90% laser power/PMT. Median and mean fluorescences (Fl) were considered along with subtraction of background (BG) subtraction. Log-transformation was applied. Differences between median and mean fluorescences are marked red.

Incubation	Median FL at 70%				Mean FL at 70%				Median FL at 80%				Mean FL at 80%				Median FL at 90%				Mean FL at 90%			
	Fl	log(Fl)	Fl-BG	log(Fl-BG)	Fl	log(Fl)	Fl-BG	log(Fl-BG)	Fl	log(Fl)	Fl-BG	log(Fl-BG)	Fl	log(Fl)	Fl-BG	log(Fl-BG)	Fl	log(Fl)	Fl-BG	log(Fl-BG)	Fl	log(Fl)	Fl-BG	log(Fl-BG)
PBS vs Negative	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PBS vs Atopic	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PBS vs CM high	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
PBS vs CM med	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
PBS vs CM low	***	***	*	**	***	***	**	**	**	***	*	**	***	**	*	**	**	**	*	*	ns	*	ns	ns
PBS vs HE high	***	***	ns	***	***	***	ns	***	***	***	ns	***	***	***	ns	***	***	***	***	***	ns	***	ns	***
PBS vs HE med	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
PBS vs HE low	***	***	ns	ns	***	***	ns	*	*	**	ns	ns	**	*	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
Negative vs Atopic	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Negative vs CM high	***	***	**	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Negative vs CM med	***	***	*	***	***	*	**	***	***	***	**	***	***	***	**	**	***	***	***	***	*	**	**	***
Negative vs CM low	*	*	ns	ns	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Negative vs HE high	***	***	ns	***	***	***	ns	***	***	***	ns	**	***	***	ns	**	***	***	***	***	ns	**	ns	***
Negative vs HE med	***	***	**	***	***	***	**	***	***	***	**	***	***	***	**	***	***	***	***	***	**	**	**	***
Negative vs HE low	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Atopic vs CM high	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Atopic vs CM med	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Atopic vs CM low	ns	ns	*	**	ns	ns	**	**	*	*	*	**	*	*	*	**	**	**	**	**	ns	*	ns	*
Atopic vs HE high	***	***	ns	***	***	***	ns	***	***	***	ns	***	***	***	ns	***	***	***	***	***	ns	***	ns	***
Atopic vs HE med	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
Atopic vs HE low	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns	ns	ns	ns	ns
CM high vs CM med	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM high vs CM low	**	**	ns	*	**	**	ns	*	**	**	ns	*	**	**	ns	*	**	**	**	**	ns	*	ns	**
CM high vs HE high	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	*	ns
CM high vs HE med	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM high vs HE low	***	***	ns	**	***	***	ns	**	***	***	ns	**	***	***	ns	**	***	***	***	***	ns	***	ns	***
CM med vs CM low	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM med vs HE high	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM med vs HE med	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM med vs HE low	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM low vs HE high	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM low vs HE med	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CM low vs HE low	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HE high vs HE med	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
HE high vs HE low	ns	ns	ns	ns	*	*	ns	ns	*	*	ns	ns	*	*	ns	ns	*	*	*	*	ns	ns	ns	ns
HE med vs HE low	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Supplement Table 16: Conversion of median IgE-specific fluorescence into "kU/l" by means of linear and logarithmic equations. Sera are arranged with increasing sIgE to HE and CM. Pairs of HE and CM results are arbitrary. n=82

HE	egg white			CM	skim milk		
CAP	Microarray	calc. "kU/l"		CAP	Microarray	calc. "kU/l"	
kU_A/l	Median FI	lin model	log model	kU_A/l	Median FI	lin model	log model
<0.35	59.6	-7.8	6.4	<0.35	102.6	0.7	12.8
<0.35	97.0	-8.6	8.7	<0.35	156.4	1.8	15.8
<0.35	122.2	-7.0	9.7	<0.35	236.8	3.6	18.8
<0.35	803.0	1.7	18.5	<0.35	88.0	0.3	11.7
<0.35	167.4	-6.5	11.2	<0.35	658.4	12.7	26.0
<0.35	101.2	-7.3	8.9	<0.35	39.0	-0.7	6.0
<0.35	425.2	-3.1	15.5	<0.35	190.0	2.6	17.2
<0.35	53.6	-7.9	5.9	<0.35	80.8	0.2	11.1
<0.35	231.0	-5.6	12.7	<0.35	44.8	-0.6	6.9
<0.35	68.6	-7.7	7.1	<0.35	40.6	-0.7	6.2
<0.35	70.4	-7.7	7.2	<0.35	170.0	2.1	16.4
<0.35	90.4	-7.4	8.3	<0.35	70.2	0.0	10.1
<0.35	56.2	-7.9	6.1	<0.35	76.6	0.1	10.7
<0.35	42.0	-8.1	4.8	<0.35	55.2	-0.4	8.4
<0.35	38.2	-8.1	4.3	<0.35	121.0	1.1	14.0
<0.35	60.4	-7.8	6.5	<0.35	41.6	-0.7	6.4
<0.35	58.6	-7.9	6.3	<0.35	75.2	0.1	10.6
<0.35	49.4	-8.0	5.5	<0.35	121.4	1.1	14.0
<0.35	145.4	-6.7	10.6	<0.35	110.6	0.8	13.4
<0.35	33.2	-8.2	3.7	<0.35	65.8	-0.1	9.7
0.00	77.2	-7.6	7.6	<0.35	49.0	-0.5	7.6
<0.35	451.2	-2.8	15.8	<0.35	60.2	-0.3	9.0
<0.35	106.8	-7.2	9.1	<0.35	50.2	-0.5	7.7
<0.35	115.0	-7.1	9.5	<0.35	45.2	-0.6	7.0
<0.35	102.2	-7.3	8.9	<0.35	246.0	3.8	19.0
<0.35	206.2	-6.0	12.2	<0.35	87.8	0.3	11.7
<0.35	92.0	-7.4	8.4	<0.35	47.4	-0.5	7.3
<0.35	149.4	-6.7	10.7	<0.35	54.6	-0.4	8.3
<0.35	227.2	-5.7	12.6	<0.35	65.6	-0.1	9.6
<0.35	62.2	-7.8	6.6	<0.35	57.4	-0.3	8.7
0.01	50.4	-8.0	5.6	<0.35	55.0	-0.4	8.4
0.02	103.4	-7.3	9.0	<0.35	57.6	-0.3	8.7
0.02	228.2	-5.7	12.7	<0.35	991.4	19.9	28.9
0.03	158.8	-6.6	11.0	0.01	72.8	0.0	10.4
0.09	179.8	-6.3	11.5	0.01	88.4	0.4	11.8
0.39	225.2	-5.7	12.6	0.02	152.2	1.7	15.6
0.82	229.6	-5.7	12.7	0.07	56.8	-0.3	8.6
0.78	119.6	-7.1	9.6	0.07	80.4	0.2	11.1
0.86	118.4	-7.1	9.6	0.15	91.0	0.4	12.0
0.95	95.4	-7.4	8.6	0.27	133.8	1.3	14.7
0.97	258.6	-5.3	13.2	0.36	50.2	-0.5	7.7
1.17	52.8	-7.9	5.8	0.39	51.4	-0.4	7.9
1.35	181.2	-6.3	11.6	0.44	24.0	-1.0	2.5
1.54	170.0	-6.4	11.3	0.59	2012.0	42.0	33.9
1.55	69.6	-7.7	7.1	0.60	51.4	-0.4	7.9
1.66	406.0	-3.4	15.3	0.66	46.6	-0.5	7.2
1.95	398.2	-3.5	15.2	0.71	55.2	-0.4	8.4
1.98	1327.6	8.5	20.9	0.81	55.2	-0.4	8.4
2.08	801.8	1.7	18.5	0.83	42.2	-0.6	6.5
2.15	202.8	-6.0	12.1	0.89	51.8	-0.4	8.0
2.19	221.2	-5.8	12.5	0.92	46.2	-0.6	7.2
2.46	212.6	-5.9	12.3	1.11	279.6	4.5	19.9
2.56	589.0	-1.0	17.1	1.14	50.6	-0.5	7.8
2.75	400.4	-3.5	15.3	1.45	77.0	0.1	10.8
2.81	291.2	-4.9	13.8	1.55	137.4	1.4	14.9
2.96	222.8	-5.7	12.5	1.91	180.0	2.3	16.8
3.57	208.4	-5.9	12.2	2.06	341.2	5.8	21.3
4.06	210.4	-5.9	12.3	2.98	76.4	0.1	10.7
5.16	223.0	-5.7	12.5	3.19	246.8	3.8	19.0
5.41	570.2	-1.3	16.9	3.97	85.8	0.3	11.5
5.56	203.4	-6.0	12.1	4.42	103.6	0.7	12.9

5.56	299.8	-4.7	13.9	4.55	1880.6	39.1	33.5
5.90	834.0	2.1	18.7	5.00	342.4	5.9	21.4
6.49	701.4	0.4	17.9	6.21	641.0	12.3	25.8
6.92	1029.2	4.7	19.7	6.29	140.2	1.5	15.0
8.29	1750.6	13.9	22.1	7.49	46.6	-0.5	7.2
8.32	800.2	1.7	18.5	10.20	60.0	-0.3	9.0
8.35	2745.6	26.8	24.2	10.70	388.0	6.8	22.3
8.40	418.4	-3.2	15.5	11.70	48.0	-0.5	7.4
8.59	1895.4	15.8	22.5	12.00	133.8	1.3	14.7
12.60	70.0	-7.7	7.1	12.90	5900.8	126.1	41.6
13.40	1890.2	15.7	22.5	13.50	288.0	4.7	20.1
13.70	3915.4	41.8	25.9	13.70	97.0	0.5	12.4
15.70	461.2	-2.7	15.9	15.40	112.8	0.9	13.5
16.40	401.0	-3.4	15.3	16.50	1431.2	29.4	31.5
24.60	1561.8	11.5	21.6	17.20	373.2	6.5	22.0
30.20	1603.0	12.0	21.7	19.30	1179.0	24.0	30.1
33.90	239.6	-5.5	12.9	23.10	53.6	-0.4	8.2
39.90	12560.2	153.2	31.3	28.00	60.8	-0.2	9.1
42.20	555.4	-1.5	16.8	46.30	21376.8	461.0	50.7
77.70	7416.0	87.0	28.9	66.20	576.8	10.9	25.1
>100	29958.2	377.4	35.4	67.20	6065.6	129.7	41.8

Supplement Table 17: Correlations of (semi)quantitative results for chosen allergens with differently processed results from fluorescent detection. Results for HE, CM and components (left column) were correlated with Median and mean fluorescence of spotted allergens (right column). Abbreviations: FI=fluorescence (cleared), CCU=Calibration Curve Units, FL intra norm=fluorescence (cleared) normalized to averaged intra assay values, FI inter norm= fluorescence (cleared) normalized to averaged inter assay values; CCU intra norm= Calibration Curve Units normalized to averaged intra assay variation, CCU inter norm= Calibration Curve Units normalized to averaged inter assay variation. Allergen names according to the World Health Organization and International Union of Immunological Societies (WHO/IUIS) are given in brackets.

n=91	(semi)quantified analyte [unit]	FI Median	FI Mean	FI intra norm Median	FI intra norm Mean	FI inter norm Median	FI inter norm Mean	CCU Median	CCU Mean	CCU intra norm Median	CCU intra norm Mean	CCU inter norm Median	CCU inter norm Mean	spotted analyte
IgE specific results	Hen's egg [kU_A/l]	0.813**	0.814**	0.739**	0.736**	0.739**	0.736**	0.692**	0.705**	0.675**	0.680**	0.622**	0.678**	egg white
	Cow's milk [kU_A/l]	0.747**	0.749**	0.737**	0.735**	0.737**	0.735**	0.749**	0.765**	0.761**	0.769**	0.714**	0.761**	skim milk
	Bos d4 [ISU]	0.758**	0.758**	0.746**	0.745**	0.746**	0.745**	0.774**	0.778**	0.776**	0.779**	0.772**	0.790**	nat α-lactalbumin
		0.536**	0.602**	0.337**	0.503**	0.337**	0.503**	0.475**	0.575**	0.327**	0.517**	0.284**	0.472**	rec α-lactalbumin
	Bos d5.0102 [ISU]	0.702**	0.718**	0.718**	0.729**	0.718**	0.729**	0.734**	0.712**	0.608**	0.641**	0.522**	0.590**	rec β-lactoglobulin (A)
	Bos d5.0101 [ISU] Bos d5.0102 [ISU]	0.777**	0.779**	0.768**	0.770**	0.733**	0.770**	0.842**	0.858**	0.693**	0.705**	0.679**	0.688**	nat β-lactoglobulin (A and B)
	Bos d8 [ISU]	0.745**	0.747**	0.727**	0.724**	0.727**	0.724**	0.737**	0.733**	0.706**	0.702**	0.651**	0.705**	tot casein
	Bos d8as1 [ISU]	0.729**	0.731**	0.719**	0.717**	0.719**	0.717**	0.751**	0.764**	0.722**	0.744**	0.689**	0.757**	rec αs1-casein
	Bos d8bcas [ISU]	0.719**	0.721**	0.660**	0.671**	0.660**	0.671**	0.763**	0.759**	0.623**	0.626**	0.640**	0.702**	nat β-casein
		0.612**	0.613**	0.581**	0.583**	0.581**	0.583**	0.711**	0.700**	0.530**	0.570**	0.476**	0.588**	rec β-casein
	Bos d8kcas [ISU]	0.721**	0.720**	0.691**	0.697**	0.691**	0.697**	0.681**	0.682**	0.691**	0.685**	0.614**	0.684**	nat κ-casein
		0.694**	0.692**	0.674**	0.670**	0.674**	0.670**	0.771**	0.782**	0.756**	0.749**	0.732**	0.784**	rec κ-casein
	Gal d1 [ISU]	0.802**	0.805**	0.799**	0.798**	0.799**	0.798**	0.821**	0.828**	0.760**	0.811**	0.757**	0.804**	nat ovomucoid
		0.805**	0.804**	0.806**	0.805**	0.806**	0.805**	0.846**	0.846**	0.791**	0.757**	0.735**	0.797**	rec ovomucoid
	Gal d2 [ISU]	0.722**	0.715**	0.728**	0.731**	0.728**	0.731**	0.651**	0.667**	0.623**	0.628**	0.559**	0.592**	nat ovalbumin
		0.225*	0.241*	0.071	0.079		0.079	0.071	0.035	-0.226*	-0.152	0.127	-0.135	rec ovalbumin
IgG4 specific results	α-lactalbumin (Bos d4) [mg_A/l]	0.924**	0.916**	0.911**	0.912**	0.911**	0.912**	0.905**	0.895**	0.841**	0.914**	0.855	0.912**	nat α-lactalbumin
		0.716**	0.691**	0.628**	0.667**	0.628**	0.667**	0.685**	0.662**	0.592**	0.681**	0.590**	0.663**	rec α-lactalbumin
	β-lactoglobulin (Bos d5) [mg_A/l]	0.849**	0.843**	0.822**	0.814**	0.822**	0.814**	0.818**	0.816**	0.811**	0.805**	0.796**	0.808**	nat β-lactoglobulin (A and B)
	casein (Bos d8) [mg_A/l]	0.885**	0.884**	0.871**	0.869**	0.871**	0.869**	0.891**	0.896**	0.894**	0.888**	0.886**	0.886**	total casein

Supplement Table 18: Correlation of sIgE to CM and HE with sums of components on ISAC and silicon microarray. IgE-specific fluorescence at 80% laser power/PMT was considered.

Immuno CAP	ISAC Glass Slide allergens	ISU	FI	internorm FI	intranorm FI	CCU	internorm CCU	intranorm CCU	Copoly(DMA-NAS-MAPS) Silicon microarray allergens
CM (kU$_A$/l)	Bos d8, Bos d4, Bos d5.0101, Bos d5.0102	0.852**	0.880**	0.869**	0.866**	0.882**	0.841**	0.832**	tot casein, nat a-lact, nat b-lacto
			0.871**	0.858**	0.846**	0.872**	0.846**	0.829**	nat a-, b-, k-casein, nat a-lact, nat b-lacto
	Bos d8, Bos d4, Bos d5.0102	0.844**	0.731**	0.717**	0.718**	0.762**	0.739**	0.758**	rec as1-, as2-, b-, k-casein, rec a-lact, rec b-lacto
	Bos d8as1, Bos d8b, Bos d8k, Bos d4, Bos d5.0102	0.786**	0.740**	0.721**	0.722**	0.776**	0.744**	0.760**	rec as1, b, k, rec a-lact, rec b-lacto
	Bos d8b, Bos d8k, Bos d4, Bos d5.0101, Bos d5.0102	0.835**	0.853**	0.834**	0.830**	0.886**	0.814**	0.800**	nat b-, k-casein, nat a-lact, nat b-lacto
	Bos d8b, Bos d8k, Bos d4, Bos d5.0102	0.782**	0.710**	0.681**	0.679**	0.741**	0.559**	0.696**	rec b, k-casein, rec a-lact, rec b-lacto
HE (kU$_A$/l)	Gal d1, Gald d2	0.788**	0.829**	0.817**	0.820**	0.741**	0.776**	0.774**	nat ovalbumin, nat ovomucoid
			0.765**	0.652**	0.650**	0.752**	0.726**	0.675**	rec ovalbumin, rec ovomucoid

Supplement Table 19: Results of intra (n=10) and inter assay (n=13) variation for IgE and IgG$_4$ specific FI by application of HE medium pool serum.

spotted analyte	IgE-specific fluorescence at 80% LP/PMT				IgG4-specific fluorescence at 60% LP/PMT			
	intra assay variation [%]		inter assay variation [%]		intra assay variation [%]		inter assay variation [%]	
	Median FI	Mean FI	Median FI	Mean FI	Median FI	Mean FI	Median FI	Mean FI
IgG$_4$ 300 µg/ml	19.9	18.0	27.8	24.6	14.7	14.4	32.7	27.5
IgG$_4$ 120 µg/ml	22.2	20.9	41.5	36.7	15.1	14.4	27.3	24.0
IgG$_4$ 60 µg/ml	21.1	18.6	145.4	133.3	12.6	12.0	29.2	29.6
IgG$_4$ 35 µg/ml	20.6	20.9	35.5	30.9	11.9	12.0	25.1	22.8
IgG$_4$ 15 µg/ml	21.4	18.8	43.2	41.9	10.1	9.6	21.5	19.7
IgG$_4$ 3 µg/ml	24.6	21.5	40.8	38.0	2.8	4.1	12.4	14.3
IgE 240 µg/ml	8.9	8.6	18.9	14.4	11.5	12.2	35.9	33.7
IgE 60 µg/ml	6.4	6.1	23.9	22.6	5.7	5.5	29.0	27.4
IgE 35 µg/ml	9.7	10.0	22.4	21.7	7.7	7.0	25.6	25.3
IgE 15 µg/ml	5.1	5.3	24.1	23.7	5.1	4.5	25.5	24.0
IgE 35 µg/ml	7.7	7.7	26.2	25.5	6.7	6.9	20.8	19.8
IgE 3 µg/ml	14.9	14.2	24.7	23.9	2.9	3.2	8.2	11.0
1% HSA	26.4	23.6	30.2	27.4	2.6	2.9	8.2	15.5
skim milk	12.2	12.4	26.8	26.8	16.1	16.0	37.7	37.1
rec αs1-casein	14.4	14.0	18.6	17.0	15.3	15.5	40.7	30.2
rec αs2-casein	13.4	13.1	60.7	55.0	13.4	13.0	37.8	35.4
rec ß-casein	9.8	8.2	45.3	33.9	24.9	17.4	67.3	34.9
rec κ-casein	13.4	11.7	28.0	27.6	15.8	14.9	32.8	29.4
nat α-casein	9.2	9.1	14.1	14.3	10.5	11.1	14.1	13.4
nat ß-casein	14.2	13.1	29.5	24.8	16.8	15.5	39.3	28.2
nat κ-casein	9.5	9.1	38.7	35.9	10.5	10.4	64.0	57.7
total casein	6.7	6.9	19.9	19.6	10.6	10.3	18.1	17.7
rec α-lactalbumin	24.2	24.0	93.8	56.8	40.2	38.1	91.1	41.0
rec ß-lactoglobulin A	15.1	14.6	38.2	31.9	16.6	16.9	59.4	45.1
nat α-lactalbumin	7.4	6.8	16.5	16.2	13.2	9.1	36.0	30.6
nat ß-lactoglobulin A+B	16.5	16.0	20.8	20.2	19.3	19.2	37.1	37.4
egg white	10.9	11.0	37.3	36.8	12.9	12.8	40.9	46.5
rec ovomucoid	14.9	14.4	33.2	32.3	14.0	13.6	32.7	33.2
rec ovalbumin	13.2	12.0	57.7	52.8	5.8	8.4	16.9	24.9
nat ovomucoid	20.9	19.6	26.4	26.1	21.6	22.8	24.0	23.9
nat ovalbumin	12.0	11.1	23.1	24.8	16.1	17.1	23.4	25.1
PBS	25.9	22.1	71.4	62.1	11.5	13.7	118.9	113.7
BLANK	31.8	27.7	35.8	31.4	9.4	11.0	63.7	63.2

Supplement Table 20: Ranges of IgE-specific CCU's and missed values substituted with "0" or "4"; N=91.

spotted analytes	Median FI IgE-specific		Counts abs.		Counts %		Mean FI IgE-specific		Counts abs.		Counts %	
	Min	Max	0	4	0	4	Min	Max	0	4	0	4
rec αs1 casein	0.00123	2.06280	48	0	52.7	0.0	0.05857	2.05644	49	0	53.8	0.0
rec β-casein	0.03949	1.81620	56	0	61.5	0.0	0.06149	1.80881	55	0	60.4	0.0
rec κ-casein	0.00099	2.01830	55	0	60.4	0.0	0.09203	1.96735	57	0	62.6	0.0
rec αs2 casein	0.03654	1.78996	67	0	73.6	0.0	0.06041	1.69818	67	0	73.6	0.0
nat β-casein	0.02800	1.42504	67	0	73.6	0.0	0.18846	1.42867	69	0	75.8	0.0
nat κ-casein	0.07547	2.45630	8	0	8.8	0.0	0.05253	2.38362	8	0	8.8	0.0
nat α casein	0.06555	2.16852	39	0	42.9	0.0	0.00726	2.11603	39	0	42.9	0.0
rec α-lactalbumin	0.02491	1.54039	73	0	80.2	0.0	0.00974	1.55252	66	0	72.5	0.0
rec β-lactoglobulin A	0.01865	1.81584	75	0	82.4	0.0	0.07949	1.81238	76	0	83.5	0.0
total casein	0.00069	1.92134	34	0	37.4	0.0	0.03678	1.88809	35	0	38.5	0.0
nat α-lactalbumin	0.06570	1.91674	35	0	38.5	0.0	0.08952	1.88416	36	0	39.6	0.0
nat β-lactoglobulin A+B	0.01068	1.83630	52	0	57.1	0.0	0.05861	1.80296	54	0	59.3	0.0
rec ovomucoid	0.09517	1.81201	56	0	61.5	0.0	0.11629	1.78661	56	0	61.5	0.0
rec ovalbumin	0.00000	0.00000	91	0	100.0	0.0	0.04531	0.04531	90	0	98.9	0.0
skim milk	0.07859	2.00700	33	0	36.3	0.0	0.07339	1.95696	35	0	38.5	0.0
nat ovomucoid	0.02211	2.08441	49	0	53.8	0.0	0.01833	2.04577	50	0	54.9	0.0
nat ovalbumin	0.02847	1.51537	44	0	48.4	0.0	0.00410	1.50064	46	0	50.5	0.0
egg white	0.00402	1.71828	35	0	38.5	0.0	0.01508	1.69712	37	0	40.7	0.0
PBS	0.00869	0.00869	90	0	98.9	0.0	0.00000	0.00000	91	0	100.0	0.0
BLANK	0.02575	0.04659	89	0	97.8	0.0	0.03107	0.03107	90	0	98.9	0.0
IgG$_4$ 300 µg/ml	0.00556	0.24031	83	0	91.2	0.0	0.10561	0.23706	87	0	95.6	0.0
IgG$_4$ 120 µg/ml	0.00457	0.05393	88	0	96.7	0.0	0.08130	0.08130	90	0	98.9	0.0
IgG$_4$ 60 µg/ml	0.66419	0.87840	79	0	86.8	0.0	0.00108	0.89459	78	0	85.7	0.0
IgG$_4$ 35 µg/ml	0.00000	0.00000	91	0	100.0	0.0	0.15413	0.15413	90	0	98.9	0.0
IgG$_4$ 15 µg/ml	0.00000	0.00000	91	0	100.0	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG$_4$ 3 µg/ml	0.00000	0.00000	91	0	100.0	0.0	0.00000	0.00000	91	0	100.0	0.0

Supplement Table 21: Ranges of IgE-specific CCU's after inter assay normalization and missed values substituted with "0" or "4". N=91.

spotted analytes	Median FI IgE-specific Min	Max	Counts abs. 0	4	Counts % 0	4	Mean FI IgE-specific Min	Max	Counts abs. 0	4	Counts % 0	4
rec αs1 casein	0.00941	1.97261	47	0	51.6	0.0	0.04425	1.97638	47	0	51.6	0.0
rec β-casein	0.04960	1.80695	54	0	59.3	0.0	0.02231	1.76475	52	0	57.1	0.0
rec κ-casein	0.09700	1.92435	59	0	64.8	0.0	0.00746	1.85535	59	0	64.8	0.0
rec αs2 casein	0.00195	1.88877	58	0	63.7	0.0	0.02495	1.83173	62	0	68.1	0.0
nat β-casein	0.00086	1.49020	66	0	72.5	0.0	0.02698	1.49551	66	0	72.5	0.0
nat κ-casein	0.04397	2.15531	11	0	12.1	0.0	0.07799	2.12052	14	0	15.4	0.0
nat α casein	0.00660	2.03078	36	0	39.6	0.0	0.01545	2.00832	39	0	42.9	0.0
rec α-lactalbumin	0.00233	1.31216	61	0	67.0	0.0	0.00776	1.38221	66	0	72.5	0.0
rec β-lactoglobulin A	0.06424	1.60059	72	0	79.1	0.0	0.07456	1.62105	73	0	80.2	0.0
total casein	0.00485	1.91788	34	0	37.4	0.0	0.00303	1.89507	33	0	36.3	0.0
nat α-lactalbumin	0.06785	1.92296	39	0	42.9	0.0	0.06494	1.91107	36	0	39.6	0.0
nat β-lactoglobulin A+B	0.02323	1.87693	47	0	51.6	0.0	0.02119	1.86403	46	0	50.5	0.0
rec ovomucoid	0.01422	2.05258	52	0	57.1	0.0	0.01884	2.02663	57	0	62.6	0.0
rec ovalbumin	0.00390	0.08727	87	0	95.6	0.0	0.04262	0.07156	89	0	97.8	0.0
skim milk	0.00796	2.05371	26	0	28.6	0.0	0.00526	2.01925	28	0	30.8	0.0
nat ovomucoid	0.01601	1.96718	49	0	53.8	0.0	0.00036	1.95290	51	0	56.0	0.0
nat ovalbumin	0.00338	1.69588	34	0	37.4	0.0	0.02859	1.67358	33	0	36.3	0.0
egg white	0.01351	1.95087	27	0	29.7	0.0	0.09994	1.89811	30	0	33.0	0.0
PBS	0.06501	0.44290	87	0	95.6	0.0	0.09719	0.35854	89	0	97.8	P.0
BLANK	0.00000	0.00000	91	0	100.0	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG₄ 300 µg/ml	0.00312	0.21794	83	0	91.2	0.0	0.08228	0.16141	88	0	96.7	0.0
IgG₄ 120 µg/ml	0.07625	0.08811	89	0	97.8	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG₄ 60 µg/ml	0.01261	0.68017	57	0	62.6	0.0	0.00815	0.65581	62	0	68.1	0.0
IgG₄ 35 µg/ml	0.14544	0.14544	90	0	98.9	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG₄ 15 µg/ml	0.08075	0.08075	90	0	98.9	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG₄ 3 µg/ml	0.00000	0.00000	91	0	100.0	0.0	0.00973	0.00973	90	0	98.9	0.0

Supplement Table 22: Ranges of IgE-specific CCU's after intra assay normalization and missed values substituted with "0" or "4". N=91.

spotted analytes	Median FI IgE-specific Min	Max	Counts abs. 0	4	Counts % 0	4	Mean FI IgE-specific Min	Max	Counts abs. 0	4	Counts % 0	4
rec αs1 casein	0.07850	2.10186	42	0	46.2	0.0	0.05224	2.10523	41	0	45.1	0.0
rec β-casein	0.06418	1.92200	48	0	52.7	0.0	0.03514	1.84035	49	0	53.8	0.0
rec κ-casein	0.05438	2.01593	52	0	57.1	0.0	0.01115	1.93973	52	0	57.1	0.0
rec αs2 casein	0.01153	1.78094	74	0	81.3	0.0	0.00689	1.75346	74	0	81.3	0.0
nat β-casein	0.01631	1.55259	66	0	72.5	0.0	0.00028	1.55807	60	0	65.9	0.0
nat κ-casein	0.03306	1.95158	2	2	2.2	2.2	0.22187	2.44440	1	0	1.1	0.0
nat α casein	0.02110	2.08199	38	0	41.8	0.0	0.03867	2.06792	37	0	40.7	0.0
rec α-lactalbumin	0.00213	1.55016	37	0	40.7	0.0	0.02003	1.53138	53	0	58.2	0.0
rec β-lactoglobulin A	0.00113	1.80113	55	0	60.4	0.0	0.04842	1.80257	59	0	64.8	0.0
total casein	0.00435	1.88562	38	0	41.8	0.0	0.03503	1.88291	37	0	40.7	0.0
nat α-lactalbumin	0.01538	1.97097	33	0	36.3	0.0	0.06343	1.97157	35	0	38.5	0.0
nat β-lactoglobulin A+B	0.00361	1.94430	45	0	49.5	0.0	0.01214	1.94171	44	0	48.4	0.0
rec ovomucoid	0.05613	2.20133	44	0	48.4	0.0	0.02741	2.16268	45	0	49.5	0.0
rec ovalbumin	0.01540	0.42792	81	0	89.0	0.0	0.01173	0.39814	81	0	89.0	0.0
skim milk	0.05452	2.21465	19	0	20.9	0.0	0.05209	2.16562	19	0	20.9	0.0
nat ovomucoid	0.05689	1.98496	50	0	54.9	0.0	0.00675	1.97915	47	0	51.6	0.0
nat ovalbumin	0.00501	1.77728	30	0	33.0	0.0	0.00752	1.76921	28	0	30.8	0.0
egg white	0.01536	2.12457	22	0	24.2	0.0	0.13960	2.08997	22	0	24.2	0.0
PBS	0.01070	0.40337	88	0	96.7	0.0	0.00658	0.29703	87	0	95.6	0.0
BLANK	0.00347	0.06801	89	0	97.8	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG₄ 300 µg/ml	0.01738	0.26941	79	0	86.8	0.0	0.03923	0.29835	76	0	83.5	0.0
IgG₄ 120 µg/ml	0.04250	0.21220	88	0	96.7	0.0	0.04494	0.11439	89	0	97.8	0.0
IgG₄ 60 µg/ml	0.01957	0.19387	86	0	94.5	0.0	0.10018	0.30654	87	0	95.6	0.0
IgG₄ 35 µg/ml	0.07565	0.10709	89	0	97.8	0.0	0.00000	0.00000	91	0	100.0	0.0
IgG₄ 15 µg/ml	0.00763	0.08001	88	0	96.7	0.0	0.03811	0.03811	90	0	98.9	0.0
IgG₄ 3 µg/ml	0.02873	0.02873	90	0	98.9	0.0	0.07768	0.07768	90	0	98.9	0.0

Supplement Table 23: Ranges of IgG$_4$-specific CCU's and missed values substituted with "0" or "4". N=91.

spotted analytes	Median FI IgG4-specific		Counts abs.		Counts %		Mean FI IgG4-specific		Counts abs.		Counts %	
	Min	Max	0	4	0	4	Min	Max	0	4	0	4
rec αs1 casein	0.34189	2.57430	28	2	30.8	2.2	0.10774	2.57670	28	2	30.8	2.2
rec β-casein	0.55584	2.49117	45	2	49.5	2.2	0.15980	2.61969	36	5	39.6	5.5
rec κ-casein	0.50218	2.60469	45	1	49.5	1.1	0.16475	2.59425	44	0	48.4	0.0
rec αs2 casein	0.47696	2.43522	56	1	61.5	1.1	0.09145	2.39456	51	1	56.0	1.1
nat β-casein	0.33952	2.31880	37	0	40.7	0.0	0.35812	2.37292	32	0	35.2	0.0
nat κ-casein	0.13230	2.58416	7	5	7.7	5.5	0.45754	2.59311	5	5	5.5	5.5
nat α casein	0.69439	2.67336	31	1	34.1	1.1	0.38452	2.64152	30	1	33.0	1.1
rec α-lactalbumin	0.04022	2.50501	43	0	47.3	0.0	0.45699	2.47708	33	1	36.3	1.1
rec β-lactoglobulin A	0.55913	2.49857	49	0	53.8	0.0	0.19523	2.43548	45	0	49.5	0.0
total casein	0.24599	2.56097	28	2	30.8	2.2	0.05774	2.55334	28	2	30.8	2.2
nat α-lactalbumin	0.08748	3.48412	13	26	14.3	28.6	0.42810	3.12462	14	25	15.4	27.5
nat β-lactoglobulin A+B	0.38847	2.58371	22	6	24.2	6.6	0.56501	2.61056	21	6	23.1	6.6
rec ovomucoid	0.26799	2.49267	37	1	40.7	1.1	0.29689	2.46939	37	1	40.7	1.1
rec ovalbumin	0.15059	1.36138	81	0	89.0	0.0	0.17874	1.37900	77	0	84.6	0.0
skim milk	0.30124	2.57577	21	5	23.1	5.5	0.11833	2.55716	19	5	20.9	5.5
nat ovomucoid	0.52972	2.74441	31	2	34.1	2.2	0.34723	2.67749	30	2	33.0	2.2
nat ovalbumin	0.19262	2.78005	19	3	20.9	3.3	0.53621	2.77768	19	3	20.9	3.3
egg white	0.27592	3.03234	14	3	15.4	3.3	0.47705	2.89378	16	3	17.6	3.3
IgE 240 µg/ml	1.56977	1.95388	0	0	0.0	0.0	1.58869	1.94610	1	0	1.1	0.0
IgE 120 µg/ml	1.41650	1.81481	0	0	0.0	0.0	1.42369	1.78913	1	0	1.1	0.0
IgE 60 µg/ml	1.13917	1.64761	0	0	0.0	0.0	1.20835	1.61676	1	0	1.1	0.0
IgE 35 µg/ml	0.73540	1.48845	1	0	1.1	0.0	0.76159	1.47255	1	0	1.1	0.0
IgE 15 µg/ml	0.00862	1.30986	24	0	26.4	0.0	0.27497	1.25403	16	0	17.6	0.0
IgE 3 µg/ml	0.00946	0.71315	79	0	86.8	0.0	0.02723	0.88320	73	0	80.2	0.0
PBS	0.29080	1.78370	79	0	86.8	0.0	0.16823	1.69737	78	0	85.7	0.0
BLANK	0.31984	0.58938	86	0	94.5	0.0	0.19596	0.49937	85	0	93.4	0.0

Supplement Table 24: Ranges of IgG$_4$-specific CCU's after inter assay normalization and missed values substituted with "0" or "4"; N=91.

spotted analytes	Median FI IgG4-specific Min	Max	Counts abs. 0	4	Counts % 0	4	Mean FI IgG4-specific Min	Max	Counts abs. 0	4	Counts % 0	4
rec αs1 casein	0.53425	2.60237	21	3	23.1	3.3	0.28279	2.60972	23	3	25.3	3.3
rec β-casein	0.65380	2.54931	22	3	24.2	3.3	0.32348	2.61052	28	6	30.8	6.6
rec κ-casein	0.11876	2.54392	38	1	41.8	1.1	0.05417	2.50289	33	1	36.3	1.1
rec αs2 casein	0.61761	2.44968	47	1	51.6	1.1	0.57548	2.41716	46	1	50.5	1.1
nat β-casein	0.70102	2.42267	33	0	36.3	0.0	0.61136	2.42855	33	0	36.3	0.0
nat κ-casein	0.57402	2.63775	10	7	11.0	7.7	0.75925	2.61911	10	6	11.0	6.6
nat α casein	0.16515	2.61562	33	3	36.3	3.3	0.33970	2.68393	30	1	33.0	1.1
rec α-lactalbumin	0.44895	2.13050	17	2	18.7	2.2	0.43040	2.43981	29	1	31.9	1.1
rec β-lactoglobulin A	0.34780	2.32097	28	1	30.8	1.1	0.61050	2.27092	33	1	36.3	1.1
total casein	0.01014	2.65055	27	2	29.7	2.2	0.12672	2.63458	25	2	27.5	2.2
nat α-lactalbumin	0.44097	3.07579	12	27	13.2	29.7	0.21819	3.55343	10	28	11.0	30.8
nat β-lactoglobulin A+B	0.46507	2.71683	17	12	18.7	13.2	0.66052	3.11108	18	12	19.8	13.2
rec ovomucoid	0.16593	2.52747	32	2	35.2	2.2	0.20232	2.50461	30	2	33.0	2.2
rec ovalbumin	0.20395	1.38194	74	0	81.3	0.0	0.00057	1.40528	64	0	70.3	0.0
skim milk	0.71823	2.65709	18	7	19.8	7.7	0.30793	2.63461	17	5	18.7	5.5
nat ovomucoid	0.47398	2.55772	29	2	31.9	2.2	0.00608	2.56504	26	2	28.6	2.2
nat ovalbumin	0.15515	2.62909	21	5	23.1	5.5	0.05691	2.67401	21	5	23.1	5.5
egg white	0.40108	2.60381	14	8	15.4	8.8	0.67196	2.88709	14	7	15.4	7.7
IgE 240 µg/ml	1.60328	1.91053	0	0	0.0	0.0	1.60229	1.95544	0	0	0.0	0.0
IgE 120 µg/ml	1.29491	1.78305	0	0	0.0	0.0	1.31925	1.79289	0	0	0.0	0.0
IgE 60 µg/ml	1.18183	1.60993	1	0	1.1	0.0	1.21434	1.59495	0	0	0.0	0.0
IgE 35 µg/ml	0.70243	1.51422	4	0	4.4	0.0	0.92171	1.47878	4	0	4.4	0.0
IgE 15 µg/ml	0.21593	1.21525	29	0	31.9	0.0	0.14351	1.19520	23	0	25.3	0.0
IgE 3 µg/ml	0.01998	0.89252	73	0	80.2	0.0	0.10586	0.72729	69	0	75.8	0.0
PBS	0.33300	1.97132	39	0	42.9	0.0	0.21258	1.90799	41	0	45.1	0.0
BLANK	0.15733	0.96399	58	0	63.7	0.0	0.21125	0.89684	60	0	65.9	0.0

Supplement Table 25: Ranges of IgG$_4$-specific CCU's after intra assay normalization and missed values substituted with "0" or "4"; N=91.

spotted analytes	Median FI IgG4-specific		Counts abs.		Counts %		Mean FI IgG4-specific		Counts abs.		Counts %	
	Min	Max	0	4	0	4	Min	Max	0	4	0	4
rec αs1 casein	0.13564	2.88013	12	3	13.2	3.3	0.24966	2.58159	11	4	12.1	4.4
rec β-casein	0.49390	2.59476	17	3	18.7	3.3	0.22924	2.62665	16	6	17.6	6.6
rec κ-casein	0.23099	2.58060	34	1	37.4	1.1	0.56819	2.53558	34	1	37.4	1.1
rec αs2 casein	0.41266	2.63555	37	0	40.7	0.0	0.33077	2.58070	35	0	38.5	0.0
nat β-casein	0.37329	2.43635	30	0	33.0	0.0	0.32556	2.43511	28	0	30.8	0.0
nat κ-casein	0.83382	2.66846	3	11	3.3	12.1	0.85240	2.60989	2	9	2.2	9.9
nat α casein	0.01396	2.65324	30	1	33.0	1.1	0.28528	2.62875	31	1	34.1	1.1
rec α-lactalbumin	1.00059	2.37585	6	2	6.6	2.2	0.18418	2.54381	15	2	16.5	2.2
rec β-lactoglobulin A	0.30170	2.47744	9	1	9.9	1.1	0.23946	2.41725	13	1	14.3	1.1
total casein	0.18354	2.57099	31	1	34.1	1.1	0.10398	2.56126	31	1	34.1	1.1
nat α-lactalbumin	0.40979	3.36904	18	18	19.8	19.8	0.60746	2.78598	18	19	19.8	20.9
nat β-lactoglobulin A+B	0.21223	2.69111	23	7	25.3	7.7	0.07049	2.73304	20	7	22.0	7.7
rec ovomucoid	0.43644	2.64895	22	1	24.2	1.1	0.04224	2.53428	23	2	25.3	2.2
rec ovalbumin	0.23212	1.35930	74	0	81.3	0.0	0.25190	1.37258	77	0	84.6	0.0
skim milk	0.50012	2.65029	14	3	15.4	3.3	0.50024	2.71120	13	4	14.3	4.4
nat ovomucoid	0.34086	2.61899	32	1	35.2	1.1	0.07306	2.52131	31	2	34.1	2.2
nat ovalbumin	0.31160	2.60647	21	3	23.1	3.3	0.22431	2.62799	20	3	22.0	3.3
egg white	0.50383	2.54550	17	7	18.7	7.7	0.52585	2.55575	12	9	13.2	9.9
IgE 240 µg/ml	1.49658	1.84025	1	0	1.1	0.0	1.49109	1.87463	0	0	0.0	0.0
IgE 120 µg/ml	1.15320	1.71675	1	0	1.1	0.0	1.17795	1.72125	0	0	0.0	0.0
IgE 60 µg/ml	0.96385	1.54434	4	0	4.4	0.0	0.12431	1.54292	2	0	2.2	0.0
IgE 35 µg/ml	0.05012	1.34020	25	0	27.5	0.0	0.49828	1.33381	21	0	23.1	0.0
IgE 15 µg/ml	0.13039	1.05360	48	0	52.7	0.0	0.15324	1.20995	40	0	44.0	0.0
IgE 3 µg/ml	0.03437	0.89137	69	0	75.8	0.0	0.05477	0.71538	73	0	80.2	0.0
PBS	0.08742	1.86214	69	0	75.8	0.0	0.30171	1.81669	74	0	81.3	0.0
BLANK	0.24137	0.67037	83	0	91.2	0.0	0.02193	0.66774	85	0	93.4	0.0

Supplement Table 26: Results of MALDI-TOF MS analysis of digested native and recombinant allergens. Theoretical *in silico* digests were applied to selected proteins and are indicated by their full name instead of Uniprot code.

Protein	Band No.	Peaks	ppm	partials	Score	Uniprot entry	SC [%]
nat α-caseine	1	75	50	0	84	CASA2_BOVIN	30.6
	2	85	600	2	69	CASA1_BOVIN	52.8
	3	72	750	1	50	CASA1_BOVIN	44.4
rec αs1-casein	1	86	600	3	56	CASA1_BOVIN	37.4
	2	93	200	2	66	CASA1_BOVIN	30.4
	3	70	200	2	56	CASA1_BOVIN	26.6
rec αs2-casein	1	64	200	2	147	CASA2_BOVIN	66.2
	2	49	50	2	127	CASA2_BOVIN	47.7
nat β-casein		109	200	1	29	CASB_BOVIN	n/a
rec β-casein	1	98	150	0	64	CASB_BOVIN	29.5
	2	73	600	0	30	CASB_BOVIN	29.5
			600	0		β-casein	32.1
nat κ-casein	1	130	200	2	37	CASK_BOVIN	38.4
	2	118	200	4	58	CASK_BOVIN	28.4
	3	105	200	2	59	CASK_BOVIN	32.6
rec κ-casein	1	88	50	4	28	CASK_BOVIN	19.5
	2	23	200	4	-	CASK_BOVIN	25.4
tot casein	1	69	50	2	108	CASA2_BOVIN	48.2
	2	91	50	2	94	CASA1_BOVIN	44,9
	3	58					
	4	91	150	2	24	CASK_BOVIN	n/a
			150	2	-	κ-casein	31,4
skim milk	1	72	50	1	79	CASA1_BOVIN	33.2
	2	49	100	0			
	3	67	50	2	95	LACB_BOVIN	64.6
	4	43					
nat α-lactalbumin		44	300	3	58	LALBA_BOVIN	21.1
rec α-lactalbumin	1	90					
	2	103					
	3	74	200	1	49	LALBA_BOVIN	44.4
			200	1	-	α-lactalbumin	39.8
rec α-lactalbumin (nativ)		64	100	0	-	α-lactalbumin	34.1
nat β-lactoglobulin		104	100	1	99	LACB_BOVIN	68,5
rec β-lactoglobulin		52	300	2	31	LACB_BOVIN	39.3
			300	2	-	β-lactoglobulin A	63.6
nat ovomucoid		26	300	1	87	IOVO_CHICK	43.3
rec ovomucoid	1	51	500	1	58	IOVO_CHICK	43.3
	2	33	350	0	31	IOVO_CHICK	21.4
nat ovalbumin	1	111	550	1	66	OVAL_CHICK	58.0
	2	97	600	1	64	OVAL_CHICK	44.7
rec ovalbumin	1	74	150	1	136	OVAL_CHICK	52.3
	2	69					
egg white	1	84	150	1	155	TRFE_CHICK	42.4
	2	104	550	1	75	OVAL_CHICK	58.0
	3	74	50	0	91	OVAL_CHICK	32.6
				0	38	IOVO_CHICK	28.6
	3	74	100	1	84	OVAL_CHICK	40.9
	4	67	100	0	100	LYSC_CHICK	59.9

9.2 Figures

Supplement Figure 1: Settings in acquisition of m/z spectra with MALDI-TOF MS. Other mass ranges than indicated here were used and are readable in the respective m/z spectra.

Supplement Figure 2: Comparison of IgE- and IgG₄-specific fluorescences for spotted allergens between different sera and application of ABs.

9.3 Alignments and sequence coverages of recombinant components

9.3.1   αs1-casein

9.3.1.1 Alignment of sequenced forward and reverse strand

```
Score      Expect    Identities    Gaps      Strand
1367 bits(740)  0.0    749/753(99%)   2/753(0%)    Plus/Minus
Query   11   AGCTT-TCTTTCGGGCTTTGTTAGCAGCCGGATCTCAgtggtggtggtggtggtgCTCGA   69
             |||||  |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  763   AGCTTCCCTTTCGGGCTTTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGA   704

Query   70   GCCACAGTGGCATAGTAGTCTTTTCACTGTTCTCAGAGCCAATGGGATTAGGGATGTCAG   129
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  703   GCCACAGTGGCATAGTAGTCTTTTCACTGTTCTCAGAGCCAATGGGATTAGGGATGTCAG   644

Query  130   AGAATGATGGGGCATCAGTGTATTGTGTGCCTAGTGGAACGTAATACCAGGCACCAGATG   189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  643   AGAATGATGGGGCATCAGTGTATTGTGTGCCTAGTGGAACGTAATACCAGGCACCAGATG   584

Query  190   GATAGGCATCCAGCTGGTAGAATTGTCTGAAAAGCTCAGGGTAGAAGTAGGCCAGTTCCT   249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  583   GATAGGCATCCAGCTGGTAGAATTGTCTGAAAAGCTCAGGGTAGAAGTAGGCCAGTTCCT   524

Query  250   GATTCACTCCTATCATAGGTTCTTTCTGTTGGGCATGGATTCCCTCTTTCATACTGTGAA   309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  523   GATTCACTCCTATCATAGGTTCTTTCTGTTGGGCATGGATTCCCTCTTTCATACTGTGAA   464

Query  310   GTCGTTCCTCAGCACTATTGGGAACAATTTCCAGCTGGGGTACTTTGTATTTTTTCAGTC   369
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  463   GTCGTTCCTCAGCACTATTGGGAACAATTTCCAGCTGGGGTACTTTGTATTTTTTCAGTC   404

Query  370   TGAGAAGCTGTTCCAGATAACCCAGGTAACGCTCAGAGGGCACATCTTCCTTTTGAATGT   429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  403   TGAGAAGCTGTTCCAGATAACCCAGGTAACGCTCAGAGGGCACATCTTCCTTTTGAATGT   344

Query  430   GCTTCTGCTCAACACTATTGGGAACAATTTCCTCACTTGACGAAATGCTTTCAGCTTCCA   489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  343   GCTTCTGCTCAACACTATTGGGAACAATTTCCTCACTTGACGAAATGCTTTCAGCTTCCA   284

Query  490   TTTGCTTAATATCTTCCATGGCTTGATCCTCAGTTGATTCACTCCCAATATCCTTGCTCA   549
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  283   TTTGCTTAATATCTTCCATGGCTTGATCCTCAGTTGATTCACTCCCAATATCCTTGCTCA   224

Query  550   GTTCATTGACCTTCTCCTTTCCAAACACTTCTGGAAAAGGTGCCACAAAAAACCTGAGTA   609
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  223   GTTCATTGACCTTCTCCTTTCCAAACACTTCTGGAAAAGGTGCCACAAAAAACCTGAGTA   164

Query  610   AATTTTCATTGAGGACTTCTTGAGGGAGTCCTTGGTGCTTGATAGGATGTTTAGGCCTCG   669
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  163   AATTTTCATTGAGGACTTCTTGAGGGAGTCCTTGGTGCTTGATAGGATGTTTAGGCCTCG   104

Query  670   GATCCCGACCCATTTGCTGTCCACCAGTCATGCTAGCCATATGTATATCTCCTTCTTAAA   729
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  103   GATCCCGACCCATTTGCTGTCCACCAGTCATGCTAGCCATATGTATATCTCCTTCTTAAA   44

Query  730   GTTAAACAAAATTATTTCTAGAGGGGAATTGTT     762
             |||||||||||||||| ||||||||| ||||
Sbjct   43   GTTAAACAAAATTATT-CTAGAGGGGAAATGTT     12
```

9.3.1.2 Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 1174
Range 1: 146 to 743

Score	Expect	Identities	Gaps	Strand
1092 bits(591)	0.0	596/598(99%)	1/598(0%)	Plus/Plus

```
Query  106  AGGCCTAAACATCCTATC-AAGCACCAAGGACTCCCTCAAGAAGTCCTCAATGAAAATTT  164
            |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  146  AGGCCTAAACATCCTATCAAAGCACCAAGGACTCCCTCAAGAAGTCCTCAATGAAAATTT  205


Query  165  ACTCAGGTTTTTTGTGGCACCTTTTCCAGAAGTGTTTGGAAAGGAGAAGGTCAATGAACT  224
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  206  ACTCAGGTTTTTTGTGGCACCTTTTCCAGAAGTGTTTGGAAAGGAGAAGGTCAATGAACT  265


Query  225  GAGCAAGGATATTGGGAGTGAATCAACTGAGGATCAAGCCATGGAAGATATTAAGCAAAT  284
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  266  GAGCAAGGATATTGGGAGTGAATCAACTGAGGATCAAGCCATGGAAGATATTAAGCAAAT  325


Query  285  GGAAGCTGAAAGCATTTCGTCAAGTGAGGAAATTGTTCCCAATAGTGTTGAGCAGAAGCA  344
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  326  GGAAGCTGAAAGCATTTCGTCAAGTGAGGAAATTGTTCCCAATAGTGTTGAGCAGAAGCA  385


Query  345  CATTCAAAAGGAAGATGTGCCCTCTGAGCGTTACCTGGGTTATCTGGAACAGCTTCTCAG  404
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  386  CATTCAAAAGGAAGATGTGCCCTCTGAGCGTTACCTGGGTTATCTGGAACAGCTTCTCAG  445


Query  405  ACTGAAAAAATACAAAGTACCCCAGCTGGAAATTGTTCCCAATAGTGCTGAGGAACGACT  464
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  446  ACTGAAAAAATACAAAGTACCCCAGCTGGAAATTGTTCCCAATAGTGCTGAGGAACGACT  505


Query  465  TCACAGTATGAAAGAGGGAATCCATGCCCAACAGAAAGAACCTATGATAGGAGTGAATCA  524
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  506  TCACAGTATGAAAGAGGGAATCCATGCCCAACAGAAAGAACCTATGATAGGAGTGAATCA  565


Query  525  GGAACTGGCCTACTTCTACCCTGAGCTTTTCAGACAATTCTACCAGCTGGATGCCTATCC  584
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  566  GGAACTGGCCTACTTCTACCCTGAGCTTTTCAGACAATTCTACCAGCTGGATGCCTATCC  625


Query  585  ATCTGGTGCCTGGTATTACGTTCCACTAGGCACACAATACACTGATGCCCCATCATTCTC  644
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  626  ATCTGGTGCCTGGTATTACGTTCCACTAGGCACACAATACACTGATGCCCCATCATTCTC  685
```

```
Query  645  TGACATCCCTAATCCCATTGGCTCTGAGAACAGTGAAAAGACTACTATGCCACTGTGG  702
            |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  686  TGACATCCCTAATCCTATTGGCTCTGAGAACAGTGAAAAGACTACTATGCCACTGTGG  743
```

9.3.1.3 Translated BLAST results

alpha-S1-casein precursor [Bos taurus]
Sequence ID: ref|NP_851372.1|
Length: 214

Range 1: 16 to 214

Score	Expect	Method	Identities	Positives	Gaps	Frame
370 bits(950)	8e-127	Compositional matrix adjust.	199/199(100%)	199/199(100%)	0/199(0%)	+3

```
Query  78   RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM  257
            RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM
Sbjct  16   RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM  75


Query  258  eaesissseeivpnsveQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL  437
            EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL
Sbjct  76   EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL  135


Query  438  HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS  617
            HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS
Sbjct  136  HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS  195


Query  618  DIPNPIGSENSEKTTMPLW  674
            DIPNPIGSENSEKTTMPLW
Sbjct  196  DIPNPIGSENSEKTTMPLW  214
```

alpha S1 casein, partial [Bos taurus]
Sequence ID: gb|ABW98943.1|
Length: 205

Range 1: 7 to 205

Score	Expect	Method	Identities	Positives	Gaps	Frame
369 bits(946)	2e-126	Compositional matrix adjust.	199/199(100%)	199/199(100%)	0/199(0%)	+3

```
Query  78   RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM  257
            RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM
Sbjct  7    RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM  66
```

```
Query  258  eaesissseeivpnsveQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL  437
            EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL
Sbjct  67   EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL  126


Query  438  HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS  617
            HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS
Sbjct  127  HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS  186


Query  618  DIPNPIGSENSEKTTMPLW  674
            DIPNPIGSENSEKTTMPLW
Sbjct  187  DIPNPIGSENSEKTTMPLW  205
```

alpha S1 casein, partial [Bos taurus]
Sequence ID: gb|ABW98940.1|
Length: 206

Range 1: 8 to 206

Score	Expect	Method	Identities	Positives	Gaps	Frame
369 bits(946)	2e-126	Compositional matrix adjust.	199/199(100%)	199/199(100%)	0/199(0%)	+3

```
Query  78   RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM  257
            RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM
Sbjct  8    RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQM  67


Query  258  eaesissseeivpnsveQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL  437
            EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL
Sbjct  68   EAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERL  127


Query  438  HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS  617
            HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS
Sbjct  128  HSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFS  187


Query  618  DIPNPIGSENSEKTTMPLW  674
            DIPNPIGSENSEKTTMPLW
Sbjct  188  DIPNPIGSENSEKTTMPLW  206
```

9.3.2   αs2-casein

9.3.2.1   Alignment of forward strand and clone sequence

```
Alignment statistics for match #1
Score     Expect     Identities     Gaps       Strand
1400 bits(758)    0.0    768/772(99%)    4/772(0%)    Plus/Minus
Query    15   TTCCTTTTCGGGGCTTTGTTAGCAGCCGGATCTCAgtggtggtggtggtggtgCTCGAGA   74
              ||||  ||||  ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   782   TTCC-TTTC-GGGCTTTGTTAGCAGCCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGA   725

Query    75   AGGTACCTCACATAGGGAATAACCTTTGTCTTAGGTTGAATCCATGGCTTCATAGCTTTC   134
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   724   AGGTACCTCACATAGGGAATAACCTTTGTCTTAGGTTGAATCCATGGCTTCATAGCTTTC   665

Query   135   TGATGCTGATAAACAGTCTTGAGATACTGGGGCAAGGCGAATTTCTGGTAACGCTGGCTG   194
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   664   TGATGCTGATAAACAGTCTTGAGATACTGGGGCAAGGCGAATTTCTGGTAACGCTGGCTG   605

Query   195   AtttttttCAGAAAATTTAGGCGATTCTTTTCTTCTTCAGTCAGTTTAGTTTTCTTAGTG   254
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   604   ATTTTTTTCAGAAAATTTAGGCGATTCTTTTCTTCTTCAGTCAGTTTAGTTTTCTTAGTG   545

Query   255   AATACTTCTGTTGATTCCATGTCAACGGTCTTCTTTGAATTTTCCTCACTGGTGGAGAGC   314
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   544   AATACTTCTGTTGATTCCATGTCAACGGTCTTCTTTGAATTTTCCTCACTGGTGGAGAGC   485

Query   315   TGCTCTCTGTTCAGAGTGGGAGTAATGGGAACAGCATTTCTCTTAACCTGATCCCATGGG   374
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   484   TGCTCTCTGTTCAGAGTGGGAGTAATGGGAACAGCATTTCTCTTAACCTGATCCCATGGG   425

Query   375   TTCAAAACAATTGGACCTTGATACAGATACTGGAGATACTGGGGGAACTTCTGATAAAAC   434
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   424   TTCAAAACAATTGGACCTTGATACAGATACTGGAGATACTGGGGGAACTTCTGATAAAAC   365

Query   435   TGATTGATTTCATTCAGTGCTTTCTGGTAGTGCTTATCGTCCACAGTAATCTTAACTTCC   494
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   364   TGATTGATTTCATTCAGTGCTTTCTGGTAGTGCTTATCGTCCACAGTAATCTTAACTTCC   305

Query   495   TCTGTGGCAACTTCAGCAGATTCCTCACTAGATGAGCCGATAGAATATTCCTCTTCATTT   554
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   304   TCTGTGGCAACTTCAGCAGATTCCTCACTAGATGAGCCGATAGAATATTCCTCTTCATTT   245

Query   555   GCGTTCCTTACAACTTCCTTGCAGAATGTGGAGCAAAGGTTCTCCTTGCTGGGATTAATG   614
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   244   GCGTTCCTTACAACTTCCTTGCAGAATGTGGAGCAAAGGTTCTCCTTGCTGGGATTAATG   185

Query   615   GCCATATTCTTTTCCTGCTTATATGTTTCCTGGGAGATGATAGATTCCTCACTGGAGGAG   674
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   184   GCCATATTCTTTTCCTGCTTATATGTTTCCTGGGAGATGATAGATTCCTCACTGGAGGAG   125

Query   675   ACATGTTCCATCGTATTCTTCGGATCCCGACCCATTTGCTGTCCACCAGTCATGCTAGCC   734
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   124   ACATGTTCCATCGTATTCTTCGGATCCCGACCCATTTGCTGTCCACCAGTCATGCTAGCC   65

Query   735   ATATGTATATCTCCTTCTTAAAAGTTAAACAAAATTATTTCTAGAGGGGAAT    786
              |||||||||||||||||||||||| |||||||||||||| ||||||||||||
Sbjct    64   ATATGTATATCTCCTTCTTAAA-GTTAAACAAAATTATT-CTAGAGGGGAAT    15
```

9.3.2.2   Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 1024

Range 1: 102 to 722

Score	Expect	Identities	Gaps	Strand
1136 bits(615)	0.0	619/621(99%)	0/621(0%)	Plus/Plus

```
Query  79   AAGAATACGATGGAACATGTCTCCTCCAGTGAGGAATCTATCATCTCCCAGGAAACATAT  138
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102  AAGAATACGATGGAACATGTCTCCTCCAGTGAGGAATCTATCATCTCCCAGGAAACATAT  161


Query  139  AAGCAGGAAAAGAATATGGCCATTAATCCCAGCAAGGAGAACCTTTGCTCCACATTCTGC  198
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162  AAGCAGGAAAAGAATATGGCCATTAATCCCAGCAAGGAGAACCTTTGCTCCACATTCTGC  221


Query  199  AAGGAAGTTGTAAGGAACGCAAATGAAGAGGAATATTCTATCGGCTCATCTAGTGAGGAA  258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222  AAGGAAGTTGTAAGGAACGCAAATGAAGAGGAATATTCTATCGGCTCATCTAGTGAGGAA  281


Query  259  TCTGCTGAAGTTGCCACAGAGGAAGTTAAGATTACTGTGGACGATAAGCACTACCAGAAA  318
            |||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  TCTGCTGAAGTTGCCACTGAGGAAGTTAAGATTACTGTGGACGATAAGCACTACCAGAAA  341


Query  319  GCACTGAATGAAATCAATCAGTTTTATCAGAAGTTCCCCCAGTATCTCCAGTATCTGTAT  378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  GCACTGAATGAAATCAATCAGTTTTATCAGAAGTTCCCCCAGTATCTCCAGTATCTGTAT  401


Query  379  CAAGGTCCAATTGTTTTGAACCCATGGGATCAGGTTAAGAGAAATGCTGTTCCCATTACT  438
            |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  402  CAAGGTCCAATTGTTTTGAACCCATGGGATCAGGTTAAGAGGAATGCTGTTCCCATTACT  461


Query  439  CCCACTCTGAACAGAGAGCAGCTCTCCACCAGTGAGGAAAATTCAAAGAAGACCGTTGAC  498
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462  CCCACTCTGAACAGAGAGCAGCTCTCCACCAGTGAGGAAAATTCAAAGAAGACCGTTGAC  521


Query  499  ATGGAATCAACAGAAGTATTCACTAAGAAAACTAAACTGACTGAAGAAGAAAAGAATCGC  558
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522  ATGGAATCAACAGAAGTATTCACTAAGAAAACTAAACTGACTGAAGAAGAAAAGAATCGC  581


Query  559  CTAAATTTTCTGaaaaaaaTCAGCCAGCGTTACCAGAAATTCGCCTTGCCCCAGTATCTC  618
```

```
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  582    CTAAATTTTCTGAAAAAAATCAGCCAGCGTTACCAGAAATTCGCCTTGCCCCAGTATCTC    641


Query  619    AAGACTGTTTATCAGCATCAGAAAGCTATGAAGCCATGGATTCAACCTAAGACAAAGGTT    678
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  642    AAGACTGTTTATCAGCATCAGAAAGCTATGAAGCCATGGATTCAACCTAAGACAAAGGTT    701


Query  679    ATTCCCTATGTGAGGTACCTT   699
               |||||||||||||||||||||
Sbjct  702    ATTCCCTATGTGAGGTACCTT   722
```

9.3.2.3 Translated BLAST results

alpha-S2-casein precursor [Bos taurus]
Sequence ID: ref|NP_776953.1|
Length: 222

Range 1: 16 to 222

Score	Expect	Method	Identities	Positives	Gaps	Frame
325 bits(832)	6e-106	Compositional matrix adjust.	207/207(100%)	207/207(100%)	0/207(0%)	+3

```
Query  105    KNTMEHVssseesiisQETYKQEKNMAINPSKENLCSTFCKEVVRNANeeeysigsssee    284
              KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEE
Sbjct   16    KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEE     75


Query  285    saevateeVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPIT    464
              SAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPIT
Sbjct   76    SAEVATEEVKITVDDKHYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPIT    135


Query  465    PTLNREQLSTSEENSKKTVDMEStevftkktklteeekNRLNFLKKISQRYQKFALPQYL    644
              PTLNREQLSTSEENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQKFALPQYL
Sbjct  136    PTLNREQLSTSEENSKKTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQRYQKFALPQYL    195


Query  645    KTVYQHQKAMKPWIQPKTKVIPYVRYL   725
              KTVYQHQKAMKPWIQPKTKVIPYVRYL
Sbjct  196    KTVYQHQKAMKPWIQPKTKVIPYVRYL   222
```

9.3.3 β-casein

9.3.3.1 Alignment of sequenced forward and reverse strand

```
Alignment statistics for match #1
Score       Expect      Identities      Gaps       Strand
1424 bits(771)   0.0     779/782(99%)   3/782(0%)    Plus/Minus
Query  13   TTCCCCTCTAG-AATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCATG   71
            |||||||||||  |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781  TTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCATG   722

Query  72   ACTGGTGGACAGCAAATGGGTCGGGATCCGAGAGAGCTGGAAGAACTCAATGTACCTGGT   131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  ACTGGTGGACAGCAAATGGGTCGGGATCCGAGAGAGCTGGAAGAACTCAATGTACCTGGT   662

Query  132  GAGATTGTGGAAAGCCTTTCAAGCAGTGAGGAATCTATTACACGCATCAATAAGAAAATT   191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  GAGATTGTGGAAAGCCTTTCAAGCAGTGAGGAATCTATTACACGCATCAATAAGAAAATT   602

Query  192  GAGAAGTTTCAGAGTGAGGAACAGCAGCAAACAGAGGATGAACTCCAGGATAAAATCCAC   251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  GAGAAGTTTCAGAGTGAGGAACAGCAGCAAACAGAGGATGAACTCCAGGATAAAATCCAC   542

Query  252  CCCTTTGCCCAGACACAGTCTCTAGTCTATCCCTTCCCTGGGCCCATCCCTAACAGCCTC   311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CCCTTTGCCCAGACACAGTCTCTAGTCTATCCCTTCCCTGGGCCCATCCCTAACAGCCTC   482

Query  312  CCACAAAACATCCCTCCTCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCT   371
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CCACAAAACATCCCTCCTCTTACTCAAACCCCTGTGGTGGTGCCGCCTTTCCTTCAGCCT   422

Query  372  GAAGTAATGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACAAAGAAATGCCC   431
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GAAGTAATGGGAGTCTCCAAAGTGAAGGAGGCTATGGCTCCTAAGCACAAAGAAATGCCC   362

Query  432  TTCCCTAAATATCCAGTTGAGCCCTTTACTGAAAGCCAGAGCCTGACTCTCACTGATGTT   491
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TTCCCTAAATATCCAGTTGAGCCCTTTACTGAAAGCCAGAGCCTGACTCTCACTGATGTT   302

Query  492  GAAAATCTGCACCTTCCTCTGCCTCTGCTCCAGTCTTGGATGCACCAGCCTCACCAGCCT   551
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GAAAATCTGCACCTTCCTCTGCCTCTGCTCCAGTCTTGGATGCACCAGCCTCACCAGCCT   242

Query  552  CTTCCTCCAACTGTCATGTTTCCTCCTCAGTCCGTGCTGTCCCTTTCTCAGTCCAAAGTC   611
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CTTCCTCCAACTGTCATGTTTCCTCCTCAGTCCGTGCTGTCCCTTTCTCAGTCCAAAGTC   182

Query  612  CTGCCTGTTCCCCAGAAAGCAGTGCCCTATCCCCAGAGAGATATGCCCATTCAGGCCTTT   671
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CTGCCTGTTCCCCAGAAAGCAGTGCCCTATCCCCAGAGAGATATGCCCATTCAGGCCTTT   122

Query  672  CTGCTGTACCAGGAGCCTGTACTCGGTCCTGTCCGGGGACCCTTCCCTATTATTGTCCTC   731
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CTGCTGTACCAGGAGCCTGTACTCGGTCCTGTCCGGGGACCCTTCCCTATTATTGTCCTC   62

Query  732  GAGcaccaccaccaccaccacTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAG   791
            |||||||||||||||||||||||||||||||||||||||||||||||||| |||||| ||
Sbjct  61   GAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAG-AAGCT-AG   4

Query  792  TT   793
            ||
Sbjct  3    TT   2
```

9.3.3.2   Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 1262

Range 1: 274 to 900

Score	Expect	Identities	Gaps	Strand
1158 bits(627)	0.0	627/627(100%)	0/627(0%)	Plus/Plus

```
Query  86   AGAGAGCTGGAAGAACTCAATGTACCTGGTGAGATTGTGGAAAGCCTTTCAAGCAGTGAG   145
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  274  AGAGAGCTGGAAGAACTCAATGTACCTGGTGAGATTGTGGAAAGCCTTTCAAGCAGTGAG   333


Query  146  GAATCTATTACACGCATCAATAAGAAAATTGAGAAGTTTCAGAGTGAGGAACAGCAGCAA   205
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  334  GAATCTATTACACGCATCAATAAGAAAATTGAGAAGTTTCAGAGTGAGGAACAGCAGCAA   393


Query  206  ACAGAGGATGAACTCCAGGATAAAATCCACCCCTTTGCCCAGACACAGTCTCTAGTCTAT   265
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  394  ACAGAGGATGAACTCCAGGATAAAATCCACCCCTTTGCCCAGACACAGTCTCTAGTCTAT   453


Query  266  CCCTTCCCTGGGCCCATCCCTAACAGCCTCCCACAAAACATCCCTCCTCTTACTCAAACC   325
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  454  CCCTTCCCTGGGCCCATCCCTAACAGCCTCCCACAAAACATCCCTCCTCTTACTCAAACC   513


Query  326  CCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAGTAATGGGAGTCTCCAAAGTGAAGGAG   385
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  514  CCTGTGGTGGTGCCGCCTTTCCTTCAGCCTGAAGTAATGGGAGTCTCCAAAGTGAAGGAG   573


Query  386  GCTATGGCTCCTAAGCACAAAGAAATGCCCTTCCCTAAATATCCAGTTGAGCCCTTTACT   445
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  574  GCTATGGCTCCTAAGCACAAAGAAATGCCCTTCCCTAAATATCCAGTTGAGCCCTTTACT   633


Query  446  GAAAGCCAGAGCCTGACTCTCACTGATGTTGAAAATCTGCACCTTCCTCTGCCTCTGCTC   505
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  634  GAAAGCCAGAGCCTGACTCTCACTGATGTTGAAAATCTGCACCTTCCTCTGCCTCTGCTC   693


Query  506  CAGTCTTGGATGCACCAGCCTCACCAGCCTCTTCCTCCAACTGTCATGTTTCCTCCTCAG   565
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  694  CAGTCTTGGATGCACCAGCCTCACCAGCCTCTTCCTCCAACTGTCATGTTTCCTCCTCAG   753


Query  566  TCCGTGCTGTCCCTTTCTCAGTCCAAAGTCCTGCCTGTTCCCCAGAAAGCAGTGCCCTAT   625
```

```
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  754   TCCGTGCTGTCCCTTTCTCAGTCCAAAGTCCTGCCTGTTCCCCAGAAAGCAGTGCCCTAT   813


Query  626   CCCCAGAGAGATATGCCCATTCAGGCCTTTCTGCTGTACCAGGAGCCTGTACTCGGTCCT   685
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  814   CCCCAGAGAGATATGCCCATTCAGGCCTTTCTGCTGTACCAGGAGCCTGTACTCGGTCCT   873


Query  686   GTCCGGGGACCCTTCCCTATTATTGTC   712
                  |||||||||||||||||||||||||||
Sbjct  874   GTCCGGGGACCCTTCCCTATTATTGTC   900
```

9.3.3.3 Translated BLAST results

beta-casein A3 [Bos taurus]
Sequence ID: gb|AAB29137.1|
Length: 224

Range 1: 16 to 224

Score	Expect	Method	Identities	Positives	Gaps	Frame
281 bits(719)	4e-90	Compositional matrix adjust.	208/209(99%)	208/209(99%)	0/209(0%)	+2

```
Query  86    RELEELNVPGEIVeslssseesITRINKKIEKFqseeqqqtedelqDKIHPFAQTQSLVY   265
             RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY
Sbjct  16    RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY   75


Query  266   PFPGPIPNSLPQNIppltqtpvvvppflqpEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT   445
             PFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPK KEMPFPKYPVEPFT
Sbjct  76    PFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKQKEMPFPKYPVEPFT   135


Query  446   ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFppqsvlslsqskvlpvpQKAVPY   625
             ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY
Sbjct  136   ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY   195


Query  626   PQRDMPIQAFLLYQEPVLGPVRGPFPIIV   712
             PQRDMPIQAFLLYQEPVLGPVRGPFPIIV
Sbjct  196   PQRDMPIQAFLLYQEPVLGPVRGPFPIIV   224
```

beta casein
Sequence ID: prf||1314242A
Length: 209

Range 1: 1 to 209

Score	Expect	Method	Identities	Positives	Gaps	Frame
280 bits(715)	9e-90	Compositional matrix adjust.	208/209(99%)	208/209(99%)	0/209(0%)	+2

```
Query   86    RELEELNVPGEIVeslssseesITRINKKIEKFqseeqqqtedelqDKIHPFAQTQSLVY   265
              RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY
Sbjct   1     RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY   60


Query   266   PFPGPIPNSLPQNIppltqtpvvvppflqpEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT   445
              PFPGPI NSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT
Sbjct   61    PFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT   120


Query   446   ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFppqsvlslsqskvlpvpQKAVPY   625
              ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY
Sbjct   121   ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY   180


Query   626   PQRDMPIQAFLLYQEPVLGPVRGPFPIIV   712
              PQRDMPIQAFLLYQEPVLGPVRGPFPIIV
Sbjct   181   PQRDMPIQAFLLYQEPVLGPVRGPFPIIV   209
```

beta-casein [Bos taurus]
Sequence ID: gb|AAA30431.1|
Length: 224

Range 1: 16 to 224

Score	Expect	Method	Identities	Positives	Gaps	Frame
280 bits(716)	1e-89	Compositional matrix adjust.	208/209(99%)	208/209(99%)	0/209(0%)	+2

```
Query   86    RELEELNVPGEIVeslssseesITRINKKIEKFqseeqqqtedelqDKIHPFAQTQSLVY   265
              RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY
Sbjct   16    RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY   75


Query   266   PFPGPIPNSLPQNIppltqtpvvvppflqpEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT   445
              PFPGPI NSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT
Sbjct   76    PFPGPIHNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT   135


Query   446   ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFppqsvlslsqskvlpvpQKAVPY   625
              ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY
```

```
Sbjct  136  ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY  195


Query  626  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV  712
            PQRDMPIQAFLLYQEPVLGPVRGPFPIIV
Sbjct  196  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV  224
```

beta-casein [Bos taurus]
Sequence ID: gb|AGT56763.1|
Length: 224

Range 1: 16 to 224

Score	Expect	Method	Identities	Positives	Gaps	Frame
279 bits(713)	3e-89	Compositional matrix adjust.	207/209(99%)	207/209(99%)	0/209(0%)	+2

```
Query   86  RELEELNVPGEIVeslssseesITRINKKIEKFqseeqqqtedelqDKIHPFAQTQSLVY  265
            RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY
Sbjct   16  RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVY  75


Query  266  PFPGPIPNSLPQNIppltqtpvvvppflqpEVMGVSKVKEAMAPKHKEMPFPKYPVEPFT  445
            PFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVK AMAPKHKEMPFPKYPVEP T
Sbjct   76  PFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKGAMAPKHKEMPFPKYPVEPLT  135


Query  446  ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFppqsvlslsqskvlpvpQKAVPY  625
            ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY
Sbjct  136  ESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPY  195


Query  626  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV  712
            PQRDMPIQAFLLYQEPVLGPVRGPFPIIV
Sbjct  196  PQRDMPIQAFLLYQEPVLGPVRGPFPIIV  224
```

9.3.4   κ-casein

9.3.4.1   Alignment of sequenced forward and reverse strand

```
Alignment statistics for match #1
Score     Expect      Identities      Gaps        Strand
1210 bits(655)    0.0      660/662(99%)    2/662(0%)     Plus/Minus
Query   13    ATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCAT    72
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  663    ATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCAT   604

Query   73    GACTGGTGGACAGCAAATGGGTCGGGATCCGCAGGAGCAAAACCAAGAACAACCAATACG   132
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  603    GACTGGTGGACAGCAAATGGGTCGGGATCCGCAGGAGCAAAACCAAGAACAACCAATACG   544

Query  133    CTGTGAGAAAGATGAAAGATTCTTCAGTGACAAAATAGCCAAATATATCCCAATTCAGTA   192
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  543    CTGTGAGAAAGATGAAAGATTCTTCAGTGACAAAATAGCCAAATATATCCCAATTCAGTA   484

Query  193    TGTGCTGAGTAGGTATCCTAGTTATGGACTCAATTACTACCAACAGAAACCAGTTGCACT   252
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483    TGTGCTGAGTAGGTATCCTAGTTATGGACTCAATTACTACCAACAGAAACCAGTTGCACT   424

Query  253    AATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGCTGCAGTTAGGTCACC   312
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423    AATTAATAATCAATTTCTGCCATACCCATATTATGCAAAGCCAGCTGCAGTTAGGTCACC   364

Query  313    TGCCCAAATTCTTCAATGGCAAGTTTTGTCAAATACTGTGCCTGCCAAGTCCTGCCAAGC   372
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363    TGCCCAAATTCTTCAATGGCAAGTTTTGTCAAATACTGTGCCTGCCAAGTCCTGCCAAGC   304

Query  373    CCAGCCAACTACCATGGCACGTCACCCACACCCACATTTATCATTTATGGCCATTCCACC   432
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303    CCAGCCAACTACCATGGCACGTCACCCACACCCACATTTATCATTTATGGCCATTCCACC   244

Query  433    AAAGAAAAATCAGGATAAAACAGAAATCCCTACCATCAATACCATTGCTAGTGGTGAGCC   492
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243    AAAGAAAAATCAGGATAAAACAGAAATCCCTACCATCAATACCATTGCTAGTGGTGAGCC   184

Query  493    TACAAGTACACCTACCACCGAAGCAGTAGAGAGCACTGTAGCTACTCTAGAAGATTCTCC   552
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183    TACAAGTACACCTACCACCGAAGCAGTAGAGAGCACTGTAGCTACTCTAGAAGATTCTCC   124

Query  553    AGAAGTTATTGAGAGCCCACCTGAGATCAACACAGTCCAAGTTACTTCAACTGCAGTCCT   612
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123    AGAAGTTATTGAGAGCCCACCTGAGATCAACACAGTCCAAGTTACTTCAACTGCAGTCCT    64

Query  613    CGAGcaccaccaccaccaccacTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGA   672
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||| |
Sbjct   63    CGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAG-AAGCT-A     6

Query  673    GT    674
              ||
Sbjct    5    GT     4
```

9.3.4.2   Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 850Number of Matches: 1
Range 1: 134 to 640

Score	Expect	Identities	Gaps	Strand
926 bits(501)	0.0	505/507(99%)	0/507(0%)	Plus/Plus

```
Query  78   CAGGAGCAAAACCAAGAACAACCAATACGCTGTGAGAAAGATGAAAGATTCTTCAGTGAC   137
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  CAGGAGCAAAACCAAGAACAACCAATACGCTGTGAGAAAGATGAAAGATTCTTCAGTGAC   193


Query  138  AAAATAGCCAAATATATCCCAATTCAGTATGTGCTGAGTAGGTATCCTAGTTATGGACTC   197
            |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  194  AAAATAGCCAAATATATCCCAATTCAGTATGTGCTGAGCAGGTATCCTAGTTATGGACTC   253


Query  198  AATTACTACCAACAGAAACCAGTTGCACTAATTAATAATCAATTTCTGCCATACCCATAT   257
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  AATTACTACCAACAGAAACCAGTTGCACTAATTAATAATCAATTTCTGCCATACCCATAT   313


Query  258  TATGCAAAGCCAGCTGCAGTTAGGTCACCTGCCCAAATTCTTCAATGGCAAGTTTTGTCA   317
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314  TATGCAAAGCCAGCTGCAGTTAGGTCACCTGCCCAAATTCTTCAATGGCAAGTTTTGTCA   373


Query  318  AATACTGTGCCTGCCAAGTCCTGCCAAGCCCAGCCAACTACCATGGCACGTCACCCACAC   377
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374  AATACTGTGCCTGCCAAGTCCTGCCAAGCCCAGCCAACTACCATGGCACGTCACCCACAC   433


Query  378  CCACATTTATCATTTATGGCCATTCCACCAAAGAAAAATCAGGATAAAACAGAAATCCCT   437
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434  CCACATTTATCATTTATGGCCATTCCACCAAAGAAAAATCAGGATAAAACAGAAATCCCT   493


Query  438  ACCATCAATACCATTGCTAGTGGTGAGCCTACAAGTACACCTACCACCGAAGCAGTAGAG   497
            ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  494  ACCATCAATACCATTGCTAGTGGCGAGCCTACAAGTACACCTACCACCGAAGCAGTAGAG   553


Query  498  AGCACTGTAGCTACTCTAGAAGATTCTCCAGAAGTTATTGAGAGCCCACCTGAGATCAAC   557
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  554  AGCACTGTAGCTACTCTAGAAGATTCTCCAGAAGTTATTGAGAGCCCACCTGAGATCAAC   613


Query  558  ACAGTCCAAGTTACTTCAACTGCAGTC   584
            |||||||||||||||||||||||||||
```

```
Sbjct  614   ACAGTCCAAGTTACTTCAACTGCAGTC   640
```

9.3.4.3 Translated BLAST results

kappa-casein precursor [Bos taurus]
Sequence ID: ref|NP_776719.1|
Length: 190

Range 1: 22 to 190

Score	Expect	Method	Identities	Positives	Gaps	Frame
345 bits(884)	8e-116	Compositional matrix adjust.	169/169(100%)	169/169(100%)	0/169(0%)	+3

```
Query   78   QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY   257
             QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY
Sbjct   22   QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY   81


Query  258   YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP   437
             YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP
Sbjct   82   YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP   141


Query  438   TINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV    584
             TINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV
Sbjct  142   TINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV    190
```

kappa casein [Bos taurus]
Sequence ID: gb|AAQ87923.1|
Length: 190Number of Matches: 1

Range 1: 22 to 190

Score	Expect	Method	Identities	Positives	Gaps	Frame
340 bits(871)	8e-114	Compositional matrix adjust.	167/169(99%)	167/169(98%)	0/169(0%)	+3

```
Query   78   QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY   257
             QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY
Sbjct   22   QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY   81


Query  258   YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP   437
             YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP
Sbjct   82   YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP   141


Query  438   TINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV    584
             TINTIASGEPTSTPT EAVESTVATLE SPEVIESPPEINTVQVTSTAV
```

```
Sbjct  142  TINTIASGEPTSTPTIEAVESTVATLEASPEVIESPPEINTVQVTSTAV  190
```

kappa-casein precursor [Bos taurus]
Sequence ID: gb|AAA30433.1|
Length: 190

Range 1: 22 to 190

Score	Expect	Method	Identities	Positives	Gaps	Frame
338 bits(866)	5e-113	Compositional matrix adjust.	166/169(98%)	166/169(98%)	0/169(0%)	+3

```
Query  78   QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY  257
            QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY
Sbjct  22   QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY  81


Query  258  YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP  437
            YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP
Sbjct  82   YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP  141


Query  438  TINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV  584
            TINTIASGEPTSTPT EAVESTVATLE SPEV ESPPEINTVQVTSTAV
Sbjct  142  TINTIASGEPTSTPTIEAVESTVATLEASPEVTESPPEINTVQVTSTAV  190
```

9.3.5 α-lactalbumin

9.3.5.1 Alignment of sequenced forward and reverse strand

```
Alignment statistics for match #1
Score     Expect     Identities     Gaps       Strand
937 bits(507)   0.0     512/514(99%)    2/514(0%)     Plus/Minus
Query  12   ATT-CCCTCTAG-AATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCAT   69
            |||  ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  524  ATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCAT   465

Query  70   GACTGGTGGACAGCAAATGGGTCGGGATCCGGAACAGTTAACAAAATGTGAGGTGTTCCG   129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  464  GACTGGTGGACAGCAAATGGGTCGGGATCCGGAACAGTTAACAAAATGTGAGGTGTTCCG   405

Query  130  GGAGCTGAAAGACTTGAAGGGCTACGGAGGTGTCAGTTTGCCTGAATGGGTCTGTACCAC   189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  404  GGAGCTGAAAGACTTGAAGGGCTACGGAGGTGTCAGTTTGCCTGAATGGGTCTGTACCAC   345

Query  190  GTTTCATACCAGTGGTTATGACACACAAGCCATAGTACAAAACAATGACAGCACAGAATA   249
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  344  GTTTCATACCAGTGGTTATGACACACAAGCCATAGTACAAAACAATGACAGCACAGAATA   285

Query  250  TGGACTCTTCCAGATAAATAATAAAATTTGGTGCAAAGACGACCAGAACCCTCACTCAAG   309
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284  TGGACTCTTCCAGATAAATAATAAAATTTGGTGCAAAGACGACCAGAACCCTCACTCAAG   225

Query  310  CAACATCTGTAACATCTCCTGTGACAAGTTCCTGGATGATGATCTTACTGATGACATTAT   369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  224  CAACATCTGTAACATCTCCTGTGACAAGTTCCTGGATGATGATCTTACTGATGACATTAT   165

Query  370  GTGTGTCAAGAAGATTCTGGATAAAGTAGGAATTAACTACTGGTTGGCCCATAAAGCACT   429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164  GTGTGTCAAGAAGATTCTGGATAAAGTAGGAATTAACTACTGGTTGGCCCATAAAGCACT   105

Query  430  CTGTTCTGAGAAGCTGGATCAGTGGCTCTGTGAGAAGTTGCTCGAGcaccaccaccacca   489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  CTGTTCTGAGAAGCTGGATCAGTGGCTCTGTGAGAAGTTGCTCGAGCACCACCACCACCA   45

Query  490  ccacTGAGATCCGGCTGCTAACAAAGCCCGAAAG     523
            |||||||||||||||||||||||||||||||||
Sbjct  44   CCACTGAGATCCGGCTGCTAACAAAGCCCGAAAG     11
```

9.3.5.2 *Alignment of forward strand and clone sequence*

Subject: clone sequence; Query: determined sequence

Length: 703

Range 1: 66 to 434

Score	Expect	Identities	Gaps	Strand
682 bits(369)	0.0	369/369(100%)	0/369(0%)	Plus/Minus

```
Query  58   CAACTTCTCACAGAGCCACTGATCCAGCTTCTCAGAACAGAGTGCTTTATGGGCCAACCA  117
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  434  CAACTTCTCACAGAGCCACTGATCCAGCTTCTCAGAACAGAGTGCTTTATGGGCCAACCA  375


Query  118  GTAGTTAATTCCTACTTTATCCAGAATCTTCTTGACACACATAATGTCATCAGTAAGATC  177
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  374  GTAGTTAATTCCTACTTTATCCAGAATCTTCTTGACACACATAATGTCATCAGTAAGATC  315


Query  178  ATCATCCAGGAACTTGTCACAGGAGATGTTACAGATGTTGCTTGAGTGAGGGTTCTGGTC  237
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  314  ATCATCCAGGAACTTGTCACAGGAGATGTTACAGATGTTGCTTGAGTGAGGGTTCTGGTC  255


Query  238  GTCTTTGCACCAAATTTTATTATTTATCTGGAAGAGTCCATATTCTGTGCTGTCATTGTT  297
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  254  GTCTTTGCACCAAATTTTATTATTTATCTGGAAGAGTCCATATTCTGTGCTGTCATTGTT  195


Query  298  TTGTACTATGGCTTGTGTGTCATAACCACTGGTATGAAACGTGGTACAGACCCATTCAGG  357
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  194  TTGTACTATGGCTTGTGTGTCATAACCACTGGTATGAAACGTGGTACAGACCCATTCAGG  135


Query  358  CAAACTGACACCTCCGTAGCCCTTCAAGTCTTTCAGCTCCCGGAACACCTCACATTTTGT  417
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  134  CAAACTGACACCTCCGTAGCCCTTCAAGTCTTTCAGCTCCCGGAACACCTCACATTTTGT  75


Query  418  TAACTGTTC  426
            |||||||||
Sbjct  74   TAACTGTTC  66
```

9.3.5.3 Translated BLAST results

alpha-lactalbumin precursor [Bos taurus]
Sequence ID: ref|NP_776803.1|
Length: 142

Range 1: 19 to 142

Score	Expect	Method	Identities	Positives	Gaps	Frame
230 bits(587)	1e-71	Compositional matrix adjust.	123/124(99%)	123/124(99%)	0/124(0%)	+3

```
Query   81   PEQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKI   260
              EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKI
Sbjct   19   AEQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKI   78


Query  261   WCKDDQNPHSSNICNISCdkfldddltddIMCVKKILDKVGINYWLAHKALCSEKLDQWL   440
             WCKDDQNPHSSNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWL
Sbjct   79   WCKDDQNPHSSNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWL   138


Query  441   CEKL   452
             CEKL
Sbjct  139   CEKL   142
```

Chain A, Crystal Structure Of Apo-Bovine Alpha-Lactalbumin
Sequence ID: pdb|1F6R|A
Length: 123

Range 1: 1 to 123

Score	Expect	Method	Identities	Positives	Gaps	Frame
228 bits(582)	4e-71	Compositional matrix adjust.	123/123(100%)	123/123(100%)	0/123(0%)	+3

```
Query   84   EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIW   263
             EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIW
Sbjct    1   EQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKIW   60


Query  264   CKDDQNPHSSNICNISCdkfldddltddIMCVKKILDKVGINYWLAHKALCSEKLDQWLC   443
             CKDDQNPHSSNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWLC
Sbjct   61   CKDDQNPHSSNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWLC   120


Query  444   EKL   452
             EKL
Sbjct  121   EKL   123
```

alpha lactalbumin [Bos taurus]
Sequence ID: gb|AAF63624.1|AF249896_1
Length: 142

Range 1: 19 to 142

Score	Expect	Method	Identities	Positives	Gaps	Frame
229 bits(583)	4e-71	Compositional matrix adjust.	122/124(98%)	122/124(98%)	0/124(0%)	+3

```
Query  81   PEQLTKCEVFRELKDLKGYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNKI   260
             EQLTKCEVFRELKDLKGYGGVSLPEWVCT FHTSGYDTQAIVQNNDSTEYGLFQINNKI
Sbjct  19   AEQLTKCEVFRELKDLKGYGGVSLPEWVCTAFHTSGYDTQAIVQNNDSTEYGLFQINNKI   78


Query  261  WCKDDQNPHSSNICNISCdkfldddltddIMCVKKILDKVGINYWLAHKALCSEKLDQWL   440
            WCKDDQNPHSSNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWL
Sbjct  79   WCKDDQNPHSSNICNISCDKFLDDDLTDDIMCVKKILDKVGINYWLAHKALCSEKLDQWL   138


Query  441  CEKL   452
            CEKL
Sbjct  139  CEKL   142
```

9.3.6   β-lactoglobulin

9.3.6.1   Alignment of sequenced forward and reverse strand

```
Alignment statistics for match #1
Score     Expect      Identities      Gaps        Strand
1158 bits(627)   0.0     637/641(99%)    4/641(0%)      Plus/Minus
Query  8     AAC-ATT-CCCTCTAG-AATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTA   64
             ||| ||| ||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  645   AACAATTCCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTA   586

Query  65    GCATGACTGGTGGACAGCAAATGGGTCGGGATCCGCTCATCGTCACCCAGACCATGAAGG   124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  585   GCATGACTGGTGGACAGCAAATGGGTCGGGATCCGCTCATCGTCACCCAGACCATGAAGG   526

Query  125   GCCTGGATATCCAGAAGGTGGCGGGGACTTGGTACTCCTTGGCCATGGCGGCCAGCGACA   184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  525   GCCTGGATATCCAGAAGGTGGCGGGGACTTGGTACTCCTTGGCCATGGCGGCCAGCGACA   466

Query  185   TCTCCCTGCTGGACGCCCAGAGTGCCCCCCTGAGAGTGTATGTGGAGGAGCTGAAGCCCA   244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  465   TCTCCCTGCTGGACGCCCAGAGTGCCCCCCTGAGAGTGTATGTGGAGGAGCTGAAGCCCA   406

Query  245   CCCCTGAGGGCGACCTGGAGATCCTGCTGCAGAAATGGGAGAATGATGAGTGTGCTCAGA   304
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  405   CCCCTGAGGGCGACCTGGAGATCCTGCTGCAGAAATGGGAGAATGATGAGTGTGCTCAGA   346

Query  305   AGAAGATCATTGCAGAAAAAACCAAGATCCCTGCGGTGTTCAAGATCGATGCCTTGAACG   364
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  345   AGAAGATCATTGCAGAAAAAACCAAGATCCCTGCGGTGTTCAAGATCGATGCCTTGAACG   286

Query  365   AGAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGCTCTTCTGCATGGAGA   424
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  285   AGAACAAAGTCCTTGTGCTGGACACCGACTACAAAAAGTACCTGCTCTTCTGCATGGAGA   226

Query  425   ACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAGTGCCTGGTCAGGACCCCGGAGGTGG   484
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  225   ACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAGTGCCTGGTCAGGACCCCGGAGGTGG   166

Query  485   ACGACGAGGCCCTGGAGAAATTCGACAAAGCCCTCAAGGCCCTGCCCATGCACATCCGGC   544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  165   ACGACGAGGCCCTGGAGAAATTCGACAAAGCCCTCAAGGCCCTGCCCATGCACATCCGGC   106

Query  545   TGTCCTTCAACCCAACCCAGCTGGAGGAGCAGTGCCACATCCTCGAGcaccaccaccacc   604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  105   TGTCCTTCAACCCAACCCAGCTGGAGGAGCAGTGCCACATCCTCGAGCACCACCACCACC   46

Query  605   accacTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCT    645
             |||||||||||||||||||||||||||||||||| |||||
Sbjct  45    ACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAG-AAGCT    6
```

9.3.6.2   Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 790

Range 1: 94 to 579

Score	Expect	Identities	Gaps	Strand
893 bits(483)	0.0	485/486(99%)	0/486(0%)	Plus/Plus

```
Query   81   CTCATCGTCACCCAGACCATGAAGGGCCTGGATATCCAGAAGGTGGCGGGGACTTGGTAC   140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   94   CTCATCGTCACCCAGACCATGAAGGGCCTGGATATCCAGAAGGTGGCGGGGACTTGGTAC   153


Query   141  TCCTTGGCCATGGCGGCCAGCGACATCTCCCTGCTGGACGCCCAGAGTGCCCCCCTGAGA   200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   154  TCCTTGGCCATGGCGGCCAGCGACATCTCCCTGCTGGACGCCCAGAGTGCCCCCCTGAGA   213


Query   201  GTGTATGTGGAGGAGCTGAAGCCCACCCCTGAGGGCGACCTGGAGATCCTGCTGCAGAAA   260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   214  GTGTATGTGGAGGAGCTGAAGCCCACCCCTGAGGGCGACCTGGAGATCCTGCTGCAGAAA   273


Query   261  TGGGAGAATGATGAGTGTGCTCAGAAGAAGATCATTGCAGAAAAAACCAAGATCCCTGCG   320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   274  TGGGAGAATGATGAGTGTGCTCAGAAGAAGATCATTGCAGAAAAAACCAAGATCCCTGCG   333


Query   321  GTGTTCAAGATCGATGCCTTGAACGAGAACAAAGTCCTTGTGCTGGACACCGACTACAAA   380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   334  GTGTTCAAGATCGATGCCTTGAACGAGAACAAAGTCCTTGTGCTGGACACCGACTACAAA   393


Query   381  AAGTACCTGCTCTTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAG   440
             |||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   394  AAGTACCTGCTCGTCTGCATGGAGAACAGTGCTGAGCCCGAGCAAAGCCTGGTCTGCCAG   453


Query   441  TGCCTGGTCAGGACCCCGGAGGTGGACGACGAGGCCCTGGAGAAATTCGACAAAGCCCTC   500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   454  TGCCTGGTCAGGACCCCGGAGGTGGACGACGAGGCCCTGGAGAAATTCGACAAAGCCCTC   513


Query   501  AAGGCCCTGCCCATGCACATCCGGCTGTCCTTCAACCCAACCCAGCTGGAGGAGCAGTGC   560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   514  AAGGCCCTGCCCATGCACATCCGGCTGTCCTTCAACCCAACCCAGCTGGAGGAGCAGTGC   573


Query   561  CACATC   566
             ||||||
```

9.3.6.3 *Translated BLAST results*

major allergen beta-lactoglobulin [Bos taurus]
Sequence ID: gb|ACG59280.1|
Length: 178

Range 1: 13 to 178

Score	Expect	Method	Identities	Positives	Gaps	Frame
332 bits(852)	1e-109	Compositional matrix adjust.	163/166(98%)	163/166(98%)	0/166(0%)	+1

```
Query   88   GRDPLIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI   267
             G    LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI
Sbjct   13   GAQALIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI   72


Query   268  LLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQS   447
             LLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQS
Sbjct   73   LLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQS   132


Query   448  LVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI   585
             LVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI
Sbjct   133  LVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI   178
```

beta-lactoglobulin [Bos taurus]
Sequence ID: emb|CAA32835.1|
Length: 178

Range 1: 13 to 178

Score	Expect	Method	Identities	Positives	Gaps	Frame
329 bits(844)	2e-108	Compositional matrix adjust.	162/166(98%)	162/166(97%)	0/166(0%)	+1

```
Query   88   GRDPLIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI   267
             G    LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI
Sbjct   13   GAQALIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGDLEI   72


Query   268  LLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQS   447
             LLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLL CMENSAEPEQS
Sbjct   73   LLQKWENDECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLVCMENSAEPEQS   132


Query   448  LVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI   585
             LVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI
Sbjct   133  LVCQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCHI   178
```

9.3.7 ovomucoid

9.3.7.1 Alignment of sequenced forward and reverse strand

```
Alignment statistics for match #1
Score      Expect     Identities     Gaps       Strand
1282 bits(694)    0.0     697/698(99%)    1/698(0%)    Plus/Minus
Query  1     CCTCTAG-AATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCATGACTG   59
             ||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  699   CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGGCTAGCATGACTG   640

Query  60    GTGGACAGCAAATGGGTCGGGATCCGGCTGAGGTGGATTGCAGTAGGTTTCCCAACGCTA   119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  639   GTGGACAGCAAATGGGTCGGGATCCGGCTGAGGTGGATTGCAGTAGGTTTCCCAACGCTA   580

Query  120   CAGACAAGGAAGGCAAAGATGTATTGGTTTGCAACAAGGACCTCCGCCCCATCTGTGGTA   179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  579   CAGACAAGGAAGGCAAAGATGTATTGGTTTGCAACAAGGACCTCCGCCCCATCTGTGGTA   520

Query  180   CCGATGGAGTCACTTACACCAACGATTGCTTGCTGTGTGCCTACAGCATAGAATTTGGAA   239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  519   CCGATGGAGTCACTTACACCAACGATTGCTTGCTGTGTGCCTACAGCATAGAATTTGGAA   460

Query  240   CCAATATCAGCAAAGAGCACGATGGAGAATGCAAGGAAACTGTTCCTATGAACTGCAGTA   299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  459   CCAATATCAGCAAAGAGCACGATGGAGAATGCAAGGAAACTGTTCCTATGAACTGCAGTA   400

Query  300   GTTATGCCAACACGACAAGCGAGGACGGAAAAGTGATGGTCCTCTGCAACAGGGCCTTCA   359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  399   GTTATGCCAACACGACAAGCGAGGACGGAAAAGTGATGGTCCTCTGCAACAGGGCCTTCA   340

Query  360   ACCCCGTCTGTGGTACTGATGGAGTCACCTACGACAATGAGTGTCTGCTGTGTGCCCACA   419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  339   ACCCCGTCTGTGGTACTGATGGAGTCACCTACGACAATGAGTGTCTGCTGTGTGCCCACA   280

Query  420   AAGTAGAGCAGGGGGCCAGCGTTGACAAGAGGCATGATGGTGGATGTAGGAAGGAACTTG   479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  279   AAGTAGAGCAGGGGGCCAGCGTTGACAAGAGGCATGATGGTGGATGTAGGAAGGAACTTG   220

Query  480   CTGCTGTGAGTGTTGACTGCAGTGAGTACCCTAAGCCTGACTGCACGGCAGAAGACAGAC   539
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  219   CTGCTGTGAGTGTTGACTGCAGTGAGTACCCTAAGCCTGACTGCACGGCAGAAGACAGAC   160

Query  540   CTCTCTGTGGCTCCGACAACAAAACATATGGCAACAAGTGCAACTTCTGCAATGCAGTCG   599
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159   CTCTCTGTGGCTCCGACAACAAAACATATGGCAACAAGTGCAACTTCTGCAATGCAGTCG   100

Query  600   TGGAAAGCAACGGGACTCTCACCTTAAGCCATTTTGGAAAATGCCTCGAGcaccaccacc   659
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99    TGGAAAGCAACGGGACTCTCACCTTAAGCCATTTTGGAAAATGCCTCGAGCACCACCACC   40

Query  660   accaccacTGAGATCCGGCTGCTAACAAAGCCCGAAAG   697
             ||||||||||||||||||||||||||||||||||||||
Sbjct  39    ACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAG   2
```

9.3.7.2 Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 952
Range 1: 261 to 813

Score	Expect	Identities	Gaps	Strand
966 bits(523)	0.0	548/559(98%)	6/559(1%)	Plus/Plus

```
Query  85   GGCTGAGGTGGATTGCAGTAGGTTTCCCAACGCTACAGACAAGGAAGGCAAAGATGTATT  144
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  GGCTGAGGTGGACTGCAGTAGGTTTCCCAACGCTACAGACAAGGAAGGCAAAGATGTATT  320


Query  145  GGTTTGCAACAAGGACCTCCGCCCCATCTGTGGTACCGATGGAGTCACTTACACCAACGA  204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  GGTTTGCAACAAGGACCTCCGCCCCATCTGTGGTACCGATGGAGTCACTTACACCAACGA  380


Query  205  TTGCTTGCTGTGTGCCTACAGCATAGAATTTGGAACCAATATCAGCAAAGAGCACGATGG  264
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  TTGCTTGCTGTGTGCCTACAGCATAGAATTTGGAACCAATATCAGCAAAGAGCACGATGG  440


Query  265  AGAATGCAAGGAAACTGTTCCTATGAACTGCAGTAGTTATGCCAACACGACAAGCGAGGA  324
            ||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  441  AGAATGCAAGGAAACTGTTCCTATGAACTGCAGTAGTTATGCCAACACAACAAGCGAGGA  500


Query  325  CGGAAAAGTGATGGTCCTCTGCAACAGGGCCTTCAACCCCGTCTGTGGTACTGATGGAGT  384
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  CGGAAAAGTGATGGTCCTCTGCAACAGGGCCTTCAACCCCGTCTGTGGTACTGATGGAGT  560


Query  385  CACCTACGACAATGAGTGTCTGCTGTGTGCCCACAAAGTAGAGCAGGGGGCCAGCGTTGA  444
            ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  561  CACCTACGACAATGAGTGTCTTCTGTGTGCCCACAAAGTAGAGCAGGGGGCCAGCGTTGA  620


Query  445  CAAGAGGCATGATGGTGGATGTAGGAAGGAACTTGCTGCTGTGAGTGTTGACTGCAGTGA  504
            ||||||||||||||||||||||||||||||||||||||||       |||||||||| ||
Sbjct  621  CAAGAGGCATGATGGTGGATGTAGGAAGGAACTTGCTGC------TGTTGACTGCAGCGA  674


Query  505  GTACCCTAAGCCTGACTGCACGGCAGAAGACAGACCTCTCTGTGGCTCCGACAACAAAAC  564
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  675  GTACCCTAAGCCTGACTGCACGGCAGAAGACAGACCTCTCTGTGGCTCCGACAACAAAAC  734


Query  565  ATATGGCAACAAGTGCAACTTCTGCAATGCAGTCGTGGAAAGCAACGGGACTCTCACCTT  624
```

```
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct 735  ATATGGCAACAAGTGCAACTTCTGCAATGCAGTCGTGGAAAGCAACGGGACTCTCACTTT  794


Query 625  AAGCCATTTTGGAAAATGC  643
           |||||||||||||||||||
Sbjct 795  AAGCCATTTTGGAAAATGC  813
```

9.3.7.3 Translated BLAST results

ovomucoid isoform 1 precursor [Gallus gallus]
Sequence ID: ref|NP_001295423.1|
Length: 210
Range 1: 25 to 210

Score	Expect	Method	Identities	Positives	Gaps	Frame
379 bits(974)	9e-129	Compositional matrix adjust.	186/186(100%)	186/186(100%)	0/186(0%)	+2

```
Query 86   AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSIEFGTNISKEHDG  265
           AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSIEFGTNISKEHDG
Sbjct 25   AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSIEFGTNISKEHDG  84


Query 266  ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD  445
           ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD
Sbjct 85   ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD  144


Query 446  KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL  625
           KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL
Sbjct 145  KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL  204


Query 626  SHFGKC  643
           SHFGKC
Sbjct 205  SHFGKC  210
```

ovomucoid [Gallus gallus]
Sequence ID: gb|ACJ04729.1|
Length: 210
Range 1: 25 to 210

Score	Expect	Method	Identities	Positives	Gaps	Frame
376 bits(966)	1e-127	Compositional matrix adjust.	184/186(99%)	185/186(99%)	0/186(0%)	+2

```
Query 86   AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSIEFGTNISKEHDG  265
           AEVDCSRFPNATD EGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYS+EFGTNISKEHDG
Sbjct 25   AEVDCSRFPNATDMEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSVEFGTNISKEHDG  84
```

```
Query  266   ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD   445
             ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD
Sbjct  85    ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD   144


Query  446   KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL   625
             KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL
Sbjct  145   KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL   204


Query  626   SHFGKC   643
             SHFGKC
Sbjct  205   SHFGKC   210
```

ovomucoid [Gallus gallus]
Sequence ID: prf||0807280A
Length: 186

Range 1: 1 to 186

Score	Expect	Method	Identities	Positives	Gaps	Frame
372 bits(955)	2e-126	Compositional matrix adjust.	183/186(98%)	184/186(98%)	0/186(0%)	+2

```
Query  86    AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYTNDCLLCAYSIEFGTNISKEHDG   265
             AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTY N+CLLCAYSIEFGTNISKEHDG
Sbjct  1     AEVDCSRFPNATDKEGKDVLVCNKDLRPICGTDGVTYNNECLLCAYSIEFGTNISKEHDG   60


Query  266   ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD   445
             ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD
Sbjct  61    ECKETVPMNCSSYANTTSEDGKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGASVD   120


Query  446   KRHDGGCRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL   625
             KRHDG CRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL
Sbjct  121   KRHDGECRKELAAVSVDCSEYPKPDCTAEDRPLCGSDNKTYGNKCNFCNAVVESNGTLTL   180


Query  626   SHFGKC   643
             SHFGKC
Sbjct  181   SHFGKC   186
```

9.3.8   ovalbumin

9.3.8.1   Alignment of sequenced forward and reverse strand

```
Alignment statistics for match #1
Score      Expect    Identities        Gaps        Strand
1659 bits(898)    0.0    1104/1195(92%)    47/1195(3%)    Plus/Minus
Query   98    ACGGCGTTGGTTGCGATGTG-CTTGA-TACAGA-AGAGGAA-TGGA-TGGTCA-GCCCTA   151
              ||| ||| | ||||||||||| ||||| |||||| ||| ||| || | || ||| ||||||
Sbjct   1245  ACGACGTAGTTTGCGATGTGACTTGATTACAGAGAGATGAATTGAAGTGTTCACGCCCTA   1186

Query   152   AATTCTTCAGAGACGCTTGCA-GCA-TCCACTCCAGCCTCTGCTGACCCTA-CCACC-TC   207
              |||  |||||||||| ||||  |||| ||| || | | |||||||||||||||| |||||| |
Sbjct   1185  AATCTTTCAGAGACGTCTGCAGGCACTCAATTCCAGCCTCTGCTGACCCTAACCACCTTT   1126

Query   208   TCTGCCTGCTTCATTGATTTC-TGCATGT-GCTGCATGGACAGCTTGAGATATCTTCAGG   265
              | ||||||||||||||||||| ||||||| |||||| ||||| |||||| ||||||||||||
Sbjct   1125  TTTGCCTGCTTCATTGATTTCGTGCATGTGGCTGCAT-GACAGC-TGAGATATCTTCAGG   1068

Query   266   CTCTCTGCTGAGGAGATGCCAGACAGATTGGCTGAAGAGCTAAACACGTCAGTAATGCCC   325
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1067  CTCTCTGCTGAGGAGATGCCAGACAGATTGGCTGAAGAGCTAAACACGTCAGTAATGCCC   1008

Query   326   ATAGCCATTAAGACAGATGTGAGGTTGTATTTTTCCTCCATCTTCATGCGAGGTAAGTAC   385
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   1007  ATAGCCATTAAGACAGATGTGAGGTTGTATTTTTCCTCCATCTTCATGCGAGGTAAGTAC   948

Query   386   ACTTTGATCTTCCTCTCTTCCATAACATTAGAACTGGTCCATTCAGTCAGTTTTTCAAAG   445
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   947   ACTTTGATCTTCCTCTCTTCCATAACATTAGAACTGGTCCATTCAGTCAGTTTTTCAAAG   888

Query   446   TTGATTATACTCTCAAGCTGCTCAAGGCCTGAGACTTCATCAGGCAACAGCACCAACATG   505
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   887   TTGATTATACTCTCAAGCTGCTCAAGGCCTGAGACTTCATCAGGCAACAGCACCAACATG   828

Query   506   CTCATTGTCCCACTGGCAAATGGAAGCTCCAGGATCTTCATTTTCTCAGAAGCCATTGAT   565
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   827   CTCATTGTCCCACTGGCAAATGGAAGCTCCAGGATCTTCATTTTCTCAGAAGCCATTGAT   768

Query   566   GCCACTCTAAATAAACCAATCTGGTACATCATCTGCACAGGTTTGCTTTCTTGCTCAGTC   625
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   767   GCCACTCTAAATAAACCAATCTGGTACATCATCTGCACAGGTTTGCTTTCTTGCTCAGTC   708

Query   626   ACTCTGAAAGGCATTGCTTGTGTGTCTTCATCCTTAAATGCTTTCTCCCACAGTCCTTTG   685
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   707   ACTCTGAAAGGCATTGCTTGTGTGTCTTCATCCTTAAATGCTTTCTCCCACAGTCCTTTG   648

Query   686   AAGACAATGGCATTAACCAGAACCATTGCAGTTTGAGAATCCACGGAGCTTGGCTGAAGG   745
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   647   AAGACAATGGCATTAACCAGAACCATTGCAGTTTGAGAATCCACGGAGCTTGGCTGAAGG   588

Query   746   ACATTTCTGATAATTCCATTTGTCTGACTTTCTACCCAGGAATTGATGAGCTCTCTGGCT   805
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   587   ACATTTCTGATAATTCCATTTGTCTGACTTTCTACCCAGGAATTGATGAGCTCTCTGGCT   528

Query   806   TGATCTGCAGCTGTTTGAAAGTTGATAGGTTCCAAGCCTCCTCTATACAGTTCCTTCACA   865
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct   527   TGATCTGCAGCTGTTTGAAAGTTGATAGGTTCCAAGCCTCCTCTATACAGTTCCTTCACA   468

Query   866   CACTGCAAGTATTCTGGCAGGATTGGGTATCTCTCTTTCAGCATAAAGTCTACTGGCAAG   925
              |||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||
Sbjct   467   CACTGCAAGTATTCTGGCAGGATTGGGTATCTCTCTT-CAGCATAAAGTCTACTGGCAAG   409

Query   926   GCTGA-CGAATAAACATCATTTGGTTCG-TGATTTGGTTGAGGATGTCTCTAAGTGAAGA   983
              ||||| |||||||||||||||||||| | ||||||||||||||||||||||||||||||||
Sbjct   408   GCTGAACGAATAAACATCATTTGGTTTGGTGATTTGGTTGAGGATGTCTCTAAGTGAAGA   349

Query   984   GTGA-CGTTCTACAGATGTTGCCACACTGAGCCTTCA-TCCTGGTCTCCGAATCCCTGAA   1041
              |||| |||| ||||||| |||||||||||||| |||| | ||| |||||||||||| |||
Sbjct   348   GTGAACGTT-TACAGATG-TGCCACACTGAGC-TTCAATACTG-TCTCCGAATCCTGGAA   293

Query   1042  GTT-ATCAA-GCG-ACAACCTTATTTATTTGTGTCCTTGGTGCTGTCTTTTGCCCCCAGG   1098
              ||| |||||| ||| |||||||||||||||||||||| ||||||||||||||| ||||||
Sbjct   292   GTTTATCAAAGCGAACAACCTTATTTATTTGTGTCCT-GGTGCTGTCTTTTGCACCCAGG   234

Query   1099  -ATACCACGGCTACAGCCGGACTTGATGGCA-TGGGGCAGCTGAGCACGTACTCCATGTG   1156
               ||||||| ||||| ||| | || ||||||||| |||||||| || | ||| ||| || |
Sbjct   233   TATACCATGGCTAGAGCTG-ACATGATGGCAATGGGGCAGTAGAAGATGTTCTC-ATTGG   176

Query   1157  CATGGCGGACCTTTGAGCCCCTGTGAATCCATTCATATACAGA-TTCCACGTGCCTGAT-   1214
              ||||| ||||| |||||||||| ||| ||||| ||| || | ||| | ||||| ||| || |
Sbjct   175   CATGGTGGAC-TTTGAGCTCCT-TGAATACAT-CAAA-ACAAAATTCCA--TGCTTGCTG   122

Query   1215  CGTCCCCATGGCAACCAATCGGAATCCGGACC-ATTCTGCGGTTCTCACTAGTCA   1268
              || || |||| | || |||||| ||| |||| ||| ||| || | ||| ||||||
Sbjct   121   CG-CCG-ATGG-AGCCCATCGGA-TCCCGACCCATT-TGCTGT-C-CACCAGTCA   74
```

9.3.8.2 Alignment of forward strand and clone sequence

Subject: clone sequence; Query: determined sequence

Length: 1872
Range 1: 65 to 1006

Score	Expect	Identities	Gaps	Strand
1729 bits(936)	0.0	940/942(99%)	0/942(0%)	Plus/Plus

```
Query  83    ATGGGCTCCATCGGCGCAGCAAGCATGGAATTTTGTTTTGATGTATTCAAGGAGCTCAAA  142
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65    ATGGGCTCCATCGGCGCAGCAAGCATGGAATTTTGTTTTGATGTATTCAAGGAGCTCAAA  124


Query  143   GTCCACCATGCCAATGAGAACATCTTCTACTGCCCCATTGCCATCATGTCAGCTCTAGCC  202
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  125   GTCCACCATGCCAATGAGAACATCTTCTACTGCCCCATTGCCATCATGTCAGCTCTAGCC  184


Query  203   ATGGTATACCTGGGTGCAAAAGACAGCACCAGGACACAAATAAATAAGGTTGTTCGCTTT  262
             |||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||
Sbjct  185   ATGGTATACCTGGGTGCAAAAGACAGCACCAGGACACAGATAAATAAGGTTGTTCGCTTT  244


Query  263   GATAAACTTCCAGGATTCGGAGACAGTATTGAAGCTCAGTGTGGCACATCTGTAAACGTT  322
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245   GATAAACTTCCAGGATTCGGAGACAGTATTGAAGCTCAGTGTGGCACATCTGTAAACGTT  304


Query  323   CACTCTTCACTTAGAGACATCCTCAACCAAATCACCAAACCAAATGATGTTTATTCGTTC  382
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305   CACTCTTCACTTAGAGACATCCTCAACCAAATCACCAAACCAAATGATGTTTATTCGTTC  364


Query  383   AGCCTTGCCAGTAGACTTTATGCTGAAGAGAGATACCCAATCCTGCCAGAATACTTGCAG  442
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365   AGCCTTGCCAGTAGACTTTATGCTGAAGAGAGATACCCAATCCTGCCAGAATACTTGCAG  424


Query  443   TGTGTGAAGGAACTGTATAGAGGAGGCTTGGAACCTATCAACTTTCAAACAGCTGCAGAT  502
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  425   TGTGTGAAGGAACTGTATAGAGGAGGCTTGGAACCTATCAACTTTCAAACAGCTGCAGAT  484


Query  503   CAAGCCAGAGAGCTCATCAATTCCTGGGTAGAAAGTCAGACAAATGGAATTATCAGAAAT  562
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485   CAAGCCAGAGAGCTCATCAATTCCTGGGTAGAAAGTCAGACAAATGGAATTATCAGAAAT  544


Query  563   GTCCTTCAGCCAAGCTCCGTGGATTCTCAAACTGCAATGGTTCTGGTTAATGCCATTGTC  622
```

```
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545   GTCCTTCAGCCAAGCTCCGTGGATTCTCAAACTGCAATGGTTCTGGTTAATGCCATTGTC   604


Query  623   TTCAAAGGACTGTGGGAGAAAGCATTTAAGGATGAAGACACACAAGCAATGCCTTTCAGA   682
                |||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||
Sbjct  605   TTCAAAGGACTGTGGGAGAAAACATTTAAGGATGAAGACACACAAGCAATGCCTTTCAGA   664


Query  683   GTGACTGAGCAAGAAAGCAAACCTGTGCAGATGATGTACCAGATTGGTTTATTTAGAGTG   742
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  665   GTGACTGAGCAAGAAAGCAAACCTGTGCAGATGATGTACCAGATTGGTTTATTTAGAGTG   724


Query  743   GCATCAATGGCTTCTGAGAAAATGAAGATCCTGGAGCTTCCATTTGCCAGTGGGACAATG   802
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  725   GCATCAATGGCTTCTGAGAAAATGAAGATCCTGGAGCTTCCATTTGCCAGTGGGACAATG   784


Query  803   AGCATGTTGGTGCTGTTGCCTGATGAAGTCTCAGGCCTTGAGCAGCTTGAGAGTATAATC   862
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  785   AGCATGTTGGTGCTGTTGCCTGATGAAGTCTCAGGCCTTGAGCAGCTTGAGAGTATAATC   844


Query  863   AACTTTGAAAAACTGACTGAATGGACCAGTTCTAATGTTATGGAAGAGAGGAAGATCAAA   922
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  845   AACTTTGAAAAACTGACTGAATGGACCAGTTCTAATGTTATGGAAGAGAGGAAGATCAAA   904


Query  923   GTGTACTTACCTCGCATGAAGATGGAGGAAAAATACAACCTCACATCTGTCTTAATGGCT   982
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  905   GTGTACTTACCTCGCATGAAGATGGAGGAAAAATACAACCTCACATCTGTCTTAATGGCT   964


Query  983   ATGGGCATTACTGACGTGTTTAGCTCTTCAGCCAATCTGTCT   1024
                ||||||||||||||||||||||||||||||||||||||||||
Sbjct  965   ATGGGCATTACTGACGTGTTTAGCTCTTCAGCCAATCTGTCT   1006
```

9.3.8.3 *Translated BLAST results*

RecName: Full=Ovalbumin; AltName: Full=Allergen Gal d II; AltName: Full=Egg albumin; AltName: Full=Plakalbumin; AltName: Allergen=Gal d 2
Sequence ID: sp|P01012.2|OVAL_CHICK
Length: 386
Range 1: 1 to 314

Score	Expect	Method	Identities	Positives	Gaps	Frame
654 bits(1687)	0.0	Compositional matrix adjust.	314/314(100%)	314/314(100%)	0/314(0%)	+2

```
Query  83   MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF   262
            MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF
Sbjct  1    MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF   60


Query  263  DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ   442
            DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ
Sbjct  61   DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ   120


Query  443  CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV   622
            CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV
Sbjct  121  CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV   180


Query  623  FKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM   802
            FKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM
Sbjct  181  FKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM   240


Query  803  SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA   982
            SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA
Sbjct  241  SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA   300


Query  983  MGITDVFSSSANLS   1024
            MGITDVFSSSANLS
Sbjct  301  MGITDVFSSSANLS   314
```

ovalbumin precursor [Gallus gallus]
Sequence ID: ref|NP_990483.1|
Length: 386
Range 1: 1 to 314

Score	Expect	Method	Identities	Positives	Gaps	Frame
652 bits(1683)	0.0	Compositional matrix adjust.	313/314(99%)	313/314(99%)	0/314(0%)	+2

```
Query  83   MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF   262
            MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF
Sbjct  1    MGSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRF   60


Query  263  DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ   442
            DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ
Sbjct  61   DKLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQ   120


Query  443  CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV   622
            CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV
Sbjct  121  CVKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIV   180


Query  623  FKGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM   802
            FKGLWEK FKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM
Sbjct  181  FKGLWEKTFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTM   240


Query  803  SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA   982
            SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA
Sbjct  241  SMLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMA   300


Query  983  MGITDVFSSSANLS   1024
            MGITDVFSSSANLS
Sbjct  301  MGITDVFSSSANLS   314
```

ovalbumin [Gallus gallus]
Sequence ID: prf||0705172A
Length: 385
Range 1: 1 to 313

Score	Expect	Method	Identities	Positives	Gaps	Frame
652 bits(1681)	0.0	Compositional matrix adjust.	313/313(100%)	313/313(100%)	0/313(0%)	+2

```
Query  86   GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRFD   265
            GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRFD
Sbjct  1    GSIGAASMEFCFDVFKELKVHHANENIFYCPIAIMSALAMVYLGAKDSTRTQINKVVRFD   60


Query  266  KLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQC   445
            KLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQC
Sbjct  61   KLPGFGDSIEAQCGTSVNVHSSLRDILNQITKPNDVYSFSLASRLYAEERYPILPEYLQC   120


Query  446  VKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIVF   625
            VKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIVF
Sbjct  121  VKELYRGGLEPINFQTAADQARELINSWVESQTNGIIRNVLQPSSVDSQTAMVLVNAIVF   180


Query  626  KGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMS   805
            KGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMS
Sbjct  181  KGLWEKAFKDEDTQAMPFRVTEQESKPVQMMYQIGLFRVASMASEKMKILELPFASGTMS   240


Query  806  MLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMAM   985
            MLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMAM
Sbjct  241  MLVLLPDEVSGLEQLESIINFEKLTEWTSSNVMEERKIKVYLPRMKMEEKYNLTSVLMAM   300


Query  986  GITDVFSSSANLS   1024
            GITDVFSSSANLS
Sbjct  301  GITDVFSSSANLS   313
```

10 Publikationsliste

Sievers S, Rawel HM, Ringel KP, Niggemann B, Beyer K.: Wheat protein recognition pattern in tolerant and allergic children. Pediatr Allergy Immunol. 2016 Mar;27(2):147-55.

submitted:

Gerd Huschek, Josephine Bönick, Yvonne Löwenstein, Steven Sievers, Harshadrai Rawel: Quantification of allergenic plant traces in processed food products by targeted proteomics based LC/MS/MS and isotope marked peptides

11 Curriculum vitae

For reasons of data protection,

the curriculum vitae is not included in the online version

12 Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung

der angegebenen Literatur und Hilfsmittel angefertigt habe.

Berlin, den 04.04.2016

Steven Sievers