UNIVERSITY OF NOVA GORICA
GRADUATE SCHOOL

DEVELOPMENT OF METHODS FOR IMMUNODETECTION OF FOOD ALLERGENS

Ana ČEVDEK

Dissertation

Mentor: prof. dr. Mladen FRANKO

Nova Gorica, 2010

Kadar vam se zgodi, da izgubljate kontrolo nad lastnim življenjem, kadar vam je dan s 24 urami prekratek, pomislite na velik kozarec kislih kumaric

…

in ne pozabite na kavo!

Hvala mama, hvala tata… sem kar sem po vaši zaslugi.

Hvala Nina… ne bom pozabila.

In hvala vam moje pupike, brez vas kozarec kumaric ne bi bil popoln!

POVZETEK

Alergija na hrano predstavlja velik problem tako za porabnike, proizvajalce hrane kot vladne organizacije. Slednja je definirana kot škodljiva reakcija na prisotnost specifičnih proteinov v hrani, ki lahko sprožijo pretiran odziv imunskega sistema konzumenta. Razširjenost alergij med državami se glede na vrsto alergenov razlikuje v odvisnosti od prehranskih navad državljanov, medtem ko pogostost alergijskih reakcij na hrano generacijsko variira, pri čemer je pojavost med odraslimi mnogo manjša (približno 0,17 %) kot pri otrocih, kjer le-ta prizadane 2,5 % celotne populacije otrok. Edini možni preventivni ukrep za preprečevanje alergij na hrano je popolno izogibanje nevarni hrani ter proteinom, ki jih le-ta vsebuje. Alergijo lahko sproži že nezaznavno majhna količina alergena, ki običajno ni označena na etiketah komercialno dostopnih prehrambenih izdelkov. Prav zaradi tega se zakonodaja glede njihovega označevanja nenehno spreminja in v bližnji prihodnosti obeta nedvoumno označevanje prisotnosti vseh alergenih snovi v živilskih izdelkih. Evropska unija med alergene uvršča skupno 11 živilskih sestavin, katerih označevanje na etiketah prehrambenih izdelkov postaja zakonsko obvezno. Med te sestavine spada tudi mleko, ki predstavlja enega najpomembnejših nutrientov svetovne prehrane.

Kravje mleko je najpogosteje uporabljeno hranilo na svetu, njegove proteinske frakcije pa so mnogokrat uporabljene tudi kot dodatki v komercialni hrani in sokovih. Mleko predstavlja mešanico različnih sestavin, tj. mlečnih maščob, sladkorjev in proteinov. Slednje delimo v dve veliki skupini, pri čemer se največ proteinov nahaja v t.i. kazeinski frakciji. Med najpogosteje uporabljene proteinske dodatke hrane uvrščamo vodotopne proteine sirotke, katerih največji delež (60 %) zavzema beta-laktoglobulin (BLG). Le-ta je znan kot prenašalec hidrofobnih molekul (npr. nekaterih vitaminov), a vendar kljub pomembni biološki vlogi ostaja glavni alergen mlečnih proizvodov. Le-ta je poznan kot prenašalec hidrofobnih molekul (npr. nekaterih vitaminov) v telesu, kar večina populacije zagotavlja z uživanjem mleka. Žal pa je zaradi netolerance posameznikov do beta-laktoglobulin, poznan tudi kot glavni alergen v mlečnih proizvodih. Posledično je njegovo odkrivanje v ne-mlečnih proizvodih izrednega pomena predvsem za beta.laktoglobulin občutljive posameznike. Pester izbor analiznih metod za njegovo detekcijo obsega različne metode tj. od izločitvene kromatografije pa vse do imunometričnih testov. Znano je, da slednje niso dovolj občutljive, prav tako pa veljajo tudi za časovno potratne. Med bolj občutljive metode uvrščamo t.i. imunološke metode, a vendar je odkrivanje alergenov v prehrambenih izdelkih oteženo zaradi nizkih koncentracij in vpliva sestavin na njihovo detekcijo. Prav slednje narekuje razvoj novih, bolj občutljivih metod, ki lahko premagajo oteženo dokazovanje alergenov.

V doktorski disertaciji je predstavljen razvoj in testiranje nove metode FIA-ELISA-TLS. Predstavljena metoda je rekombinacija različnih analiznih pristopov, ki združuje imunodetekcijo s pretočnimi (FIA) sistemi, pri čemer izkorišča visoko občutljivost TLS (thermal lens spectrometry) detektorja. Imunodetektiranje prispeva k specifičnosti metode, ki je omejena na kolono vključeno v pretočni sistem. Pretočni sistemi so dobro definirane analizne tehnike, ki omogočajo povečano število analiz in zmanjšano porabo reagentov, medtem ko je TLS visoko občutljiva metoda, ki omogoča detekcijo ekstremno nizkih koncentracij analitov. FIA-ELISA-TLS je enostavna metoda, pri kateri vnesemo vzorec v

pretočni sistem. Pri prehodu skozi imunokolono se alergen, ki je prisoten v vzorcu zadrži v koloni. Pri tem alergen predhodno označimo s protitelesi, ki imajo vezano hrenovo peroksidazo. Ko v pretočni sistem vnesemo reagent, se v imunokoloni tvori produkt, ki ga zaznamo s TLS detektorjem.

FIA-ELISA-TLS omogoča ponovljive rezultate, a le v primeru uporabe homogenih raztopin, ki ne povzročajo motenj pri občutljivem TLS detektorju. Kot najprimernejša nosilna raztopina se je izkazal PBS (phosphate buffer saline) pufer. Optimalni pretok za FIA-ELISA-TLS je bil 0,4 mL/min, pri tem smo dosegli mejo detekcije 2 pg beta-laktoglobulina pri 100 µL vzorca. Dosežena limita metode je 100-krat nižja od detekcijske meje, ki jo lahko dosežemo z validirano ELISA metodo. CPG (controlled pore glass) nosilec se je izkazal primeren za razvoj metode. Vendar CIM (convective interaction media) nosilec je primernejši za uporabo, ker manj privlači markerska protitelesa in pri tem se tvori nižji nespecifični signal.

FIA-ELISA-TLS sistem je univerzalna metoda, uporabna za detekcijo tudi preostalih alergenov hrane. Slednja je bila preizkušena na ovalbuminu (OVA), tj. je alergenu beljaka jajc. Metodo je možno modificirati enostavno z zamenjavo protiteles v imunokoloni. Primerjava metode za detekcijo ovalbumina in beta-laktoglobulina je pokazala, da se sistem obnaša podobno, vendar pa detekcija ovalbumina poteka pri višjih koncentracijah, kar se tudi odraža v meji detekcije. Kljub temu da smo lahko zaznali komaj 1 ng ovalbumina na 100 µL vzorca, pa je ta meja nižja kot pri validirani ELISA metodi za ovalbumin. Iz dobljenih rezultatov lahko zaključimo, da je FIA-ELISA-TLS tehnika vsesplošno uporabna za odkrivanje nizkih koncentracij raznovrstnih alergenov.

SUMMARY

Food allergies are a big concern for consumers, food industry and government. A food allergy is an adverse reaction to food proteins, which are normally present in food products. The distribution of such disease around the world changes from country to country. The range of allergies is from 0.17 % for adult people to 2.5 % for children. Food-allergic consumers must avoid the offending food, in which the allergens are present. Only a strict avoidance diet it the only strategy to prevent abnormal immunological responses to specific proteins in food. Already, trace levels of the offending food can elicit adverse reaction. Therefore, accurate and unambiguous labelling of food products is needed. The European legislation recognizes eleven ingredients, which has to be stated on food labels. The proteins of these ingredients are well known to trigger allergic reactions. Among them there is also milk, a vital nutrient in childhood.

Bovine milk is one of the most common dietary products used all over the world. Different fractions of the bovine milk are used as additive in precooked food products and fortified beverages. Milk is a mixture of different molecules such as lipids, saccharides and of course proteins. Lactoproteins can be divided into two groups, most abounding casein fraction and water soluble whey fraction. 60 % of the whey proteins consist of beta-lactoglobulin (BLG). The basic biological role of beta-lactoglobulin is to be a carrier of fat-soluble biological molecules. Beta-lactoglobulin has an important role in transportation of fat-soluble vitamins, which is in great part of population assured by consumption of milk. At the same time however, this protein is one of the major allergens in milk and milk products. Despite its important role in transportation of fat-soluble vitamins, this protein is one of the major allergens in milk and milk products. The only way to prevent allergic reaction is to avoid the allergen itself. The confirmation of its presence in food products, which are not milk-based products, is of great importance. However, its removal from the diet may lead to vitamin deficiencies. Methods for beta-lactoglobulin detection range from size-exclusion chromatography to immunometric assays. These methods are time consuming and not sensitive enough. Even though, the immunobased methods are highly specific, detecting beta-lactoglobulin in food products is made difficult due to small concentrations. Additionally, the allergen is masked by the food matrix. Therefore, novel methods, which could overcome these problems and detect trace concentrations of beta-lactoglobulin, are developed.

This thesis describes the development of a novel FIA-ELISA-TLS method for beta-lactoglobulin detection. The method developed is a combination of different analytical approaches. We have coupled immunocolumn with flow-injection analysis (FIA) and exploiting sensitive thermal lens spectrophotometer (TLS) as a detector. The immunocolumn is used for its specificity in detection of the target analyte. Flow-injection analysis method is a well-established technique, which offers increased sampling rate, lower reagent consumption and high versatility. Thermal lens spectrometry is a photothermal technique, which is well-known for its high sensitivity. The principle of the method is simple. Beta-lactoglobulin is injected into the stream of the carrier buffer. By passing the immunocolumn is retained. The marked allergen is detected by injecting a chromogenic reagent. The product formation is determined with thermal lens spectrophotometer (Ar-ion excitation laser, 457.9 nm, 100 mW).

The method resulted more reproducible if homogenous solution were used for the flow-injection analysis system. In the case of this application phosphate buffer saline (PBS) gave unperturbed signals, which were easy to evaluate. Post-immunocolumn addition of organic solvent to the carrier stream resulted into enhanced thermal lens spectrometry signals. The optimal carrier flow-rate is 0.4 mL/min. The evaluated limit of detection (LOD) for this flow-rate is approximately 2 pg of beta-lactoglobulin in 100 µL of sample. The LODs achieved with FIA-ELISA-TLS method was approximately 100-time lower in comparison ELISA for beta-lactoglobulin detection. The controlled pore glass (CPG) support is appropriate for the development of the method. However, the convective interaction media (CIM) supports perform better, showing less affinity toward horseradish peroxidase (HRP) conjugated antibodies and other molecules present in samples.

The developed method can be applied also for other allergens. The selectivity of the immunocolumn is changed by changing the antibodies immobilized on the solid support of the immunocolumn. The method was tested also for ovalbumin, the allergen of eggs' white. In comparison with the detection for beta-lactoglobulin, ovalbumin is detected in higher concentration range. This result coincides with the worse performance of IgG raised against ovalbumin compared to IgG raised against beta-lactoglobulin. However, also in this case the LOD of 1 ng is lower than the limits achieved with ELISA. Therefore, the method can be applied also for the detection of other allergens.

ABBRIVIATION

BLG	Beta-lactoglobulin
BSA	Bovine serum albumin
CIM	Convective interaction media
CPG	Controlled pore glass
DAP	2,3-diaminophenazine
ELISA	Enzyme-linked immunosorbent assay
FIA	Flow-injection analysis
HRP	Horseradish peroxidase
IgG	Immunoglobulin type G
LOD	Limit of detection
NaAc	Sodium acetate buffer
OPD	O-phenylenediamine
OVA	Ovalbumin
PBS	Phosphate buffer saline
RSD	Relative standard deviation
SFM	UV/Vis spectrophotometer
TLS	Thermal lens spectrometry

TABLE OF CONTENTS

TABLE OF FIGURES

List of tables

1 Introduction

Allergies are a widespread problem affecting a relatively large part of the population. The most concerning adverse reaction are the food mediated allergies. Food allergy is an adverse health effect provoked by the ingestion of food product and/or medicines to which subjects are sensitized (Kimber and Dearman 2001). Food allergy is mainly a problem in infancy and early childhood. Adverse reaction to food such as cow's milk, are most common in the first year life (Halken 1997a, b; Madsen 1997). Cow's milk is one of the first foreign source of proteins encountered by infants (Burks and Sampson 1993). Because of the cross-reactivity of cow's milk with sheep's and goat's milk, allergic reaction to these species are also possible (Gall et al. 1996). Anaphylactic reactions have been described also after the administration of the majority of vaccines. In this case the anaphylaxis has been attributed to the final formulation, such as gelatine, or to impurities derived from the manufacturing process, such as egg proteins (Descotes et al. 2002; Roukensa et al. 2009; Sakaguchi et al. 1996).

As a result of the continuing development of production, preservation and safety technology, humans in developed countries have the access to a huge quantity and diversity of food. In the past, only locally-produced and seasonal food was available to consumers. Modern technologies made it possible to supply food produced in countries from around the world throughout the year. The access to various food products has been reflected in a rising trend of food allergies and of course in the appearance of new allergens, to which patients in developed countries react. In order to ensure that food supplied to the public is of adequate quality, many agencies were formed to monitor food safety. These agencies are designed to protect the consumer and improve health by providing information about food constituents. Furthermore such agencies develop policies related to food safety. Moreover, they monitor relevant developments in science, technologies and other fields of knowledge related to food.

The management of food allergy concentrates on a strict avoidance diet. Accurate and unambiguous labelling of food products is therefore of paramount importance. Strategies for the assessing the food safety combine several approaches. Definitely, the most important is the investigation of the structure/function relationship for indication of allergenicity. The *in vitro* assays with enzyme, receptor proteins, or cultured cells, which mimic the *in vivo* studies, have recently gained recently a lot of popularity (Konig et al. 2004). However, just some of the *in vitro* tests are validated formally. Therefore, there is still an urge for fast, specific and sensitive methods for screening of allergens in food stuffs.

During the years, detection methods for allergen determination were developed, but they are not sufficiently sensitive, are time-consuming and are not reliable for quantitative determination of the allergen presence. Methods for allergen detection vary from immunoassays such as ELISA (enzyme-linked immunosorbent assay), RAST/EAST (radioallergosorbent test/ enzymeallergosorbent test) inhibition, dipsticks to liquid chromatography/tandem mass spectrometry LC/MS/MS, which are dealing directly with the allergenic proteins. Some of the methods confirms the allergen indirectly by detecting the presence of DNA molecules, PCR methods (Van Hengel et al. 2007). However, those methods are time demanding and have the detection limits higher than the concentration of allergens in food products. The lack of fast and accurate methods for allergen detection makes it impossible to allow a better control over the presence of allergens in food and appropriate warning labels on food product.

This work focuses on the development of immunodetection method for allergen determination in food products and medicines combined with sensitive thermal lens spectrometry (TLS). A fast and sensitive method for the confirmation of allergen presence is achieved by the coupling a system of specific immunodetection reaction, the versatility of flow injection methods and spectrophotometric detection. However, the coupling of these methods with thermal lens spectroscopy as provided even higher sensitivities.

2 Research goals

The aim of our work was to develop a method for *in vitro* detection of allergens in food products. As discussed in the introduction fast, specific and reliable methods for screening of allergen presence in food stuff are still needed. The development of the method can be divided into several steps:

In the first step sensitive and accurate detection methods possibly to be combined together are chosen. The immuno-enzymatic detection methods have the necessary specificity, velocity and sensitivity (Besler 2001; Darwish et al. 2007; Edwards et al. 1999) for recognition and quantization of chosen allergen. Moreover, flow injection methods give all the necessary characteristics to gain repeatability of measurements, faster analysis, lower contaminations of samples and small waste production for best “on-line” measurements (Gomez and Callao 2007; Hansen and Miro 2007; Wu et al. 2007; Wu and Cheng 2005).

The second goal of this research is the optimization of sample preparation prior to the injection in the detection system. Allergens in food products can be found in small quantities, therefore, difficult to detect but high enough to elicit an allergic reaction (Van Hengel et al. 2007). The most problematic is the food matrix which should be removed in as few steps possible to avoid allergen loss.

The inclusion of thermal lens spectroscopy (TLS) constitutes a final step in the method development. TLS gives the possibility to improve the sensibility of the detection system. Moreover, the improved sensibility of the methods helps to lower the consumption of reagent for detection of the allergen.

3 Theoretical background

3.1 Allergies and allergens

Allergies arise in response to certain proteins termed allergens, capable of triggering immediate hypersensitivity reactions. The development of allergy is a multistep process, and the mechanisms leading to sensitization, the production of IgE antibodies, and allergic disease are complex (Bredehorst and David 2001). The development of allergic sensitization requires that an immune response is stimulated by introducing a protein allergen (Figure 1). As Kimber and Dearman stated, the susceptibility of an individual is determined by heritable characteristics and environmental factors (Kimber and Dearman 2001). As a result of climate changes a shift in the plant population has occurred. Therefore novel food got in contact with the human population resulting in sensitization to novel proteins occurred (Noyes et al. 2009).

Adverse reactions to food are divided into toxic and non-toxic reactions. The latter are subdivided into immune-mediated and non-immune-mediated adverse reactions (Figure 2). Food allergy is part of the immune-mediated responses termed hypersensitivity reactions, which are harmful and can cause tissue injury and serious disease (Janeway et al. 2001a). Food allergy denotes an immunologic mechanism represented almost exclusively by IgE-mediated reactions (Besler 2001). IgE antibodies are the most bothersome ones. They are found mainly in tissues unlike other antibodies that are found in the blood. In contact with an allergen they activate the release of histamine from specialized blood-derived cells called mast cell, a biogenic amine involved in local immune responses (Lodish et al. 1995). Excessive activation of mast cells in the exposed tissue by IgE results in an extreme inflammatory response.

Usually, the first contact with an allergen evokes a milder reaction; however, any subsequent contacts with one of the environmental substances can provoke serious health problems. This course of events is a feature of the immune system. The immune system has the ability to learn. The first encounter of an immune cell with an allergen leads to a slowly rising synthesis of the antibody. The second encounter with the same antigen leads to a more rapid and greater response. The system has learned to recognize the antigen to which it was previously exposed, long living memory cells were formed during the first contact and they persist in the circulation (Lodish et al. 1995).

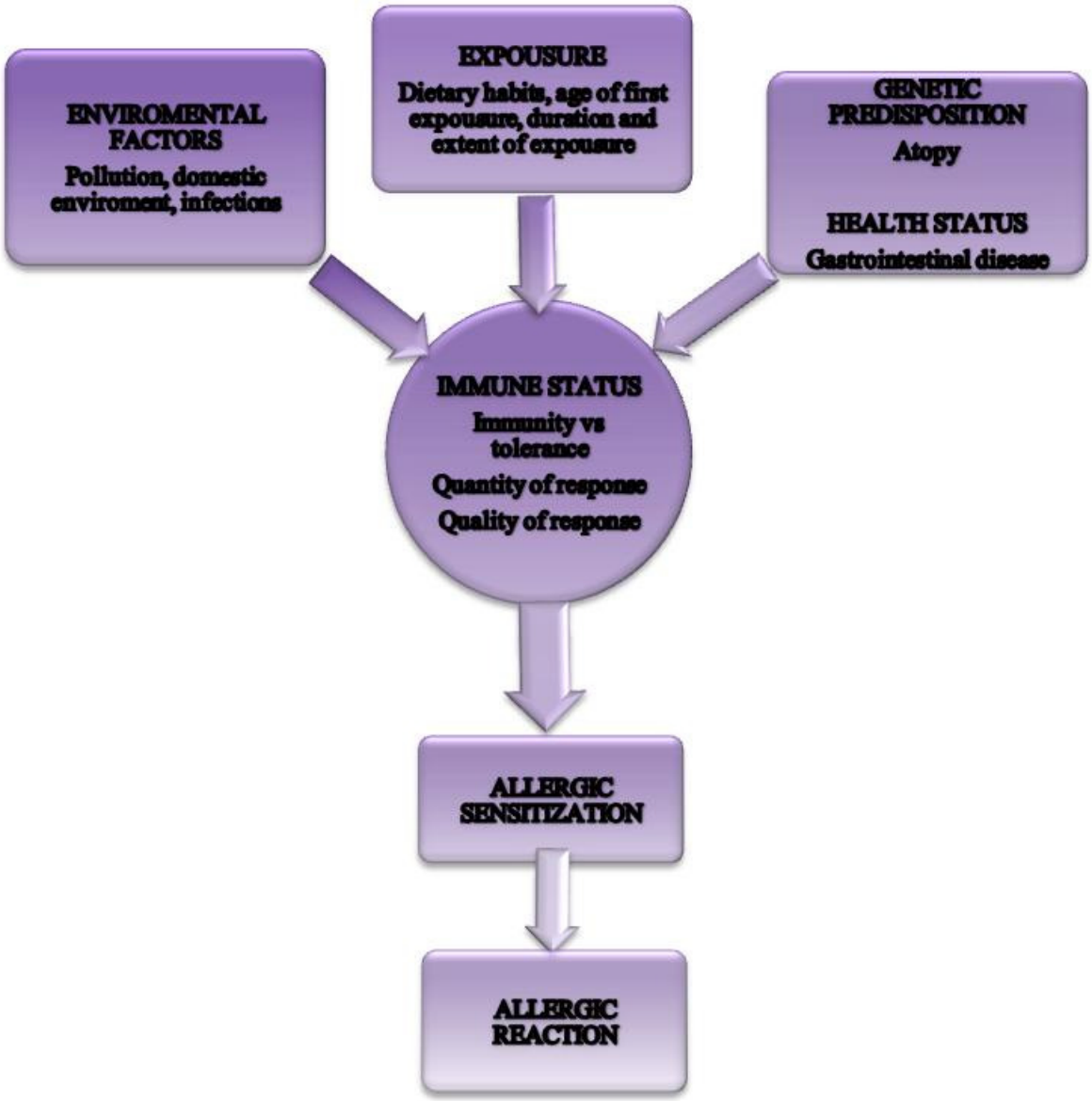

Figure 1: *Schematic representation of exposures to allergens. Effective sensitization of an susceptible individual requires that exposure is started via an appropriate rout and in a necessary extent and duration according to Kimber and Dearman (Kimber and Dearman 2001).*

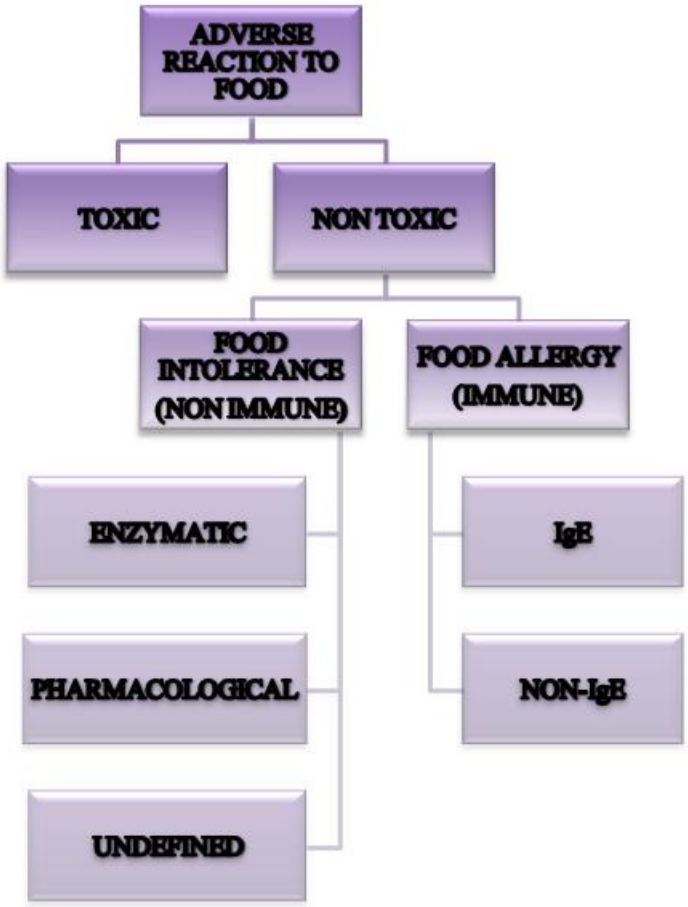

Figure 2: *Classification of adverse food reactions.*

The inflammatory response after IgE-mediated mast-cell activation occurs as an immediate reaction, within seconds, and as a late reaction, which takes up to hours. The late-phase reactions are clinically less marked than the immediate response but cause much serious long-term illness. When a re-exposure to allergen triggers an allergic reaction, the effects are focused on the site of introduction. Clinical syndrome produced by an allergic reaction depends on three variables: the amount of specific IgE present, the route by which the allergen is introduced and the dose of allergen. A direct introduction of the allergen into the bloodstream or a rapid absorption from the gut cause a very dangerous syndrome called systemic anaphylaxis which not treated with epinephrine can lead to anaphylactic shock and consequent death. Allergy to food causes symptoms limited to the gut and systemic reactions (Janeway et al. 2001a).

Allergens have a no-dose dependent toxicity as other toxicants harmful for humans. Ingestion of small amounts of an offending food can elicit adverse reactions in individuals with IgE-mediated food allergies. The threshold dose for provocation of such reactions is often

considered to be zero. However, because of various practical limitations in food production and processing, food may occasionally contain trace residues of the offending food. The thresholds dose might be defined as the lowest amount of the offending food that would elicit mild, objective symptoms in the most sensitive individuals. The amount of such food needed to elicit symptoms is variable, possibly over an order of magnitude or more between different individuals with the same type of food allergy. Consequently, the threshold doses for different allergenic food are not necessarily equal. The lowest dose of allergen needed to provoke reaction is varying from 0.2 mg of the allergic protein up to 200 mg of allergen (Taylor et al. 2002).

Food allergens are proteins from vegetable and animal sources found in very minute doses in food products. The major food allergens identified as class 1 allergens are water-soluble glycoproteins that are 10 to 70 kDa in size (Sampson 1999). The plant allergen and the food allergen from animal origin are characterised by a number of biochemical and physicochemical properties. These include thermal stability and resistance to proteolysis, which are enhanced by the ability to bind ligands, such as metal ions, lipids or steroids. (Breiteneder and Mills 2005). Hidden food allergens are problematic due to the fact that these allergens are resistant to heating, hydrolysis and enzymatic treatment (Besler 2001; Dube et al. 2004; Scheurer 2004; Sell et al. 2005; Thomas et al. 2007), therefore no cooking treatment of food ingredients can eliminate the cause of allergic reaction.

In Table 1, the antigenic protein purified from different sources and recognized as triggers of allergic reaction are listed (Becker and Reese 2001; Breiteneder and Mills 2005; Burks and Sampson 1993).

3.1.1 Clinical symptoms

The majority of allergic reactions to food occur within minutes of consuming the offensive food. The first occurring are the localized symptoms at the site of intake. Systematic anaphylaxis generally occurs within 1 to 30 minutes. It can progress rapidly or begin with mild symptoms and progress to shock and cardiorespiratory arrest.

Food allergies produce respiratory, gastro intestinal, cutaneous and cardiovascular symptoms (Ortolani and Pastorello 2006; Ring et al. 2001). The most frequent manifestation of food allergy is an acute urticaria, which is part of the symptoms related to the skin. Beside urticaria

also eczema and angioedema might occur. Moreover, gastrointestinal symptoms can be confused also with other toxic effects of food since they comprise nausea, cramping, vomiting and diarrhoea. Bronchoconstriction and rhinitis are representatives of the respiratory symptoms that might occur as an allergy reaction. As a really sever defence to the ingested allergen is an immediate allergic reaction, which might lead to death. In this case cardiovascular symptoms occur prior to the anaphylactic shock (Ring et al. 2001).

Table 1: *Purified antigens in food (Becker and Reese 2001; Breiteneder and Mills 2005; Burks and Sampson 1993).*

Protein source	**Antigenic protein**	**Molecular weight (kDa)**
Cow's milk		
(Caseinic fraction)		
	α-casein	27
	α-casein	23
	β-casein	24
	κ-casein	19
	γ-casein	21
(Whey fraction)		
	β-lactoglobulin	36
	α-lactoglobulin	14.4
	serum albumin	69
Chicken egg white		
	Ovalbumin	45
	Ovomucoid	28
	Ovotransferrin	77.7
	lysozyme	14.3

Table 1: *Purified antigens in food (Becker and Reese 2001; Breiteneder and Mills 2005; Burks and Sampson 1993).*

Protein source	Antigenic protein	Molecular weight (kDa)
Peanut		
	Ara *hI*	63.5
	Ara *hII*	17
Soybean		
	Soy bean trypsin inhibitor	20.5
Fish		
	Allergen M (*Gad c 1*)	12.3
Shrimp		
	Antigen I	42
	Antigen II	38

3.2 Policies

The prevalence of allergy is increasing in economically advanced regions of the world due to many environmental factors such as environmental pollution, allergen levels and dietary changes. The latter is the most important for food born allergies. In the past, only locally-produced and seasonal food was available to consumers. As already explained in the introduction, the development of new technologies made it possible to spread various food ingredients all over the world. The access to various food products is reflected also in rising trends of food allergies over time (Gupta et al. 2007).

Given the incurable nature of food allergy and its potential life-threatening consequences, the management of food allergy concentrates on a strict avoidance diet that has to be implemented by food allergic individuals and their care givers. The accurate and unambiguous labelling of food products is therefore of paramount importance. World-wide regulatory initiatives are aimed at the mandatory declarations of certain allergic food (Paulsen

et al. 2007; Van Hengel et al. 2007). The Food labelling Directive (Directive 2000/13/EC OJ L 109, 6.5.2000;(EUCouncil 2000)) ensures that consumers are informed of the complete contents of foodstuffs so that the sensitive consumers can identify allergenic ingredients that may be present. As previously mentioned the only way to avoid allergic reaction is to have a strict diet without proteins that cause the allergy. The European Commission proposed a complete listing of food ingredients and allergens (Table 1). At first it was not obligatory to label the components of compound ingredients that make up less than 25 % of the final food product. The full ingredient labelling ensures optimal consumer information. A label indicating the presence of allergens would enable susceptible consumers to avoid such products. The most common food allergens are found in a wide variety of processed food product. The list of allergenic substances should include the foodstuffs, ingredients and other substances recognised as those causing hypersensitivity. The European Commission recognises eleven common allergenic foods, which are discussed in more detail in the following chapter.

3.2.1 Food allergy in Europe

The distribution of allergies around Europe changes from country to country. Moreover, the prevalence to food allergens changes with age, according to the dietary habits of residents. The most common allergy in children is mainly directed against cow's milk. The range of children allergy to cow's milk is from 1.9 % in Sweden to 2.5 % in England. The second allergen most common in children is peanut allergen; it has a prevalence of 0.5 %. However, in adulthood the prevalence of food allergy is lowered to 0.17 % of the whole population. Among adults the number of offending food is enlarged to shrimps, eggs and different meats (Madsen 1997).

A precaution for allergic residents of the European Union is the correct labelling of offending food ingredients. The presence of the allergenic ingredients can be stated on the list of the ingredients in a food commodity. The other possibility is a separate statement that usually follows the list of the ingredients. In any case, allergic food has to be stated on the label (EUCouncil 2000, 2003; van Hengel 2007).

Table 2: *Ingredients recognized as allergens (EUCouncil 2003).*

Ingredient	
Cereals	Wheat, rye, barley, oats
Crustaceans	Cramps, shrimps
Fish	Different fish and seafood
Eggs	Including lysine
Peanuts	
Soybean	
Milk	Including lactose
Nuts	Almond, hazelnut, walnut, cashew, brazil nut
Celery	
Mustard	
Sesame seeds	

Unfortunately, the most common reason for sensitive individuals to ingest allergen is contamination of safe food. In Table 3 are presented some foods that might hide allergic proteins and represent a danger for sensitive people.

Table 3: *Foods that might contain hidden allergens (Anibarro et al. 2007).*

Food proteins	Foods
Egg	Noodles, puddings, salad dressing, soups, candy, wines
Milk	Fortified flours, gravies, vegetarian cheese, sausages, muesli, margarine, packed soups, custards, bread
Soy	Meat pies, cookies, chocolates, bread, cooking oils, hot dogs, infant formulas, margarine, candy, salad dressing, crackers
Wheat	Modified starch, gravy, wine, licorice, hot dogs, pies, milk shakes, soy sauce
Peanuts	Marzipan, soups, sweets, vegetable fat and oils, ice creams, chilli, candy, margarine, soups, pastry

3.3 Milk and its allergens

1.1.1 Milk proteins as nutrients

Milk is a mixture of different nutrients. Among them there are approximately 3.6 % of fats, 4.6 % of lactose and 0.7 % of different minerals. Milk is also a primary source of proteins, which takes approximately 3.4 % of the whole nutritional values of the milk's constituents. Of course, the composition of milk differs between species. Bovine milk is one of the most studied types of milk as one of the most common dietary product used all over the world.

However, milk allergens are mostly constituents of the proteinic fraction in milk. Milk proteins, also called lactoproteins, can be divided into two groups (Figure 3). The most abounding portion of proteins is the group of caseins. They represent 80 % of the total protein content in the bovine milk. The rest of the proteins are the whey proteins, called also serum proteins. The groups differ in some characteristic. The most evident is the fact that whey proteins are soluble at a pH 4.6. On the other hand the caseins group are proteins that tend to form micelles and are insoluble at the above mentioned pH insoluble (Bonizzi et al. 2009; Bordin et al. 2001; Punidadas and Rizvi 1998).

3.3.1 Beta-lactoglobulin

Beta-lactoglobulin (BLG) is a milk protein that represents approximately 60 % of whey proteins. This is equal to 10 % of total protein contents in milk (Lozano et al. 2008). This compact and soluble protein is part of the lipocalin superfamily of proteins (Sawyer and Kontopidis 2000). Different lipocalins are known allergens (Mantyjarvi et al. 2000) triggering the immediate-type immune response by IgE antibodies. All lipocalins are sharing a typical structure.

3.3.1.1 Beta-lactoglobulin structure

In the native state the BLG structure is formed of a β-barrel and 3 short α-helices. A β-barrel is typically formed by eight antiparallel β-stands (Fogolari et al. 1998b). The barrel is flanked by an additional α-helix (Forge et al. 2000; Hamada and Goto 1997; Hernandez-Ledesma et

al. 2006). Besides this secondary structures there is also an additional β-strand which is implicated in the dimerisation (Forge et al. 2000; Hamada et al. 2009), seen in Figure 4. Most of the hydrophobic and aromatic amino acid side chains, potential cleavage sites for proteolytic enzyme, are buried inside the β-barrel and therefore they are not exposed to enzymatic action. This structural feature confers to BLG its resistivity to enzymatic proteolysis (Hernandez-Ledesma et al. 2006).

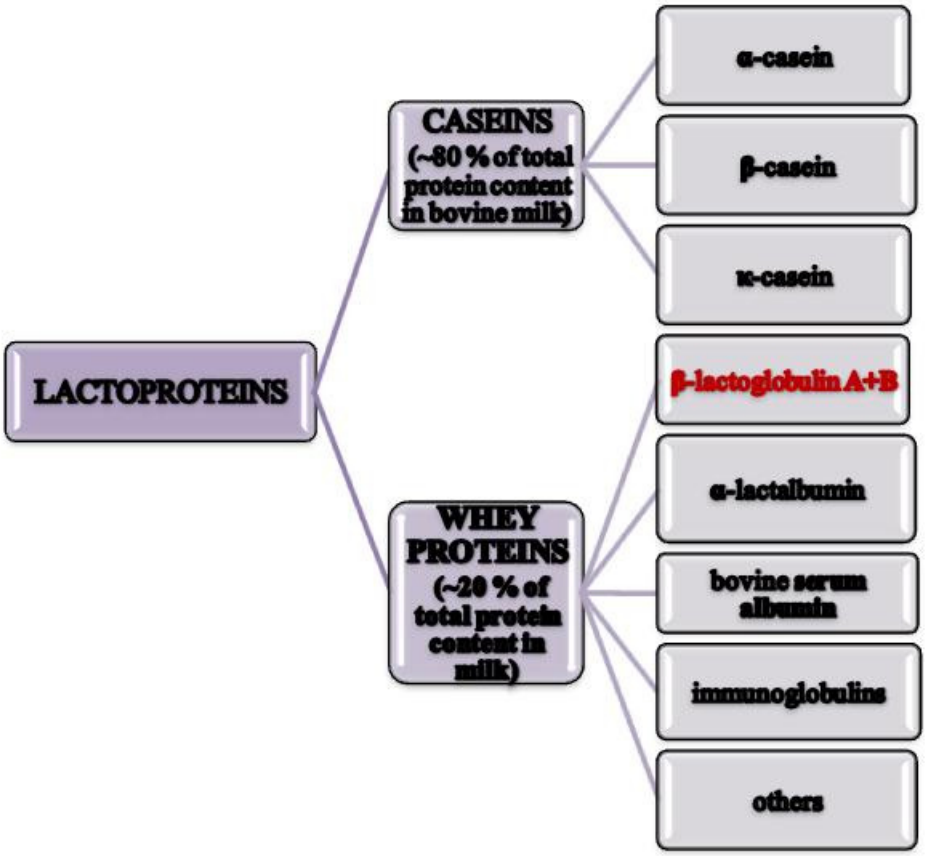

Figure 3: *The major allergens were identified among caseins and whey proteins (Goodman et al. 2007a; Goodman et al. 2007b).*

In a pH range from 5.2 to 7.5 beta-lactoglobulin is in a dimer form. These dimers are formed by two identical chains; therefore they formed a homodimer of beta-lactoglobulin. Outside this pH range the monomeric form is dominant (Apenten et al. 2002; Forge et al. 2000). However, pH also affects the secondary structure of beta-lactoglobulin causing some structural differences at a different pH. In Figure 5, structures at different pH ranging from 6.2 to 8.2 are presented. The most evident difference is the extension of the α-helix (Figure 5).

The resistance to proteolytic activity of BLG makes the protein a persistent threat, which cannot be easily avoided in commercially available food product.

Figure 4: *Dimeric native-state structure of beta-lactoglobulin (Brownlow et al. 1997). Beta sheets form the β-barrel (green strand), which are flanked by α-helices (red strands).*

1.1.1.1 Beta-lactoglobulin biological function

Since beta-lactoglobulin is a member of the lipocalin protein superfamily one of its expected functions is to be the carrier of fat-soluble vitamins in low fat or fat-free food (Lozano et al. 2008; Manderson et al. 1999a; Manderson et al. 1999b; Ragona et al. 1997). Lipocalins are capable of binding different molecules inside the β-barrel. Perez et all. have found that beta-lactoglobulin sequester free fatty acids and therefore facilitates the activity of pregastric lipase (Kontopidis et al. 2002; Perez et al. 1989). On the other hand, the heating of beta-lactoglobulin resulted in the production of bioactive peptides, which have the capacity to inhibit the activity of angiotensin converting enzymes (ACE) (Hernandez-Ledesma et al. 2006). Those enzymes have a potent role in the regulation of the arterial vasoconstriction (Lindpaintner et al. 1995). Some peptides formed from beta-lactoglobulin have also antimicrobial activity (Pan et al. 2007; Pellegrini et al. 2001).

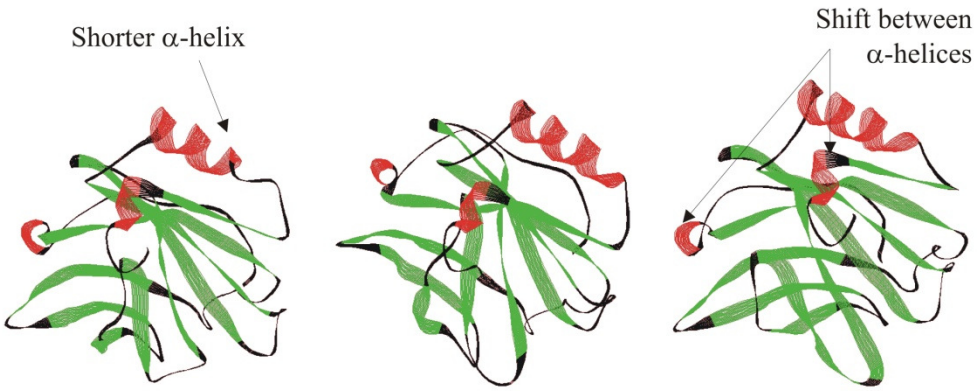

Figure 5: *Structural changes of beta-lactoglobulin at different pH, in order 6.2, 7.1 and 8.1 according to Qui and co-workers (Qin et al. 1998).*

3.4 Detection methods for BLG detection

Identification and characterization of food allergens is necessary to improve diagnosis, prediction of allergenicity of novel food proteins and therapy (Sicherer and Leung 2005). Different analytical techniques have been developed for food control. Detection methods for food allergen range from electrophoretic and mass-spectrometric detection to chromatographic techniques with immunostaining. Immunoassays are presently the method of choice for the detection and identification of a wide range of food components (Besler 2001). The Table 4 presents some of the methods applied for the chosen allergen beta-lactoglobulin.

Table 4: *Methods for BLG detection*

Method (time, LOD)	**Short description**	**Reference**
Gold cluster – linked bioassay (30 min, 100 ng/mL)	Optical biosensor based on the resonance enhanced absorption	(Hohensinner et al. 2007)
ELISA (min. 5 h, 5 ng/mL)	Immunoadsorption in the sandwich ELISA (30 min of colour development)	(de Luis et al. 2009)

Table 4: *Methods for BLG detection*

Method (time, LOD)	Short description	Reference
Size-exclusion chromatography (10 min, 2.5 mg/mL)	Size-exclusion chromatography with on-line multiangle laser light scattering, differential refractive index and UV detection	(van Dijk and Smit 2000)
Immunometric assay (1 h, 30 pg/mL)	Two enzyme immunometric assays performed in 96-well microtiter plates and based on the use of pairs of monoclonal antibodies	(Negroni et al. 1998)
ELIAC (30 min, 370 pg/mL)	Enzyme linked immunoaffinity chromatography	(Puerta et al. 2006)
starch-gel electrophoresis (7h, LOD not stated)		(Bell 1967)
LC-MS (20 min, 1 µg/mL)	A method using solid-phase extraction and liquid chromatography coupled to mass spectrometry	(Monaci and van Hengel 2008)

3.5 Milk beta-lactoglobulin and cross reactivity with other species

Cow's milk is widely consumed due to its high values of proteins and high amounts of calcium (Boyano-Martinez et al. 2009). Milk proteins are consumed also in other dairy products as cheese and butter. However, milk from other sources usually substitutes cow's milk. The comparison of the aminoacidic composition of beta-lactoglobulin from different

animals can provide an overview of the relations between those proteins. The Figure 6 presents the relationship between beta-lactoglobulins from different animals. The comparison is generated from aminoacidic sequences only. However, the sequence relatedness can be a very powerful predictor for relatedness of different species. The length of the branches of the phylogenetic tree represents the relationship distance between different proteins. The shorter is the length between two proteins the more related they are. As it is shown in Figure 6, the proteins from buffalo, goat or sheep are genetically the most akin to bovine beta-lactoglobulin. In this case, a cross-reactivity to those proteins might occur. Different studies have showed that children allergic to cow's milk have reactions to goat's milk as well (Bellioni-Businco et al. 1999).

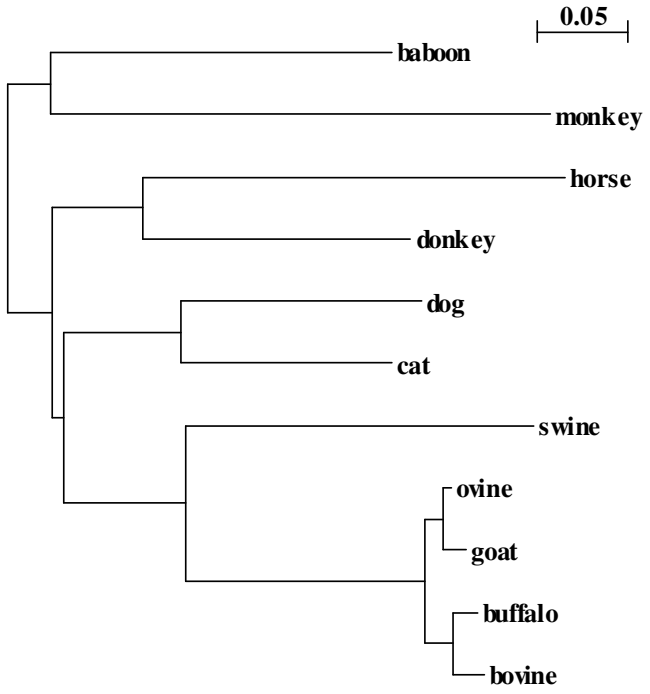

Figure 6: *Phylogenetic tree; the comparison of primary structures of beta-lactoglobulin from different animals.*

3.5.1 Ovalbumin, allergen from hen's eggs' white

Together with cow's milk and peanuts hen's egg is one of the most common foods involved in children hypersensitivity (Eigenmann 2000). Major allergenic proteins are located in the eggs' white (Hildebrandt et al. 2008). Ovalbumin (OVA) is the most abundant protein of the egg's allergens. It represents 55 – 65 % of the total protein content in eggs' white. OVA is a

glycosylated protein with the molecular mass of 45 kDa (Huntington and Stein 2001). Although, OVA is the main representative of the allergens in eggs, its function remains unknown. It was demonstrated, that OVA belongs to the serpin superfamily (Hunt and Dayhoff 1980). Serpins are serine protease inhibitors. Despite that, OVA does not show the serine protease activity as other serpins (Huntington and Stein 2001).

3.5.1.1 Ovalbumin structure

The ovalbumin structure includes four ovalbumin chains (Huntington and Stein 2001). Each chain is formed from a central core of two β-sheets. The β-sheets consist of 5 β-strands. The core of the protein is flanked with 10 α-helices. At the N-terminus of the protein, there is an exposed three turns α-helix. This helix represents the reactive centre loop of the serpins.

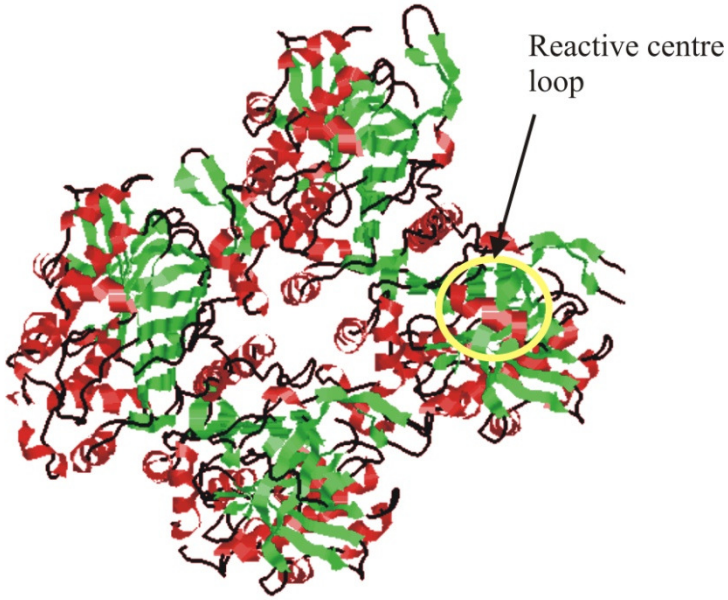

Figure 7: *OVA tetramer; the β-sheets are flanked by α-helices. The reactive centre loop of the proteins of the serpins superfamily is an three turns helix (surrounded in yellow) (Stein et al. 1991).*

During embryonic development or *in vitro* under elevated pH conditions, OVA is transformed into heat-stabilized form, S-ovalbumin (Yamasaki et al. 2003). The shift from the native OVA to S-ovalbumin is accompanied by a movement of the reactive centre loop in addition to the phosphorylation of the Serine 344 (Shinohara et al. 2007), which does not affect the

allergenicity. The heat-stabilized form represents an additional threat, since the allergen persists in thermally treated food products.

3.6 Detection methods for OVA allergen

OVA is mostly detected with immunoassays. Most of them are not just OVA specific. Most of the ELISA tests are made for whole egg proteins. However, Hefle et co-workers describes an sandwich ELISA, which can detect as low as 1 ppm of eggs' proteins (Hefle et al. 2001). Kuramitz and co-workers used an automated fluidic system bead-based immunoassay with electrochemical detection, which can detect 470 ng/ml of OVA (Kuramitz et al. 2006). However, OVA can be detected in higher concentration by using chromatographic methods. Awade et co-workers used reversed-phase HPLC for determination of egg white proteins, where OVA is totally separated from other proteins (Awade and Efstathiou 1999).

3.7 Principles applied for the development of the detection methods

3.7.1 Flow injection analysis in analytical chemistry

Flow injection analysis (FIA) is an approach to chemical analysis performed in a flowing carrier stream, where the samples and the reagents are directly injected into the system and carried to the detection point.

3.7.1.1 Evolution of FIA

FIA is a well-established technique with numerous and wide spread applications. Compared to batch mode assay it offers increased sampling rate, lower reagents consumption, better precision and high versatility (Tzanavaras and Themelis 2007a, b). Batch assays are laborious and long incubation times are necessary for sensitive detection. As a major limitation of batch systems, it is impossible to distinguish between multiple cross reactive compounds in one sample (van Bommel et al. 1999a, b). FIA involves sample determination under non-steady state conditions. Typically, FIA involves injection of a defined volume of the sample into a moving stream of a solution, which propels the sample zone upstream of the flow-through

detector. Additional processes may also occur in order to generate the detected species. A result of such a method is a transient signal (Hall 1991; Tzanavaras and Themelis 2007a). The measurements are performed under non-equilibrium conditions.

Since a transient signal is formed most of the time a tailing phenomenon appears (Figure 8). The effect of tailing has been studied by many researchers. Pai and co-workers state that the tailing or deformation of a flow injection analysis peak is caused by purely temporal effect. Therefore, the peaks are skewed when the time is short. By prolonging the time between the injection and the detector a more Gaussian-like curve is obtained (Pai 2002).

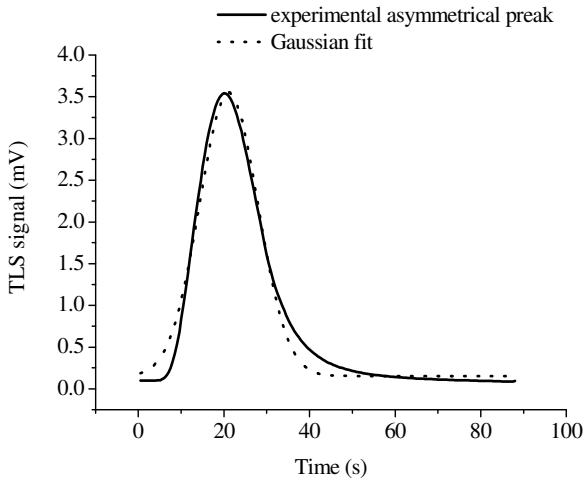

Figure 8: *Representation of the most common problem in FIA system: experimental peak with tailing effect and the reciprocal Gaussian fit to the obtained data.*

FIA evolved from initial simple form in which the reactants are hold in channels to FIA biosensors (Hall 1991). And all the variants of the FIA system are efficiently exploited in various research fields; from clinical diagnosis to environmental analysis (Table 5). FIA systems are appreciated mostly because of their accuracy, reliability, reproducibility and high sample throughput. Moreover, FIA methods can be automatized. An additional emphasis should be placed on the fact that FIA systems made it possible to lower the consumption of samples and reagent solution in order to fulfil the environmental demands for ecologically friendly analysis.

Table 5: *Basic evolution of FIA methods (Hall 1991; Hansen and Miro 2007)*

FIA generation	Components	Principles
1st generation	Pump, injection valves, Channels	Sample and reagents react in solution before passing the detector.
2nd generation	Immobilized reagent column upstream the detector, pump, injection valves, channels	The sample passes through the column in the carrier stream and the product is formed in the column and later passes through the detector.
3rd generation	Biosensor, pump, injection valves, channels	The chemical reaction is integrated directly on the detector and the product is formed when the sample pass through the biosensor.

From the three generations mentioned in Table 5, the second generation, which implies the immobilization of biological molecules, mostly enzymes, is the most developed. The major advantage of using enzymes in the analytical procedure is the amplification specifically caused by conversion of substrate molecules by each enzyme. The result of the enzymatic amplification of the product is higher sensitivity (van Bommel et al. 1999a). The immobilization on solid supports has more advantages in comparison to the reaction performed with solubilised enzymes. Immobilized enzymes are not consumed since they are attached on a support and therefore can be reused. This affects the cost of analytical procedures since the reuse of expensive enzymes is lowered. The enzymes are tightly bonded to the support hence no-leakage is observed. However, the calibration of such systems is dependent on several factors. The equilibrium between the enzyme and the substrate is changed in comparison to solubilised enzymes. The microclimate on the column changes, therefore the pH maximum is changed, which consequently affects the pH of the carrier solution and samples. Moreover, the calibration curves change because of the dispersion in

the FIA system (Hall 1991). Therefore many factors influence the outcome of our measurements.

3.7.1.2 Application of the FIA system

As already mentioned, the FIA analysis is used in many fields. For example, pharmaceutical industry applies the FIA biosensor for the detection of active pharmaceutical compounds during the production. It is also spread in agricultural field in order to monitor the usage of toxic compounds in the environment. Environmental science exploits the fast analysis for safely controls etc. Table 6 presents just some of the applications of diverse FIA systems for accurate analysis.

Table 6: *Implementation of the FIA system in analytical procedures*

	Application		**Reference**
1. generation	Chromium speciation	Simple FIA system coupled with TLS detection, LOD 0.07 µg/L of Cr(VI) in water	(Madzgalj et al. 2008)
	Lansoprazole determination	Simple FIA used in pharmaceutical analysis using UV/Vis spectrophotometric detection, LOD 0.5 µM	(Yeniceli et al. 2004)
	Hypochlorite determination	Multysyringe FIA analysis in commercial available products, LOD 1.2 g/L	(Soto et al. 2008)
2. generation	Pesticide toxicity evaluation	Immobilized enzyme FIA system coupled with TLS used for environmental analysis	(Kralj et al. 2007)
	Imidacloprid detection	FIA enzymatic analysis in environmental science, LOD 0.3 ppb	(Lagalante and Greenbacker 2007)
	Glycerol and tryglycerol determination	FIA enzymatic analysis with amperometric detection for	(Wu and Cheng 2005)

Table 6: *Implementation of the FIA system in analytical procedures*

	Application	Reference
		clinical diagnosis, $LOD_{Glycerol}$ 50 µM
	Gentamicin detection	Immunoassays couple with FIA and electrochemical detectors for food control, (Van Es et al. 2001)
	Okadaic acid	Immunosensor for FIA for toxicological analysis in mussels (Marquette et al. 1999)
	L-alanine detection	Chemiluminometric FIA detection in cultivation medium of mammalian cell, LOD 2µM (Janasek and Spohn 1999)
	Sucrose evaluation	Multi-enzyme FIA system with amperometric detection in biotechnology (Sosnitza et al. 1998)
3. generation	D-glucose determination	FIA coupled with amperometric tubular biosensors, LOD 470 µM (Alvarez-Romero et al. 2004)
	Cu^{2+} detection	FIA coupled with yeast biosensor (Tag et al. 2007)
	Phosphate determination	FIA biosensor, LOD 1.25 µM (Male and Lyong 1991)

3.7.2 ELISA principles

The enzyme-linked immunosorbent assay (ELISA) is a direct binding assay for protein detection (Janeway et al. 2001b; Voet et al. 1998). The principles of ELISA are based on the fact that antibodies against protein can be raised and later used for protein recognition.

An antibody against the protein of interest is immobilized on an inert solid material. The solution to be assayed is applied to the antibody-coated surface. The antibodies bind the

protein of interest. The other unbound proteins are washed away. The protein-antibody complex reacts with a secondary protein-specific antibody which is labelled with an enzyme. Binding of the second antibody-enzyme complex is measured by assaying the activity of the enzyme. The amount of substrate converted to products indicates the amount of protein present (Voet et al. 1998). The representation of such detection is shown in Figure 9.

One of the basic variants of said assay is a sandwich ELISA. This method needs two antibodies. This procedure is used to determine the antigen concentration in unknown samples. This makes ELISA accurate. In case where a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample.

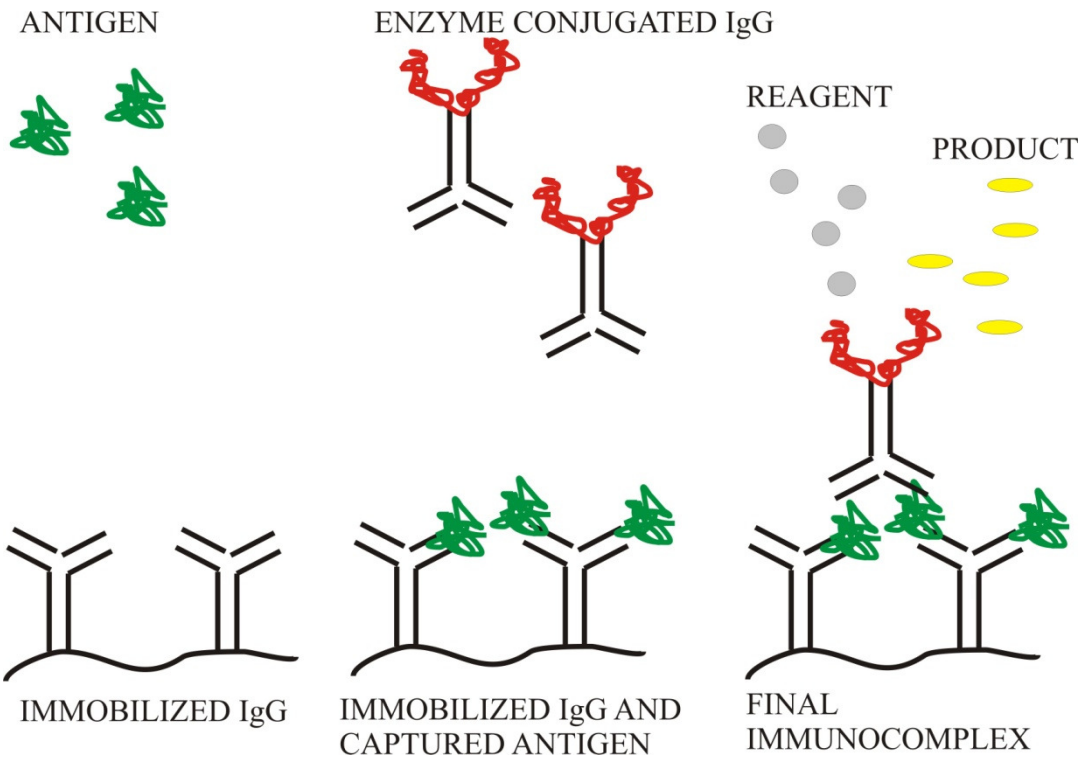

Figure 9: *Sandwich ELISA; step by step representation of the immunological detection with the ELISA method.*

The immunodetection of ELISA principles is widely used in the application of biosensors. Some of them are listed in the Table 7.

Table 7: *Overview of some applications of ELISA principles in biosensors*

Application	Reference
Detection of α-fetoprotein in human serum	(Fogolari et al. 1998a; Yu et al. 2004)
Progesterone	(Tschmelak et al. 2004)
CA19-9 tumor marker	(Fogolari et al. 1998a)
TNT (2,3,4-trinitrotoluene)	(Wilson et al. 2003)
IgE detection	(Hianik et al. 1999)
Bacteria detection	(Lee et al. 2000)
Pesticides	(Skládal and Kaláb 2005)

3.7.3 UV/Vis Absorbance spectrometry

Absorption spectroscopy is based on the interaction of electromagnetic radiation with matter, more specifically the absorption of the photons by one or more substances present in a sample. In absorption spectroscopy the absorbed photons are not re-emitted. The energy absorbed by the matter is released by non-radiative relaxation. An absorption spectrum is characteristic for a particular compound. At a wavelength typical for the matter the photons are absorbed and this results in a decrease of the measured transmission intensity. By measuring the absorption of the compound at its typical wavelength the concentration of the absorbing compound can be determined (Anderson et al. 2004).

3.7.4 TLS principles

Thermal lens spectrometry (TLS) is a photothermal technique, which relies upon the absorption of optical radiation by the sample and subsequent non-radiative relaxation of absorbed energy. TLS method is based on sample reflection changes due to sample heating caused by non-radiative relaxation of excited molecules (Snook and Lowe 1995). Laser optical radiation is used for sample excitement. As previously mentioned the sample relaxes in a non-radiative manner, resulting in a temperature change. The temperature change affects the density of the sample. Some gradients in the refractive index of the sample are made, which results in direction changes in the propagation of light and therefore in the intensity of the probe beam. If simplified model is used, the change in the probe beam intensity is described by equation 1:

$$\frac{\Delta I_{bc}}{I_{bc}} = \frac{2.303 \times P \times (-dn/dT) \times A}{\lambda \times k} \quad (1)$$

Where:

$\frac{\Delta I_{bc}}{I_{bc}}$ is the relative change in the beam centre intensity

P is the excitation laser power

dn/dT is the sample's temperature coefficient

A is the absorbance of the sample

λ is the probe beam wavelength

k is the thermal conductivity of the sample.

The assumption is that the amount of the released heat is equal to the energy absorbed by the sample. The sensitivity of the detection technique can be increased simply by increasing the laser power or improving the thermo-optical properties of the sample medium. Higher TLS signals can be obtained when the experiments with solvents with more favourable thermo-optical properties than water are carried out (Table 8). Water as a solvent has low temperature dependence of refractive index and high thermal conductivity, but in comparison with other organic solvents it has a low enhancement factor.

The enhancement factor is the ratio of the optothermal lens signal magnitude to that which would be obtained by using conventional transmission spectroscopy. The enhancement factors shows that methanol, acetic acid ethanol and others organic solvent are more appropriate for TLS measurements (Bialkowski 1996). Thermal lens spectroscopy as an analytical method has been applied for the analysis of liquids, gases and solids. Commonly used analytical methods cannot detect minute amounts of allergens in complex samples such are food products. TLS can overcome this problem. Due to its extreme sensitivity it was already applied in food analysis with success. More over in comparison with other

transmission techniques is less subjected to errors induced by light scattering (Bičanič et al. 2001; Boškin et al. 2009; Luterotti et al. 1999; Luterotti et al. 2002; Luterotti et al. 2000).

Table 8: *Some of the thermooptical properties of major organic solvents, which are used for calculation of the enhancement factor (Bialkowski 1996).*

Substance	κ (W/m/K)	C_P (J/g/K)	-(dn/dT) $x10^4K^{-1}$
Tetrachloromethane	0.099	0.85	6.12
n-hexane	0.120	2.27	5.20
Dichloromethane	0.122	1.19	5.50
Diethyl ether	0.130	2.37	6.06
Toluene	0.131	1.71	5.68
2-propanol	0.135	2.60	4.00
Benzene	0.141	1.74	6.52
Ethyl acetate	0.144	1.94	4.90
Carbon disulfide	0.149	1.00	8.09
1-propanol	0.154	2.39	3.70
Acetic acid	0.158	2.05	3.90
Acetone	0.161	2.17	5.42
Ethanol	0.169	2.44	4.00
Acetonitrile	0.188	2.23	4.50
Methanol	0.200	2.53	3.94
Water	0.598	4.18	0.91

3.7.4.1 Enhancement of the TLS signal in a FIA system

To enhance the sensitivity of the TLS signal an addition of salts, surfactants and organic co-solvents is almost obligatory. As already mentioned, the organic solvents are thermo-optically favourable compared to water (Table 8). The use of organic solvents in TLS measurements results in greater sensitivity for a given analyte concentration. Non-polar solvents are particularly preferred for trace analysis because of their high thermooptical (dn/dT) and low thermal conductivity (κ) values (Bialkowski 1996). Following the aberrant model for TLS

signal prediction, the enhancement can be calculated as given in Equation 2 (Biosca and Ramis-Ramos 1997; Georges 1994).

$$E_t = \frac{(2.303)(-dn/dT)}{1.91\kappa\lambda} \qquad (2)$$

However, the Equation (2) can be applied just for simple one component solvents. For solvents mixtures, the composition dependent TLS signal is more complex to calculate, since information on thermo-optical properties of the mixture is needed. In this case, the relationship between enhancement factor and solvent composition is estimated as a linear function of the volume fraction of each solvent (Weimer and Dovichi 1986).

However, TLS measurements of flowing samples represent a special case of TLS applications. In the FIA-TLS case the effect of the heat dissipation due to mass flow out of the excited sample should be considered. Moreover, thermal-lens detector is highly sensitive to fluctuations in flow-rates of the carrier solution (Dzyabchenko et al. 1998). The increase of the flow-rate lowers the sensitivity of TLS measurements and negatively affects the enhancement due to organic solvent presence.

3.7.4.2 Analytical applications of TLS

TLS techniques have found many application in the analysis of environmental and food samples. In the recent years the TLS measurements were coupled to bioanalytical techniques, which offer an improvement in the selectivity of the detection method. Table 9 presents some application of TLS in environmental analysis (Franko 2009).

3.7.5 Biosensors

A biosensor is broadly defined as any measuring device that contains a biological element (Buerk 1992). The principles of biosensors are very simple. A target analyte should interact with biological elements in the biosensor and make a response that can be measured. The

biological element of the biosensor can convert the analyte to another chemical species through a biochemical reaction, changes its optical, electrical or mechanical properties or any other response that can be quantified. The transducer of the signal may be a conventional electrochemical sensor or may be based on another technology such as the spectroscopic detection (Luppa et al. 2001).

Biosensors must have the following characteristics (Buerk 1992):

- The biological part must be highly specific for the purpose of the analysis,
- Good stability over a number of assays,
- The detection reaction should be independent as much as possible from physical parameters (stirring, pH, temperature),
- Accurate, precise, reproducible and linear response over useful range,
- Complete biosensor should be inexpensive, small, portable and easy to use.

Table 9: *Application of TLS in bioanalytical research*

Analyte	**Method**	
Determination of organophosphates	Implementation of Acetylcholinesterase	(Pogačnik and Franko 1999)
Oxidation of tiol-organophosphorus pesticides	Implementation chloroperoxidase	(Boškin et al. 2009)
Carotenoids detection in plasma blood	HPLC-TLS method	(Franko et al. 1998)
Fatty acid	Photothermal beam deflection spectrometer	(Bičanič et al. 1995)
Interferon-γ	MicroELISA	(Sato et al. 2004)
Phytoplankton cell lysis	Dual beam TLS	(Logar et al. 2006)

Table 10 gathers diverse application of biosensors.

Table 10: *The overview of applications of biosensors (Buerk 1992)*

Biosensor	Application
Enzyme-based electrochemical	Glucose biosensor
	Urea biosensor
	Alcohol biosensor
	Fatty acid biosensor
	Fish freshness biosensor
Immunosensors	Potentiometric immunosensors
	Amperometric immunosensors
	Enzyme-linked immunoassay
	Surface resonance
	Laser magnet immunoassay
Living biosensors	Biosensor using cultured cells
	Biosensor using intact tissues
	Biosensor using receptor elements

3.8 Solid supports for IgG immobilization

The surface of the support, on which the antibody is immobilized, has an important role in retaining the tertiary structure of the protein, which highly influences the activity and thermal stability of the immobilized protein. The choice of the support material is also of vital importance because different materials enable different accessibility of substrates to active sites of immobilized molecules. Particle size and porosity influence the mass transfer resistance and diffusion of reagents (Girelli et al. 2007a).

In the past, the controlled porosity glass (CPG) was widely used as immobilization support for various biological molecules. However, in the recent years, synthetic monoliths have gained importance as support for covalent immobilization of enzymes (Vodopivec et al. 2003b). CPG is known for high thermal stability and good resistance to acids, which makes it suitable for very harsh working conditions. In comparison to CPG, the structure of a

monolithic convective interaction media (CIM) support is more compact and therefore easy to use. Characteristics such as flow independent resolution and binding capacity, fast separation and high binding capacity for large biomolecules makes CIM discs an interesting support in comparison to conventional materials (Štrancar et al. 2002). Analytical techniques performed with CIM supports are fast, precise, and reliable (Vodopivec et al. 2003b).

The CPG and CIM supports were used for immobilization of biomolecules. The Table 11 shows some of the applications of such materials as support material for immobilized biological molecules.

Table 11: *Some of the CPG and CIM immobilized molecules*

CPG	**CIM**
α – amylase (Kahraman et al. 2007)	Glucose oxidase (Vodopivec et al. 2000)
Apricot pectinesterase (Karakus and Pekyardimci 2009)	Deoxyribonuclease (Benčina et al. 2005)
Mushroom tyrosinase (Girelli et al. 2007b)	DNase and trypsin (Benčina et al. 2007)
Chymotrypsin and β-galactosidase (Robinson et al. 1971)	Antibodies (Brne et al. 2009)
Single-stranded DNA (Walsh et al. 2001)	Citrate lyase, malate dehydrogenase, isocitrate dehydrogenase and lactate dehydrogenase (Vodopivec et al. 2003a)
Laccase (Rogalski et al. 1999)	Albumin (Zacharis et al. 2007)

4 Experimental

4.1 Material and instruments

For the new detection method development for the presence of beta-lactoglobulin (BLG) in food the bovine BLG A+B (Sigma, L-0130, EC 232.928.9), affinity purified anti-bovine BLG A+B antibodies raised in rabbit unlabelled and labelled with HRP (Bethyl Labs) in the commercial solutions of 1 mg/mL were used. O-phenylenediamine (99 %, Acros Organics) was used as a substrate for the HRP conjugated to antibodies.

The rabbit ovalbumin antibody (AB1221, 90 % PAGE) was obtained as a solution of 10 mg protein/mL PBS. Polyclonal ovalbumin antibodies raised in rabbit were used as primary antibodies. The analyte was incubated prior the analysis with ion-exchanged purified ovalbumin antibodies raised in rabbit and labelled with HRP (AB20415). The immunoglobulins were purchased from Abcam (Cambridge, UK).

Other chemicals used for buffer solutions and other chemical reagent use for the experiments: ethanol (absolute, puriss.), acetonitrile (99.9 %), Bradford reagent, glycine (99 %), sodium periodate (99 %), 3-(decyldimethyl-ammonio)propanesulphonate inner salt, (3-aminopropyl)triethoxysilane (98 %), chicken egg albumin (EC 232.692.7), BSA (EC 232.936.2), controlled pore glass (CPG, PG-240-120) from Sigma; sodium chloride (99 %), sodium dihydrogenphosphate dihydrate (99 %), disodium hydrogenphosphate anhydrous (99 %), Tween20 (molecular biology grade), Coomassie Brilliant blue G-250, Ponceau S (3-hydroxy-4-[2-sulfo-4-(4-sulfophenylazo)-phenylazo]-2,7-naphthalenedisulfonic acid, 80 %), sodium dodecilsulfate (96 %), ammonium persulfate (pure 100 %), bromphenol blue, glycerol (99 %), TEMED (n,n,n',n'-tetramethylethylenthylenediamine, 100 %) from AppliChem; TRIZMA base (99 %) from Riedle de Haen; metanil yellow, potassium oxalate monohydrate, beta mercaptoethanol (99 %), succinic acid (99 %), acetic acid (99.8 %) and sodium acetate anhydrous (98.5 %) from Fluka; methanol (99 %), 2-propanol (99.7 %) from JT Baker; sodium borohydride (98 %), sodium cianoborohydride (95 %) from Acros Organics; potassium chloride (99 %) from Carlo Erba; hydrochloric acid (30 %), nitric acid (65 %), hydrogen peroxide (30 %), pyrogallol (99 %), sodium molybdate dehydrate (99.5 %), trichloroacetic acid (99 %), glutardialdehyde (50 %) from Merck; ECL from Amersham, CIM diol from BIA (BIA Separations, Villach, Austria).

Instruments used for experiments: excitation Ar-ion laser working at 457.9 nm emission line (100 mW, Coherent CR4; Moorpark, CA, USA), probe Helium-Neon laser working at 632.8 nm emission line (2 mW, Uniphase 1102P, Milpitas, CA, USA), SR830 DSP lock-in amplifier (Stanford Research System, Sunnyvale, CA USA), optical chopper (SciTech Instruments, Wiltshire, UK), UV/Vis spectrophotometer 8453 from HP (Waldbronn, Germany), QE65000 spectrophotometer from Ocean Optics (Dunedin, FL, USA), light source HPX-2000 from Micropack (Avantes, Germany), HPLC pump from Knauer (Berlin, Germany), HPLC pump LC10-Ai from Shimadzu (Tokyo, Japan), injection valve (Rheodyne, San Francisco, USA), injection valve (Knauer, Berlin, Germany), electrophoretic cell and blotting cell from Clever, Infinte F200 from Tecan (Mannedorf, Germany), centrifuge Centrifuge 54152 and Thermomixer Compact from Eppendorf, Vortex TopMix FB15024 from Fisher Scientific, pH meter HI8417 from Hanna, sonicator Sonics 4 from Iskra PIO and analytical balance ABJ by KERN.

The figures of proteins structure were reproduced from sequences in the Protein Data Bank using molecular viewing program RasWin (open source).

4.1.1 Solutions and buffers

Solution	**Composition**
Phosphate solution A	0.2 M NaH_2PO_4
Phosphate solution B	0.2 M Na_2HPO_4
Phosphate buffer pH 7.4 (0,1M)	9.5 % (v/v) phosphate solution A 40.5 % (v/v) phosphate solution B deionised H_2O
Phosphate buffer pH 5.7 (0,1M)	1.5 % (w/v) NaH_2PO_4 $2H_2O$ 0.02 % (w/v) Na_2HPO_4 deionised H2O
PBS	0.01 M phosphate buffer pH 7.4 0.88 % (w/v) NaCl

Solution	Composition
	0.02 % (w/v) KCl
	pH 7.4
Column storage buffer	PBS
	0.02 % (w/v) NaN_3
Borohydride solution	0.16 % (w/v) $NaBH_4$
	0.1 M phosphate buffer pH 7.4
Antibody binding buffer	0.3 % NaCNBH
	0.1 M phosphate buffer pH 5.7
	antibody solution
Diol oxidation solution	5 % $NaIO_4$
	acetic acid (v/v= 50/50)
Binding group reduction solution	0.19 % $NaBH_4$
	0.1 M phosphate buffer pH 7.4
Pre-cleaning solution for CPG glass	5 % nitric acid
	deionised H2O
Aminoalkylating solution for CPG glass	10 % (v/v) 3-aminopropyltriethoxysilane
	deionised H2O
	pH 3.5 adjusted with HCl
TRIS buffer pH 7	1.2 % TRIS
	deionised H2O
	pH adjusted with HCl
Cross-linking agent	2.5 % (v/v) glutaraldehyde
	0.1 M phosphate buffer pH 7.4
Westrn Blotting buffer	20 % methanol
	25 mM TRIS

Solution	Composition
	192 mM glycine
	deionised H2O
	pH 8.3
10X Ponceau solution	2 % (w/v) Ponceau-S
	30 % (w/v) CCl_3COOH
	30 % (w/v) sulfosalicylic acid
	deionised H2O
TBS	10 mM Tris/HCl pH 7.4
	150 mM NaCl
Peroxidase substrate for film detection	
Peroxidase substrate OPD for spectrophotometric detection (stock solution)	1 % o-phenylenediamine
	in methanol
Peroxidase substrate OPD for spectrophotometric detection (working solution)	1 % (v/v) OPD stock solution
	0.1 % H_2O_2
	in acetate buffer pH 4.5
Acetate buffer pH 4.5	CHCOONa pH 4.5 adjusted with acetic acid
Coomassie washing solution	30 % methanol
	10 % acetic acid
	deionised H2O
Coomassie fixing solution	10 % acetic acid
	5 % glycerol
	deionised H2O
Stacking gel (2 gels)	0.5 M Tris/HCl pH 6.8
	acrylamide/bis-acrylamide solution (30 %)
	SDS solution (10 %)

Solution	Composition
	APS (10 %)
	TEMED
	deionised H2O
Resolving gel (2 gels)	1.5 M Tris/HCl pH 8.8
	acrylamide/bis-acrylamide solution (30 %)
	SDS solution (10 %)
	APS (10 %)
	TEMED
	deionised H2O
5X SDS PAGE running buffer	1.5 % (w/v) TRIS
	0.5 % (w/v) SDS
	0.7 % (w/v) glycine
	deionised H2O
4x SDS PAGE loading buffer	8 % (w/v) SDS
	40 % (v/v) glycerol
	20 % (v/v) β-mercaptoethanol
	in 250 mM TRIS/HCl buffer pH 6,8
Pyrogallol red precipitation reagent	0.0006 % (w/v) pyrogallol
	0.004 % (w/v) sodium molybdate dihydrate
	0.018 % (w/v) potassium oxalate monohydrate
	0.59 % (w/v) succinic acid
Metanil yellow	1 % (w/v) in PBS buffer

4.2 Methods

4.2.1 The immunoaffinity FIA method

The FIA set up consists of a HPLC pump pumping the buffer through a bioanalytical column built of a non-labelled antibody bind on a column support of choice. The sample is incubated with labelled antibodies, which recognized the model allergen prior the injection on the column. Hundred µL of sample is injected at a flow rate of 0.1 mL/min. After the sample injection the column is washed with buffer in order to eliminate the unbind proteins present in the sample and to remove waste. The signal derived from a labelled antibody captured on the column is detected spectrophotometrically by a UV/Vis spectrophotometer measuring the absorbance at 450 nm. The antibodies are labelled with the horseradish peroxidase. Horseradish peroxidase (HRP) catalyzes the oxidative coupling of o-phenylenediamine (OPD) to form 2,3-diaminophenazine (DAP) in the presence of hydrogen peroxide. Figure 10 presents the structure changes from OPD into DAP. 20 µL – 5 µL of HRP substrate is injected at a flow rate of 0.1 mL/min to 0.4 mL/min. After signal detection, captured protein can be removed by two consecutive injections of Glycine/HCl buffer at pH 2.5 at flow rate of 0.3 mL/min – 0.4 mL/min. The column is ready to be reused for another sample analysis.

Figure 10: *O-phenylenediamine and 2,3-diaminophenazine*

4.2.1.1 Antibody immobilization on column support

In the process of the analytical column selection three different column supports were used for unlabelled antibody immobilization.

4.2.1.2 Antibody immobilization on agarose

Agarose is one of the basic materials used as a filling material for protein separation column. Therefore, this material could be used as an immobilizing agent for unlabelled antibodies used in the detection methods.

4.2.1.2.1 The procedure of immobilization of unlabelled antibodies on 1.5 % agarose gel:

Agarose preparation: 15 mg of agarose powder were dissolved in 1 mL of PBS buffer in a boiling bath. As soon as the powder dissolves the gel was cooled for a minute to reach an approximate temperature of 50˚C.

Antibody intercalation: 200 μL of cooling gel was added to the antibody solution, quickly mixed and filled in a column cartridge. The gel was left at 4˚C for additional cooling and consequent gelling.

Column washing: previously to the usage of the column for detection procedures the column was washed with the working solution.

4.2.1.2.2 Antibody immobilization on CPG glass

Immobilization on activated glass is a procedure relying on cross-linking with glutaraldehyde (Pogačnik and Franko 2001). The previously described method (Pogačnik and Franko 2001) was slightly modified by removing the air from the pores of the carrier by putting the system under vacuum. The antibody immobilization was performed under a constant stream of a reducing gas (Kadima and Pickard 1990).

Pre-cleaning step: CPG-240 glass was boiled in a pre-cleaning solution for 30 min. The glass was centrifuged for glass sedimentation and the solution was removed from the glass. The glass was washed with water and centrifuged again. After centrifugation water was removed and the whole procedure with water washing was repeated for three times.

Aminoalkylating step: the dried CPG-240 glass was added to the aminoalkylating solution and swirled for 150 min at 75˚C in a thermomixer. After that time the aminoalkylating

solution was changed by centrifuging the solution and removing it. The whole aminoalkylating step was repeated again to ensure complete activation of the glass.

Antibody-immobilization: antibody solution was added to cold (4˚C) 0.1 M phosphate buffer pH 7.4 and mixed with pre-treated glass. The glass was swirled in a thermo mixer at room temperature and nitrogen was bubbled from time to time through the solution. After 1 hour the solution was centrifuged to remove the solution with non-bonded protein. The glass was washed several times with cold phosphate buffer in order to remove any unlinked proteins.

The glass beads with immobilized antibodies were stored in PBS buffer at 4˚C after immobilization.

4.2.1.2.3 Antibody immobilization on CIM disc

CIM discs used in these experiments were kindly donated for this research by BIA (BIA Separations, Villach, Austria). Immobilization was performed through the transformation of the diol-CIM into aldehyde-CIM and the subsequent reaction of the activated CIM with the amino groups of antibody (Puerta et al. 2005). The method was slightly modified by performing the immobilization in a FIA system in order to achieve fast immobilization procedure. The reagents were pumped through the immobilization column at a flow rate of 0.1 mL/min. The 0.1 M phosphate buffer pH 5.7 was used as antibody binding buffer instead of the 0.5 M phosphate buffer pH 6.9.

4.2.1.2.4 Immobilization procedure for CIM disc:

Oxidation of diol groups: the oxidizing solution was pushed through the discs to change the environments inside the pores and then lightly swirled at room temperature in a thermomixer for 2 hours. After, the discs were washed firstly with water and subsequently with phosphate buffer pH 5.7.

In the flowing mode the reagents were pumped through the system and sequentially added after a certain amount of time.

Antibody immobilization: The antibodies were mixed with the antibody binding solution. The mixture was pushed through the CIM disc and after the disc was put in this solution, gently swirled at 4˚C in a thermomixer for 60 hours. After immobilization the disc was first washed with phosphate buffer pH 5.7 in order to remove any unlinked antibody and phosphate buffer pH 7.4.

In the flowing system the reagents were pumped through the system and sequentially added after a certain amount of time.

Reduction of free binding groups: a borohydride solution was pushed through the column to clean the pores and swirled in this solution at room temperature for 1 hour. Consequently, the disc was washed with phosphate buffer pH 7.4 and PBS respectively.

In the flowing system the reagents were pumped through the system and sequentially added after a certain amount of time.

After immobilization, the disc was stored in PBS at 4˚C.

4.2.2 ELISA Procedure for Beta-Lactoglobulin detection

The ELISA was performed as the antibody producer recommended. OPD was used instead of TMB as the enzymatic reagent.

Plate coating and blocking: The primary antibody was diluted into a coating buffer to a mass concentration of 10 µg/mL. The 100 µL of diluted coating antibody was added to the wells of the plate and incubated for 60 min at room temperature or over night at 4°C. After the incubation, the plate was washed 5 times with the washing solution. The blocking for 30 minutes with the blocking solution (room temperature) followed the procedure. After wards, the plate was washed 5 times with the washing solution.

Standards and samples application: the standard solutions were prepared from a stock solution at the concentration of 250 ng BLG/mL according to the Table 12. Subsequently, they were

diluted to expected concentration (further called sample solutions). 100 µL of standard or sample solution was transferred to each well and incubated over night at 4°C or 60 min at room temperature. After incubation, the solutions were removed and the wells were washed 5 times with the washing solution.

Table 12: *Dilution protocol for standard solution for ELISA detection of BLG*

Standard	**Mass c (ng/mL)**		**Sample diluent**
initial	250	//	//
1	125	500 µL from initial	500 µL
2	62.5	500 µL from standard 1	500 µL
3	31.25	500 µL from standard 2	500 µL
4	15.625	500 µL from standard 3	500 µL
5	7.8	500 µL from standard 4	500 µL
6	3.9	500 µL from standard 5	500 µL
7	1.95	500 µL from standard 6	500 µL
8	0	blank	500 µL

HRP conjugated antibody application: the HRP conjugated antibodies were diluted in a ratio 1:10000 in the diluent solution. 100 µL of the diluted antibody is transferred to each well and incubated for 60 minutes at the room temperature. After incubation, the solution was removed and the wells were washed 5 times with the washing solution.

OPD substrate incubation: 0.4 mg/mL solution of o-phenylenediamine (OPD) was prepared in acetate buffer pH 4.5. Prior to application, 6 µL of 30 % H_2O_2 was added to 10 mL of OPD solution. 200 µL of activated OPD solution was added to each well and left (for 30 minutes at room temperature in the dark) for the colour development. After incubation, 50 µL of 3 M HCl was added to block the reaction prior to the measurements.

Absorbance measurement: The absorbance was measured on a microtiter plate reader INFINITE 200 (Tecan, Männedorf, Switzerland).

4.2.3 Sample preparation

The sample preparation consisted of two different extraction procedures:

Procedure nr. 1:

1g of food product (triturated if necessary) was mixed with 10 mL of 0.3 M NaCl solution containing 0.2 % of TritonX-100. The mixture was extensively shaken for 2 hours at the room temperature in a thermomixer. The next step was the centrifugation for 45 minutes at a speed of 16000 rpm at 4˚C. The aqueous layer was filtered and the centrifugation process was repeated. After centrifugation the liquid was acidified with HCl to pH 2. Afterward, the aqueous layer was incubated for 20 min at 37˚C in a thermomixer, which was followed by another centrifugation for 2 minutes at a speed of 16000 rpm at 4˚C. The aqueous layer was filtered again and the solution was stored at -20˚C for further analysis.

Procedure nr. 2:

Ten grams of food product (triturated if necessary) was mixed with 10 mL of PBS/ether (v/v, 4:1). The mix was extensively shaken for 5 minutes at the room temperature and afterwards centrifuged for 15 minutes at the maximum speed in order to get rid of larger pieces and to accelerate the formation of the double layer between PBS and ether. The ether was removed and the aqueous layer was centrifuged again at the maximum speed. The aqueous layer of the treated sample was used for further analysis.

4.2.4 Protein quantization

4.2.4.1 Spectrophotometric absorbance measurements

4.2.4.1.1 Absorbance at 280 nm

The concentration of protein can be calculated from the relative absorbance at the wavelength 280 nm and of the absorbance coefficient at 280 nm typical for the protein in question. The extinction coefficient can be evaluated out of a known quantity of protein and its absorbance at the selected wavelength (Equation 3).

$$A_{280} = \varepsilon d c \qquad \textbf{(3)}$$

4.2.4.1.2 Bradford method

The Bradford method was used for an approximate evaluation of the antibody immobilization efficiency on CIM disc. The principle of this method is the formation of coloured complexes between the acidic solution of the colour agent Coomassie Blue and amino acids. In a plastic cuvette 200 μL of sample and 800 μL of Bradford reagent were mixed. The mixture was left for 5 min at the room temperature for colour development. The absorbance of the sample was measured at 595 nm against a blank consistent of a mixture of Bradford reagent with the buffer without proteins. The concentration of proteins was calculated out of a calibration curve, which was made with BSA protein, Equation 4.

$$A_{595nm} = 0.03c + 0.05 \qquad \left(R^2 = 0.99\right) \qquad \textbf{(4)}$$

4.2.4.2 SDS-PAGE detection of IgG quantity

The immobilization efficiency was estimated by checking protein content in immobilization buffer prior and after the immobilization procedure. Since extensive washing was required in order to remove the excess of unbound antibodies, the Bradford method for protein quantization based on absorbance measurements was not sensitive enough for quantification of antibodies in buffer after immobilization. Therefore, proteins in immobilization buffer were precipitated and quantified by SDS-PAGE under reducing condition (90 min at 100 V) on an electrophoretic gel (15 % resolving gel), based on comparison to standards of different concentration. Protein bands were visualized by Coomassie Brilliant Blue stain. The antibody density (A/area) was calculated out of the results obtained by SDS-PAGE electrophoresis and data obtained from the support producers.

4.2.4.3 Westrn blot for evaluation of the efficiency

For the purpose of the efficiency of the immunodetection FIA system, two fractions were collected. The first fraction was the eluent ready after the sample injection; the second fraction was the eluent after Glycin/HCl buffer injection on the column. This fraction was chosen in order to determine the presence of the model allergen on the column. The whole

detection procedure was divided into three parts: SDS PAGE, blotting on membrane and chemiluminiscent detection.

4.2.4.4 SDS PAGE

The SDS PAGE was performed according to SDS PAGE protocol (Baines 2001). Vertical system Clever for electrophoresis was used for SDS PAGE. The first step was the preparation of acrylic gel. A 12 % resolving gel was poured between two glasses and let to solidify before a second stacking gel, 4 %, was added at the top of the resolving gel. A loading buffer was added to the samples and boiled at 95ºC for 5 min. The mixture was loaded on the gel and soaked in the SDS running buffer. The electrophoresis took 50 min at a constant current of 35 mA/gel. The next step is the blotting from the gel to the nitrocellulose membrane.

4.2.4.5 Wet Westrn blot

The Westrn blot was performed according to Westrn blot protocol (Baines 2001). After SDS PAGE the gel was washed with deionized H_2O and soaked in the blotting buffer. The nitrocellulose membrane and the filter papers were cut to the size of the gel and soaked in the blotting buffer. The membrane and the gel were put together between filter papers and put in the blotting cell. The blotting took approximately 1.5 hour at constant current of 0.8 mA/cm^2 of gel. After the blotting the membrane was washed with deionized H_2O and dried for a few minute at the room temperature. The subsequent step consisted of the colouring with Ponceau solution to assure the blot of the protein and to mark the protein ladder on the membrane. The colour was easily washed away with TBS buffer. After that the membrane was used for protein detection.

To assure the perfect blot, an additional colouring of the gel with Coomassie Brilliant Blue was performed. This method allows the perception at about 0.5 μg of proteins. The protocol procedure is presented in Table 13.

Table 13: *Procedure of Coomassie staining*

Step	Solution	Time (min)
Colouring of the gel	Coomassie colouring solution	10
Uncolouring of the bulk coloration	Coomassie washing solution	5
Uncolouring of the bulk coloration	Coomassie washing solution	8
Uncolouring of the bulk coloration	Coomassie washing solution	10
Fixing of the colour bands on gel	Coomassie fixing solution	5

4.2.4.5.1 Chemiluminiscence detection after Westrn blot

The first step in the immunodetection of protein on membrane was the blocking of non-specific binding site that can be recognized by the used antibodies. The membrane was lightly shacked for 1 hour in a blocking solution at room temperature. Afterwards, the membrane was washed for three times with TBS buffer. After the washing step the immuno detection protocol was applied.

4.2.5 Detection of model allergen

The washed membrane was incubated in a solution of primary antibodies and constantly shaken for about 1 hour at the room temperature. After incubation with primary antibodies, the membrane was washed with TBS buffer for three times and reincubated in a solution of secondary antibodies labelled with HRP. After the second incubation the membrane was washed with TBS for three times in order to remove any unbound labelled antibodies. The membrane was poured with ECL reagent. The reagent was mixed right before the detection, the solution I and solution II were mixed together in a proportion 1:1. The ECL reagent was left for 5 min at room temperature and then the whole system was moved in a dark chamber, where the membrane was put in a developing cassette together with a sensitive film. The ECL reagent consists of luminol a chemical that is oxidized by HRP. Oxidized luminol emits light, which consequently irradiates the film. After irradiation the film was put in a developing solution for 3 min and subsequently washed with water and kept in a fixing solution for another 3 min. At the end the film was washed and let to dry.

4.2.6 Detection of labelled antibodies raised against the model allergen

The detection procedure for labelled antibody detection follows the same steps as the detection procedure against model allergen.

4.2.7 Protein precipitation for concentration and buffer changing

The proper choice of buffer solution in which the proteins are kept, assures flawless experiment performance. It is of great importance to precipitate the proteins, remove the old buffer and to change it with a different one. There are several protocols for protein precipitation. Since the buffer used in antibody immobilization are not adequate for protein detection by absorption measurements and Bradford quantification, the change in buffer becomes an urge. Pyrogallol colour has the ability to bind to the proteins and under the centrifugal force the complex protein/Pyrogallol precipitates at the bottom of the tubes.

4.2.7.1 Precipitation protocol used:

Sample was mixed with Pyrogallol red reagent in a ratio 1:3. The mixture was left for 1 hour at 4˚C. After colour binding to the protein the mixture was centrifuged for 10 - 15 min at a maximum speed. The supernatant was removed and the protein was diluted in another buffer to the volume of 20 µL.

4.2.8 TLS manifold

The detector consisted of dual-beam laser system (Figure 11). The excitation source was an Argon-ion laser (Ar-ion) operating at 457.9 nm and 100 mW power. The probe beam was provided by a Helium-Neon laser (He-Ne) operating at 632.8 nm. A variable-speed mechanical chopper modulated the pump laser beam at 37 Hz. For correct operation and optimum measuring sensitivity, collinear propagation of the two beams was obtained using dichroic mirror. The change in the probe beam intensity due to TLS effect was monitored by a

photodiode placed behind a filter, which filters out the excitation laser beam. The signal from the photodiode was fed into a lock-in amplifier with a 1 s time constant, which was connected to a computer for data processing and storage.

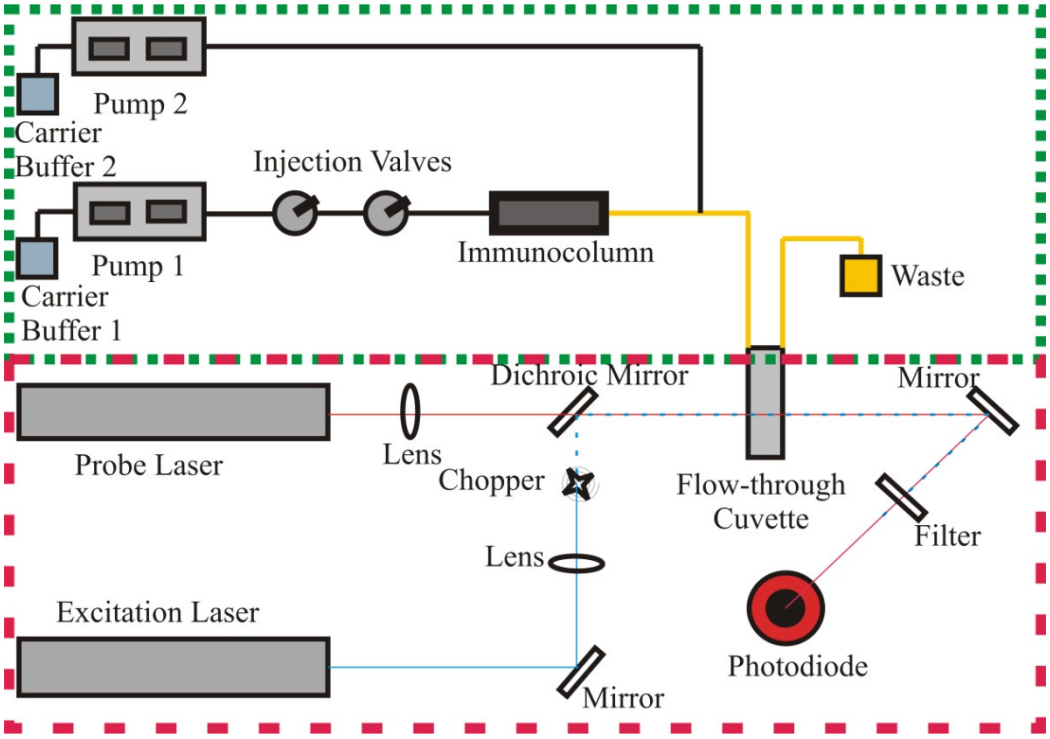

Figure 11: *The FIA-ELISA-TLS set-up. In the green bracket there is the scheme of the FIA-immuno system. In the red bracket, there is the schematic representation of the TLS system.*

4.2.9 FIA system for FIA-ELISA-TLS

The FIA manifold assembled for the purposes of this work consisted of a HPLC pump, two injection valves (injection loops of 5 µL and 100 µL), immunocolumn and TLS detection unit previously described (Figure 11). The carrier buffer used was PBS solution of pH 7.4, pumped through the system at a flow rate of 0.4 mL/min.

The FIA manifold assembled for experiments involving organic solvent consisted of two HPLC pumps, two injection valves (injection loops of 5 µL and 100 µL), immunocolumn followed by mixing column and TLS detection unit previously described. The two pumps were connected through a low-volume T-connector between the immunocolumn and mixing column. The PBS pH 7.4 carrier buffer was pumped through the first pump at a flow rate of 0.4 mL/min. The pure organic solvent was pumped at a flow rate of 0.4 mL/min.

4.2.10 Detection procedure

The principles of the detection procedure are presented in Figure 12.

In the first step of the detection procedure, the sample was preincubated with enzyme conjugated IgG. The addition of the immunoglobulins took place approximately 10 minutes prior to the injection into the stream of the carrier buffer. In the second step, the sample was injected into the stream through an injection valve of a defined volume. The third step constituted of the catching of the allergen in the immunocolumn (Figure 13). Subsequent step was an injection of the enzyme substrate o-phenylenediamine (OPD) in the FIA system. The product was detected during the passage through to the flow-through cuvette. The last step was the injection of the Glycine/HCl buffer.

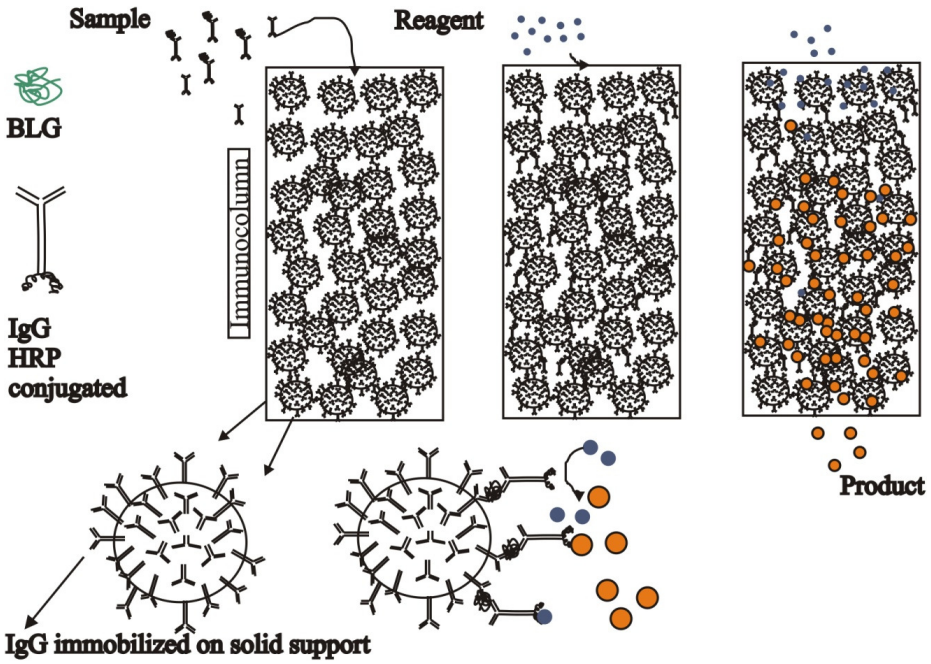

Figure 12: *Scheme of the immunological detection in a closed system i.e. an immunocolumn.*

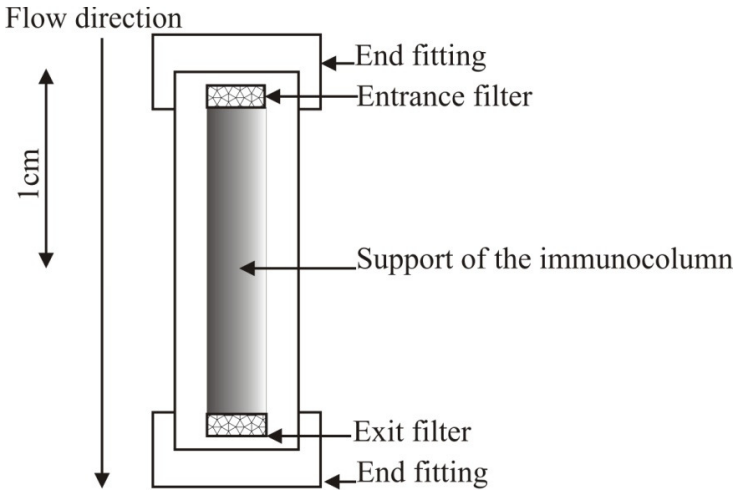

Figure 13: *Scheme of the immunocolumn; the filters at the entrance adsorb the pigments of the coffee.*

4.2.11 Excitation laser alignment evaluation

The excitation Ar-ion laser stability was checked by using a solution of Metanil Yellow, a reagent found to be stable over a long period time. The laser alignment was checked by injection of a standard solution of yellow colour each experimental day (Figure 14). The reagent was kept in dark as stock solution of 100 mg/mL. Prior to analysis the reagent was diluted 100-times and directly injected into the FIA-TLS system. The immunocolumn was not present in FIA system during the stability measurements.

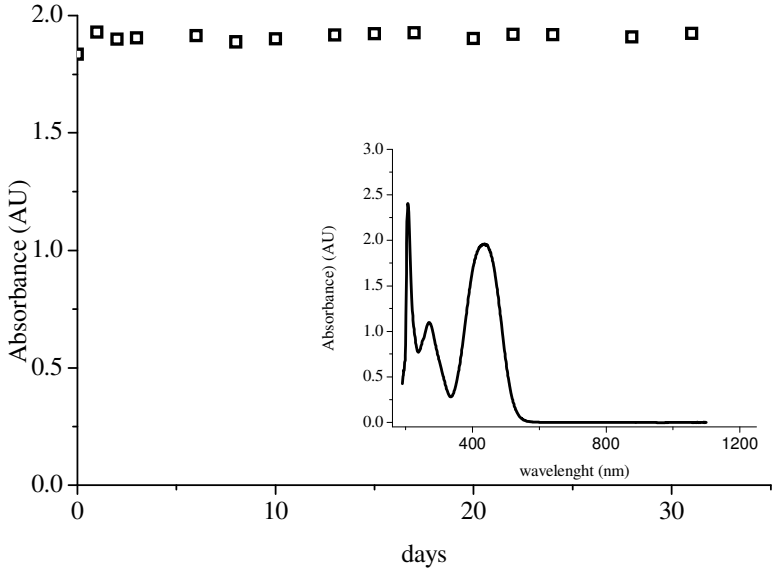

Figure 14: *Absorbance spectrum of Metanil Yellow and the stability of this compound measured as the change in absorbance with time.*

4.2.12 LOD calculation

The LOD were calculated as in Equation 5:

$$LOD = \frac{3 \cdot \sigma_{blank}}{k} \quad ; \qquad (5)$$

where σ is the standard deviation from the blank signal and k is the slope of the calibration curve (Mocak et al. 1997).

5 Results and discussion

5.1 Method description

The method described in this work is a combination of different detection principles. Altogether, it combines immunodetection, flow-injection analysis (FIA) and spectrometric detection. The milk allergen beta-lactoglobulin (BLG) was used as an analyte for method development. The allergen in question was chosen because of the widespread use of milk proteins as additives to processed food and medical products such as vaccines, tablets etc.

5.1.1 IgG raised against beta-lactoglobulin and immunodetection

The first part of this work has been the choice of antibodies raised against the allergen beta-lactoglobulin. Antibodies give specificity to the detection method. The immunological detection was based on sandwich ELISA principles. The detection procedure needs two different antibodies. One set of antibodies are immunoglobulins, which recognize the allergen. They are usually immobilized on a solid support such as PDVF membrane, ELISA plates and glass beads and are of IgG type, hence more easily producible and have a vast range of applications. The second type of antibodies belongs to the group of immunoglobulins raised against the same protein. However, the secondary antibodies are usually marked. The marker is an enzyme, which enables to perform enzymatic reactions to confirm their presence in the system. There are two different enzymes available, horseradish peroxidase (HRP) and alkaline phosphatase (AP). In this research the HRP enzyme was chosen due to its working parameters. The enzyme conjugated antibodies recognize and bind the allergen. In this way, the allergen is tagged and therefore can be easily detected by performing the enzymatic reaction.

The recognition of the allergen occurs on the support, where the immobilized IgGs are present. The derivatised support is packed into a small column. Due to the presence of immunoglobulins in the interior the column is called immunocolumn. The allergen is captured by the immobilized immunoglobulin and extracted out of other substances present in the sample. To complete the recognition the second immunoglobulin is applied, which labels the captured allergen. To confirm the presence of the allergen, the substrate for the marker

enzyme is added. The formed product is detected with one of the spectrometric detection methods available. In this research, the thermal lens spectrometry was applied as spectrophotometric detection method.

5.1.2 The implementation of the immunological detection into the flow-injection system

In this research, the immunological detection was implemented into a FIA system. The FIA system enables fast procedures such as application of the sample, washing the excess material etc. The implementation of the immunological detection is possible due to the column immobilization of the antibodies. The immunocolumn is placed in the flowing system and all the reagents can be applied directly into the stream. The FIA set-up is made of a pump, which pumps the carrier buffer through the system. The carrier buffer moves the solutions, which are injected though injection valves, along the system. The number of injection valves varies depending on the exigency of the detection method. The method described in this work requires two different injection valves. Each of these valves has an injection loop of defined volume (different volumes were tested ranging from 5 µL to 200 µL). The injected solution passes through the analytical column, where the detection reaction occurs. Then, the carrier buffer washes the formed product of the column and moves it to the flow-through cuvette. The flow-through cuvette is placed either into a UV/Vis spectrophotometer or in a thermal lens (TLS) detection system (Figure 11).

5.2 Evaluation of the IgG immobilization

As part of the method development, we tested the suitability of CPG and CIM supports for immobilization of immunoglobulin. The evaluation of the binding efficiency of antibodies to the support showed that the amounts of antibodies bound in a single immunocolumn differed by ca. 50 % in favour of CIM disk supports. However, when taking into account the specific surface area of the supports, much larger difference between CIM and CPG supports becomes evident, which was reflected in 350 times higher density of antibodies on the CIM support (Table 14).

Table 14: *Estimated values of immobilized primary antibodies*

Support	Immobilized antibodies per column (µg)	Antibody/ area (µg/m^2)
CIM	55	4900
CPG	35	13

5.3 Development of the method on the UV/Vis spectrophotometer

5.3.1 O-phenylenediamine absorbance spectra and the chosen wavelengths for method development

Possibility of a broad range wavelength application makes a UV/Vis spectrophotometer (SFM) a suitable instrument for many spectrophotometric applications. For TLS measurements, the wavelengths are defined by the type of the laser used as an excitation laser. These bands are restricted to few lines in the case of Argon ion laser (Table 15) used in this research. The most powerful line emitted by this instrument is the 488 nm. However, the wavelength chosen for this research is 457.9 nm, which is the closest to the maximum absorbance of the reagent used in this research (Figure 15) and gives the highest calculated values for the TLS signal, shown in Table 1 and calculated from the Equation 1. The TLS signal is totally dependent on the chosen wavelength, the absorption of the compound at this wavelength and of the power from the emitted line.

Horseradish peroxidase, the enzyme coupled to the antibodies, catalyzes the oxidative coupling of OPD to form 2,3-diaminophenazine (DAP) in the presence of hydrogen peroxide (Figure 15). The enzymatic product has a very distinctive yellow coloration in comparison to the transparency of the initial OPD solution.

Table 15: *Emission lines from the Ar-ion excitation laser, their corresponding powers and calculated TLS signals (for 1 mg/mL OPD).*

Wavelength (nm)	Maximum power (mW)	$\Delta I_{bc}/I_{bc}$	$\Delta I_{bc}/I_{bc}$ (for P=100 mW)
501.7	150	22.7	15.1
496.5	300	52.7	17.5
488.0	800	191.8	24.0
476.5	400	132.8	33.2
472.7	200	71.6	35.8
465.8	500	198.7	39.7
457.9	120	54.3	45.2

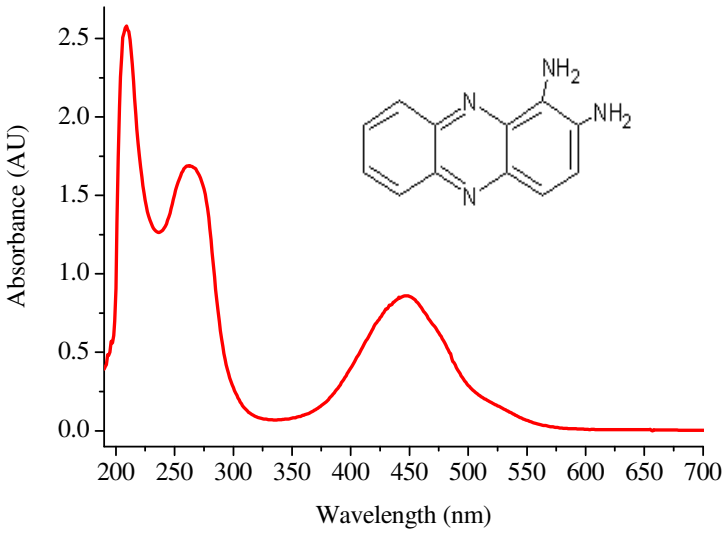

Figure 15: *Absorbance spectrum of the enzymatic product DAP*

The enzymatic product of OPD absorbs at a range of 350 to 550 nm, having the maximum absorbance at 450 nm. Comparing the absorbance of the product with the available emission wavelengths of the excitation laser (Figure 16) it is obvious that just four of the emission lines

457.9, 465.8, 476.5 and 488 nm) can be used for the development of the detection method. However, by shifting off the maximum absorbance for the product the possible interferences in the measurements can be more disturbing.

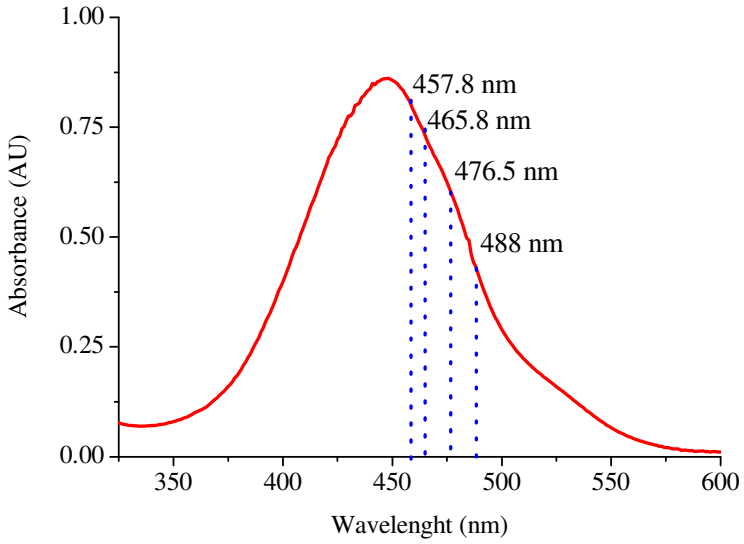

Figure 16: *Absorption spectrum of DAP (OPD concentration 1mg/mL) and wavelengths available for detection in TLS system.*

While performing measurements of the absorption spectra of the enzymatic product DAP in different media, we observed slight differences in the position of the absorption maximum, which was visible as a colour change (Figure 17). The wavelength of the absorbance maximum of the enzymatic product in water is similar to the spectra in acetate buffer pH 4.5 (Figure 18). The maximum absorbance is at 450 nm. However, the sensitivity (based on the yield of the reaction product) is lower. It was equal to only 20 % of the value reached in acetate buffer. In contrast, the spectrum of the enzymatic product in PBS buffer pH 7.4 is different. It has an additional peak at approximate 300 nm (signed with a blue arrow in the Figure 18). The absorption range in buffer pH 7.4 is wider and the maximum is shifted towards higher wavelengths (466 nm).

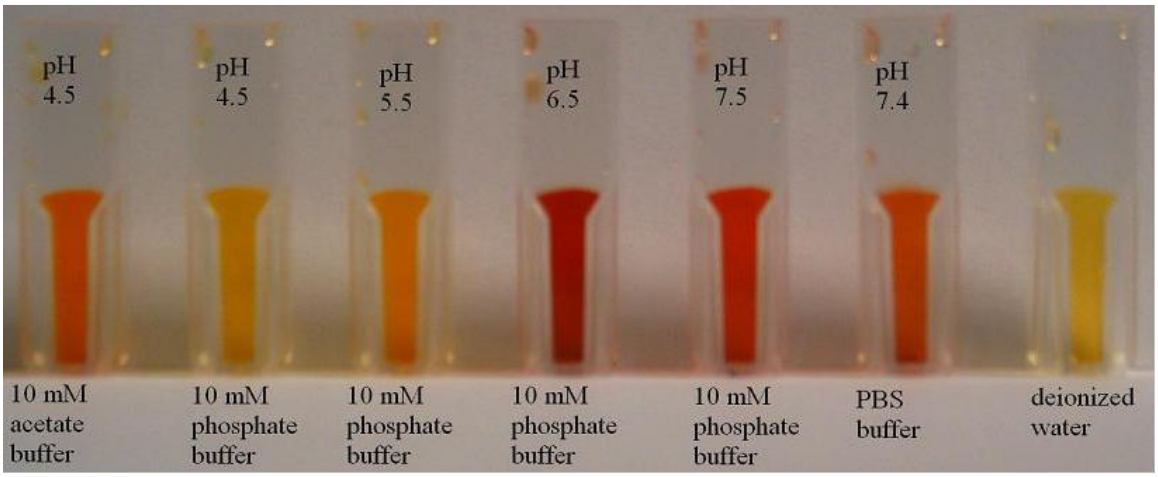

Figure 17: *Comparison of the colour development during enzymatic reaction in different solutions and at different pH.*

The additional peak at 300 nm (marked with the arrow in Figure 18), was formed at pH values higher than 6 and no such peak was present in absorbance spectra of DAP below this pH. In this experiment, we observed the shift of the absorbance maximum at pH 7.5 and 6.5 indicating that the pH has the biggest influence on the difference in absorbance spectrum (Figure 19). The changes in protonated and deprotonated form of the product probably affect the structure of the whole compound and therefore cause the shift in the absorption spectra.

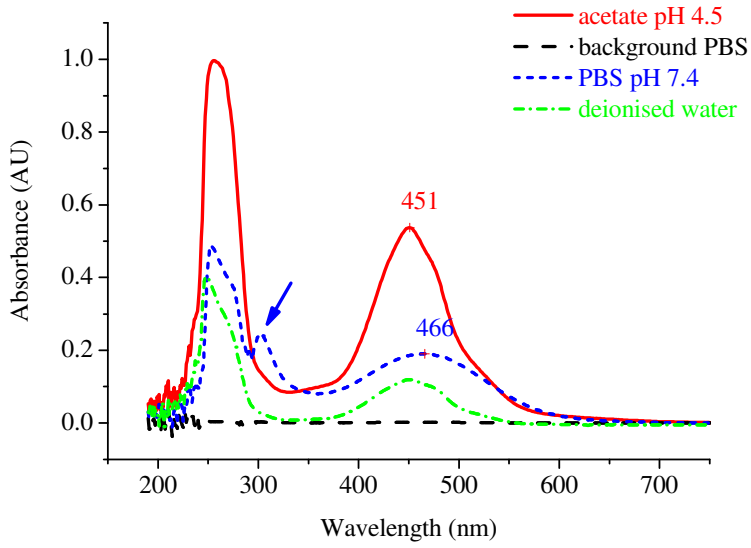

Figure 18: *Absorbance spectra of the enzymatic product DAP obtained in different buffered solution.*

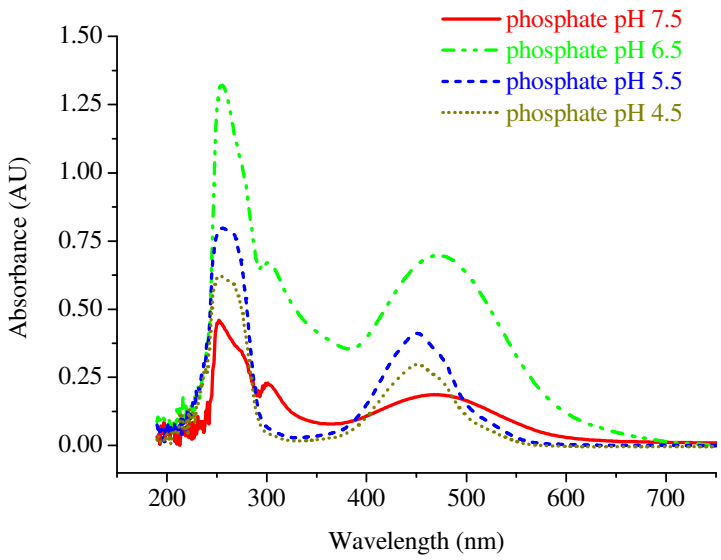

Figure 19: *Absorbance spectra of DAP obtained in phosphate buffers at different pH values.*

5.3.2 Influence of buffer composition on the enzymatic activity of conjugated horseradish peroxidase

As already mentioned, the pH optimum of the horseradish peroxidase is 6.0-6.5. However, the optimal pH for immunological reaction is around 7.5. Therefore, the proposed buffer for the detection method was a PBS buffer as a carrier and sodium acetate buffer as a dissolution buffer for the o-phenylenediamine reagent. Hence, the enzymatic activity was tested for both cases - where the reagent was dissolved in pure PBS and where it was dissolved in sodium acetate buffer and additionally diluted with PBS (resembling the condition during an injection into the FIA system). The results showed better activity in the case, where the reagent was dissolved in acetate buffer and then diluted in PBS. Therefore, the OPD dissolution in acetate buffer was preferred (Figure 20).

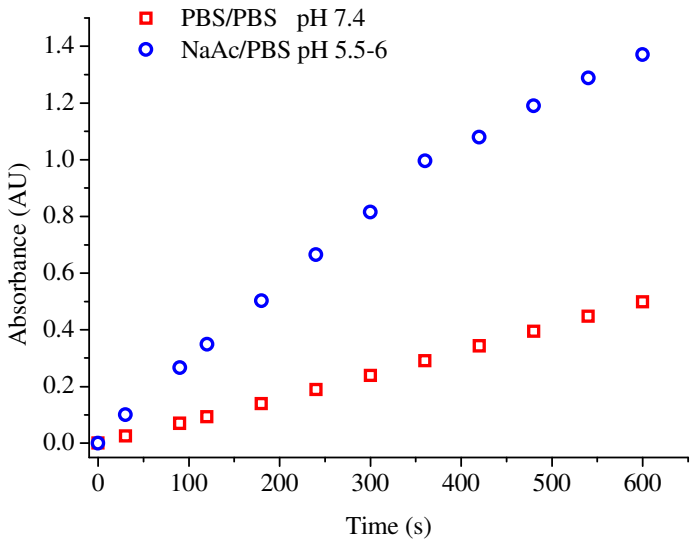

Figure 20: *Changes of absorbance in time due to enzymatic action on OPD substrate in different combinations of buffer for OPD solution and dilution.*

5.3.3 Concentration of IgG HRP conjugated for spectrophotometric detection

One of the major parameters that had to be established for the spectrometric detection was the proper concentration of the HRP conjugated IgG. These antibodies should be in excess in comparison with the analyte to assure binding of all molecules of the analyte. At the same time, the concentration of immunoglobulins should not be too high. In order to prevent undesired coagulation of proteins and subsequent sedimentation, which may lead to the depletion of available antibodies, the concentration has to be carefully chosen. As it can be seen in Figure 21, the reaction rate of the product formation drops drastically at dilutions higher than 20 000-time dilution of the initial concentration of the HRP conjugated antibodies (initial concentration 1 mg/mL). In the FIA-ELISA experiments the magnitude of the signal is proportional to the amount of analyte bound in the immunocolumn. Lower is the concentration of the analyte less HRP conjugated antibodies are kept inside the immunocolumn. Fewer antibodies inside the immunocolumn mean also less detectable product. The HRP enzyme has a limited amount of time to convert the substrate (for the FIA-ELISA system less than one second). On Figure 21, the situation where low concentration of detectable product was formed is represented. Detectable product formed in less than a second was observed just at lower dilutions of IgG HRP (higher concentrations of HRP conjugated IgG). We expected, that minute concentration of allergens will produce

lower concentration of enzymatic product, which will be difficult to detect with UV/Vis spectrophotometer. As a conclusion, UV/Vis spectrometric detection is suitable for preliminary experiments but cannot be used for detection of minute concentration of the analyte. The immunoglobulin producers recommend working volume dilutions of HRP conjugated antibodies for ELISA test in the range of 1: 10 000 to 1: 200 000. However, such high dilution did not work for the FIA system with the UV/Vis spectrophotometer and lower dilutions were tested. After preliminary experiments, the chosen dilution for UV/Vis spectrophotometric measurements was 1:3300 (Figure 22). For a FIA system with UV/Vis detection higher concentration of HRP conjugated antibodies performed better. However, lower dilution of 1: 10 000 was chosen for TLS measurements. As already mentioned, TLS technique is known to be more sensitive than UV/Vis spectrometry (Franko 2009) and therefore lower concentration of the generated product can be detected reliably.

As shown in Figure 22, fast screening of the antibody concentration showed that the higher the dilution, the smaller background signal was achieved. The results pointed out that the dilution of 1: 3300 for FIA-ELISA-SFM was appropriate. The signal to blank ratio was definitely better when the antibodies were in a lower concentration.

After the selection of a proper concentration for the experiments, the stability of diluted antibodies solution was verified. The experiments showed that 50 % of the activity is lost during overnight storage at 4°C (Figure 23). Therefore, fresh dilutions of antibodies dilution were prepared on a daily basis.

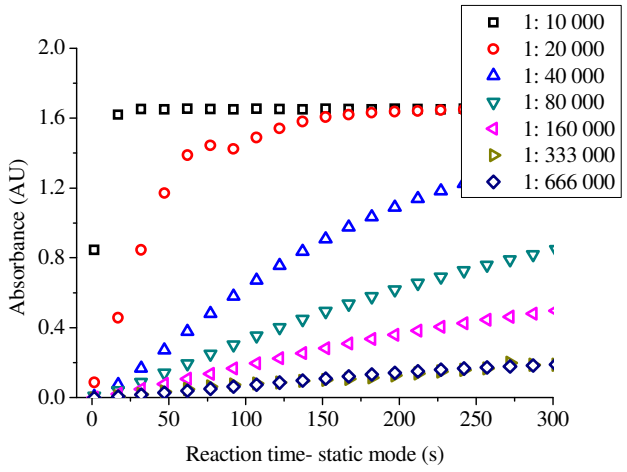

Figure 21: *Activity of HRP at different dilutions.*

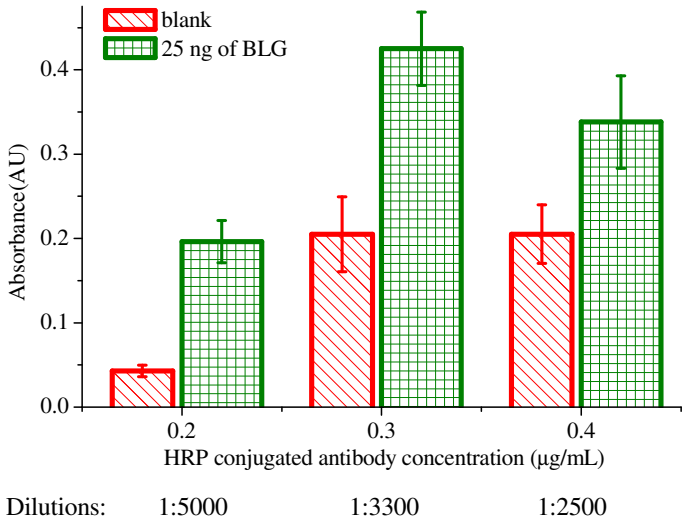

Figure 22: *Differences in the sample signal and blank signal at different concentration of IgG.*

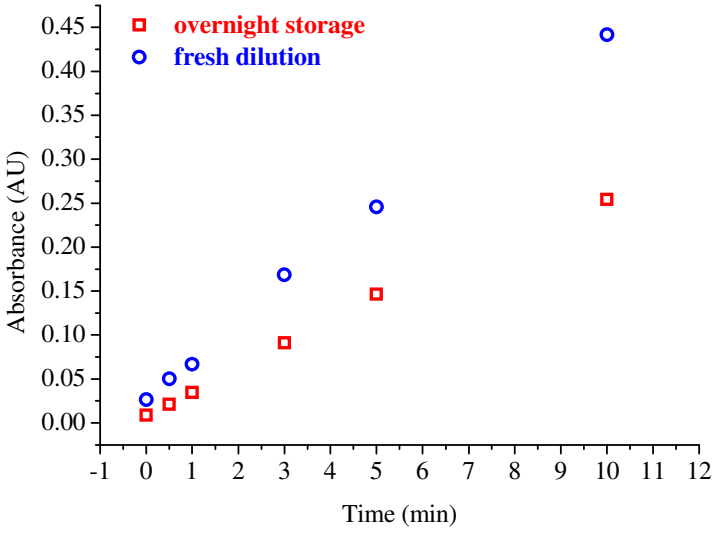

Figure 23: *Comparison of HRP activity of freshly made HRP conjugate IgG dilution and overnight stored dilution.*

5.3.4 Flow-injection system working parameters

The reaction parameters are highly affected by the flowing stream. Enzymatic reactions for immunological detection never reach an equilibrium state since the reagent and the product

are constantly washed away from the point of reaction. The parameters that have to be defined are:

- Flow rate (affects the peak broadening and apparent enzymatic activity)
- Injection volumes (affects the sensitivity of the detection methods).

5.3.4.1 Flow rate

The flow rate directly influences the formation of the product in an enzymatic reaction. The product is formed due to the presence of the HRP enzyme co-bound on the captured allergen inside the immunocolumn. The substrate for the enzyme is injected into the stream of the carrier buffer at a certain flow rate. If the flow rate is faster less product is formed. The enzyme captured in the system does not have time to convert the reagent into the product; therefore is expected that the un-reacted substrate is washed out of the column. The tested flow rate ranged from 0.05 mL/min to 0.5 mL/min (Figure 24). Higher rates were avoided in order to preserve the column from leaking and loosing the captured proteins (Ruzicka et al. 2006). Moreover, at higher flow rates the reaction time between the enzyme and the substrate is shorter, which influences the intensities of the detected signals and consequently the limits of detection. A weak protein-protein interaction is formed between immobilized antibodies and the allergen, therefore, it is expected that at higher flow rates of the carrier buffer the proteins can be washed away. On the other hand, the slower flow rates were avoided in order to shorten the detection procedure and to prevent the saturation of the TLS signal due to excess concentrations of product in case of higher allergen concentration.

At slower flow rates the signals originating from the presence of the allergens and the signal resulting from non-specific antibody retention were high. Moreover, a clear difference was observed between the signal formed due to the allergen presence in the immunocolumn and the signal formed due to non-specific retention of HRP conjugated antibody. By increasing the flow the signal for allergen presence dropped. This confirmed that the enzyme did not have enough time to act on the reagent and therefore lower product formation occurred. By accelerating the flow from 0.05 to 0.5 mL/min, the difference in the ration signal/blank decrease. The blank signal, which is the indicator of non-specific retention of HRP conjugated antibodies, was 70 % lower at the flow rate of 0.5 mL/min in comparison to the blank signal

obtained for the flow rate of 0.05 mL/min, while the signal for the allergen presence dropped by 80 %. The stability of the flow rate has a direct impact on LODs. At lower flow rates the stability is rather poor, which resulted differences in LOD up to 40 %. For example at 0.05 mL/min the blank signal was 0.04 with an RSD of 20 % and the LOD of 33 pM corresponding to 60 pg in 100 µL sample. While at 0.5 mL/min the blank signal was 0.01 with an RSD of 3 %. The achieved LOD was 25 pM corresponding to 47 pg in 100 µL sample. Moreover, the elution time of the product was halved at higher flow rates, which directly influence the times of the analysis. In regard to these results, the flow rate of 0.3 mL/min for the spectrophotometric detection was chosen, since it gave an adequate (detectable) signal, low LODs and a fast detection procedure (approximate 10 min). Higher flow rates were avoided in this case.

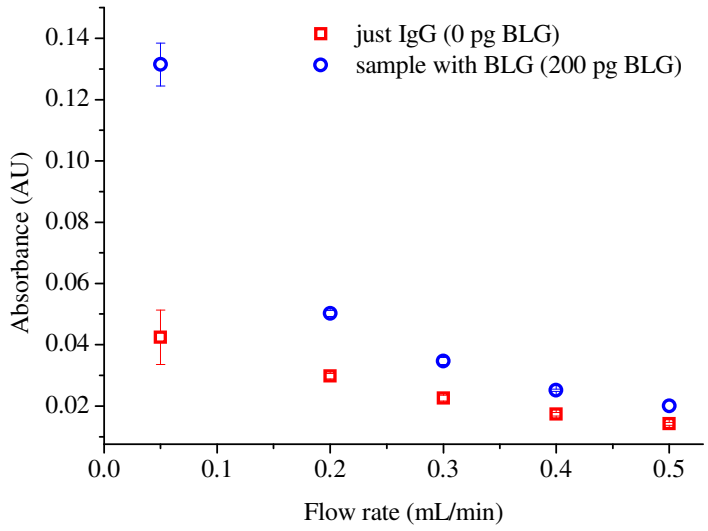

Figure 24: *Comparison of signal vs. blank signal at different flow rates.*

5.3.4.2 Injection volume

Injection volume of the HRP substrate is also an important parameter regarding the consumption of the reagent and effectiveness of the product formation. The tested injection loops for the HRP substrate for the spectrophotometric detection varied from 500 µL to 20 µL. The reagent volume affects the sensitivity of the method and influences also the detection time. The volume of 500 µL produced a signal, in which DAP was eluting for 5 minutes. Therefore, such large volume was not acceptable for a fast detection method. As shown on

Figure 25, the elution time of the formed product depends on the volume of the injection loop and the flow rate used for the injection of the HRP substrate.

The elution times varied from 2 minutes in the case of 200 µL sample loop to less than a minute for the volume of 20 µL (using the same flow rate of 0.3 mL/min). Since shorter times were preferred, the 20 µL sample loop was chosen for further investigation. In the Figure 25, it is evident, that double peak shaped signals were formed, which were not expected in the FIA-ELISA-SFM system. The DAP product should be eluting in an uniformly Gaussian-shaped signal, larger (higher signal and wider areas) for higher volumes of injection loops. The double peak shaped signals are most probably formed due the poor dispersion and non-uniformly moving of the reagent through the system (Baraguan et al. 2002). Since the double peaks signals are difficult to evaluate, the area of both peaks should be calculate. As seen in Figure 26, the larger is the injection loop larger areas are observed, which is expected.

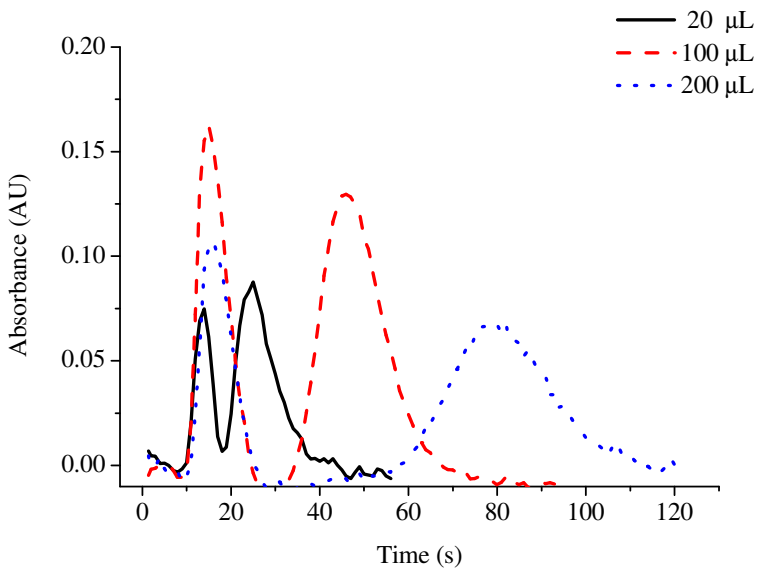

Figure 25: *Elution time of the signal for different reagent volumes; smaller the volume shorter is the time and less pronounced is double shaped peak (flow rate 0.3 mL/min, OPD concentration 360 µg/mL, IgG HRP conjugated 1:3300).*

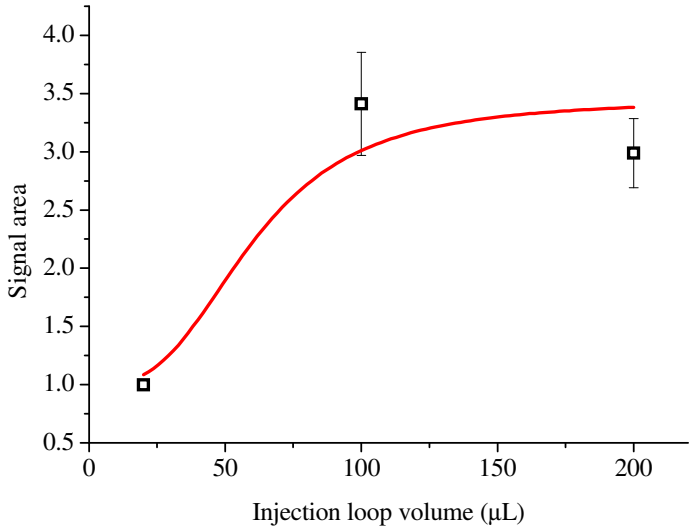

Figure 26: *Peak areas of the signal for different volumes of injected samples.*

5.3.5 Peroxide influence on product formation

Peroxide is the oxidizing agent needed for horseradish peroxidase (HRP) activity (Veitch 2004). Without it, the enzymatic product formation does not occur. As showed in Figure 27, the concentration of around 0.02 % peroxidase results in a quick response of the enzyme. The OPD solution was freshly prepared each experimental day and the H_2O_2 was added to it right before the injection onto the immunocolumn. The optimal concentration of H_2O_2 is in the range from 0.01 % - 0.06 %. In order to keep the volume of the reagent used as low as possible and to have more precise concentration of the peroxide, the concentration chosen for the methods was 0.06 % of H_2O_2, which provided sufficient activation of the enzyme and at the same time did not negatively influence the enzymatic activity, which is observed as a drop in the formation rate at concentration higher than 0.06 %.

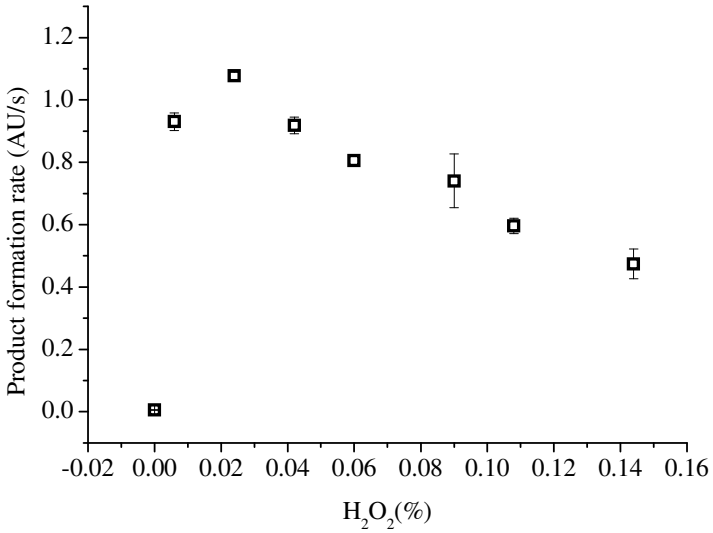

Figure 27: *Different concentration of the H_2O_2 as the activators of the reaction processes.*

5.3.6 Calibration curve for FIA-ELISA-SFM

Figure 28 presents the calibration curve obtained by the selected parameters (Table 16). The observed saturation of the signal can be attributed to the small capacity of the column. Moreover, the deviation from linearity is a consequence of the porosity of the support and the diffusion of the OPD reagent into the pores. The preincubated solution of the analyte has time to interact with the immobilized antibodies due to slower flow rates of the carrier buffer during sample injection. A certain amount of analyte is retained by the primary antibodies at the surface of the glass beads. However, when the surface sites are all occupied, the rest of analyte can diffuse also into the pores. Therefore, at higher concentrations the amount of analyte inside the pores is proportional to the amount of the analyte, which was not retained on the surface. On the other hand, the OPD reagent is injected onto the column at higher flow rates in order to limit the broadening of the signal due to diffusion. The higher is the flow rate, less OPD regent reaches the HRP in the pores and therefore the formation of the enzymatic product is mainly the consequence of HRP enzyme retained on the surface of the glass beads, which leads to relatively lower analytical signals at higher BLG concentrations. The limit of detection achieved with FIA-ELISA-SFM was 10 pM (S/N= 3) corresponding to 18.3 pg of BLG in a 100 µL sample.

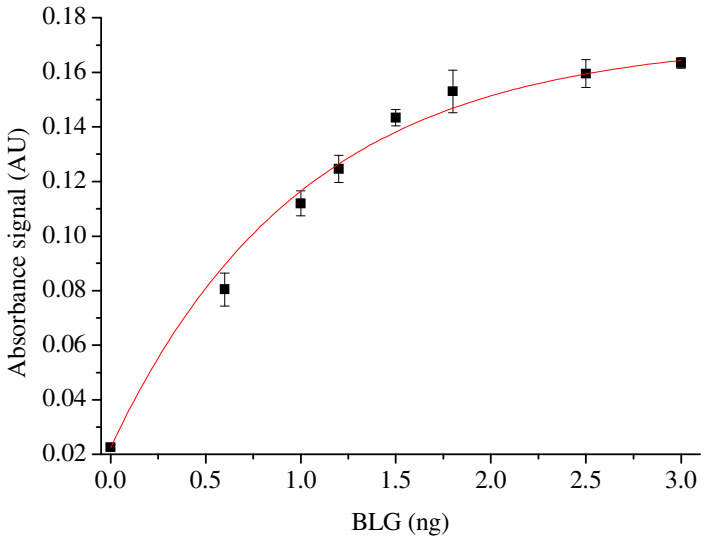

Figure 28: *Calibration curve for BLG obtained using CPG as support material (20 µL injection loop, 0.3 mL/min flow rate, 360 µg/mL OPD).*

Table 16: *Parameters used in the spectrophotometric FIA-ELISA method.*

	Buffered solution	**Injection volume (µL)**	**Flow rate of carrier buffer (PBS) (mL/min)**
Sample	PBS	100	0.1
Reagent (with 0.06 % H_2O_2)	Sodium Acetate	20	0.3
Cleaning solution	Glycine/HCl	100	0.3

5.4 Optimization of the method on the thermal lens spectrometer (TLS)

5.4.1 Photolability and thermolability of the OPD reagent

In TLS detection method two powerful lasers were used. The probe laser beam has a power of 2 mW. On contrary to the probe laser, the so-called excitation laser could achieve the light power of 100 mW. The excitation light might cause non-enzymatic changes of the used reagent due to photoconversion. In order to evaluate the possible thermo- and photolability of the reagent OPD, temperature dependent experiments were performed.

One of the experiments consisted of the evaluation of signal changes after heating at different temperatures. OPD (180 µg/mL) was incubated for 20 minutes at diverse temperatures

(Figure 28). The sampling (1 mL) was performed with a time interval of 5 minutes. The reagent changes were checked in the FIA-TLS system. The results in Figure 30 clearly show differences between samples incubated at lower temperatures and those kept at higher temperatures. At a temperature above 60 °C the deterioration rate of OPD was fast and the changes in the OPD solution could be already noted by a naked eye.

An approximate calculation indicates that the temperature of an 8 µL aqueous sample exposed to laser power of 100 mW would increase at maximum by 1.8 degrees. However, this calculation is based on the assumption, that all the energy is absorbed by a non-fluorescing sample and that the sample is static. In the case of FIA systems the sample is carried away from the irradiation point, which lowers the effect of sample heating. Moreover, the sample does not absorb all laser energy, but rather a small fraction (< 1 %), since the absorbance measured by TLS is usually below 10^{-7}.

It is evident, that even at temperature changes as high as 30 °C, the changes in TLS signal are not significant despite much longer period of heating (up to 25 minutes) as compared to sample residence times in the flow-through cuvette (about 1 s). Therefore, the temperature raise expected in the TLS measurements of DAP is negligible and thermalconversion of the reagent shall not influence the TLS signal.

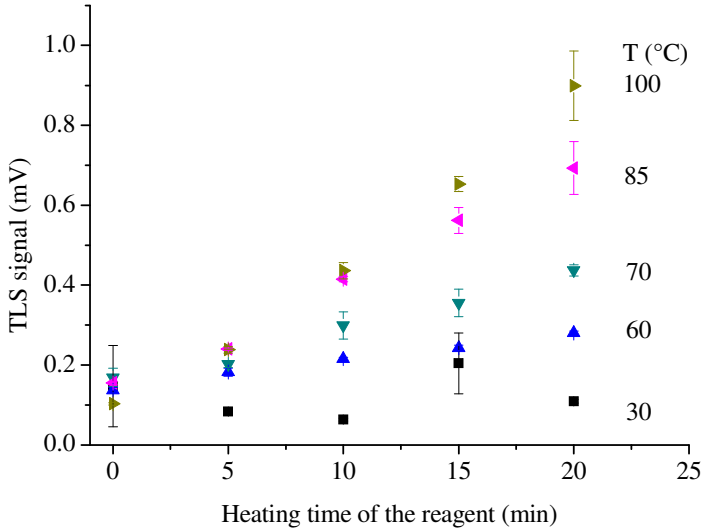

Figure 29: *Signal increasing due heating of the reagent and consequent changes (substrate dissolved in PBS).*

However, the excitation laser beam works at high light power, therefore also the photoconversion has to be determined. We performed some experiments in a steady state mode in order to establish the influence of the laser beam power on the reagent changes. We observed the signal increasing during the exposition of OPD to the laser beam. This photoconversion was not observed previously on the UV/Vis spectrophotometer, since the sensitivity of that instrument is not comparable to the sensitivity of the TLS detection and the emitted light of the spectrophotometer does not reach such high powers. The reagent was exposed to direct light beam of the excitation laser at different powers. The irradiation under higher powers of the laser beams resulted in higher rates of changes in absorbance of the irradiated solution. Therefore, we could propose that the higher power could already change rapidly the starting solution without any enzyme presence (Figure 30). A rough estimation out of these results led to the conclusion that the excitation laser affect the solution in the same manner as if the solution was exposed to higher temperature (above 100 °C). However, in a FIA system, the samples' residence time in the irradiated sample cuvette is short (around 1.2 second for 0.4 mL/min flow rate of the carrier buffer). Therefore, the energy implied in the non-enzymatic conversion of the OPD reagent is approximately 0.12 J (if 100 mW laser power is used).

The impact of the photoconversion was calculated by taking into the account that the sample time residence in the flow-through cuvette is 1.2 s at a flow rate of 0.4 ml/min. The photoconversion rate at 100 mW is approximate 1.5 µV/s. The estimated raise of the signal due to photoconversion is approximately 0.8 %. Therefore, the impact of photoconversion on the signal for higher concentration of allergens is negligible. However, when the concentration of the allergen is in the limits of quantification, the photoconversion has a higher impact on the signal. In this case, photoconversion represents 4 % of the actual signal.

Thus, the actual contact time between the reagent passing through a flow cuvette and the excitation beam should be as short as possible in order to avoid the degradation of the reagent due to laser activity. This could be achieved with higher flow rates. However, it should be taken into account that also the immobilized enzyme need as long as possible contact time with the reagent. The two processes have to be in proper balance.

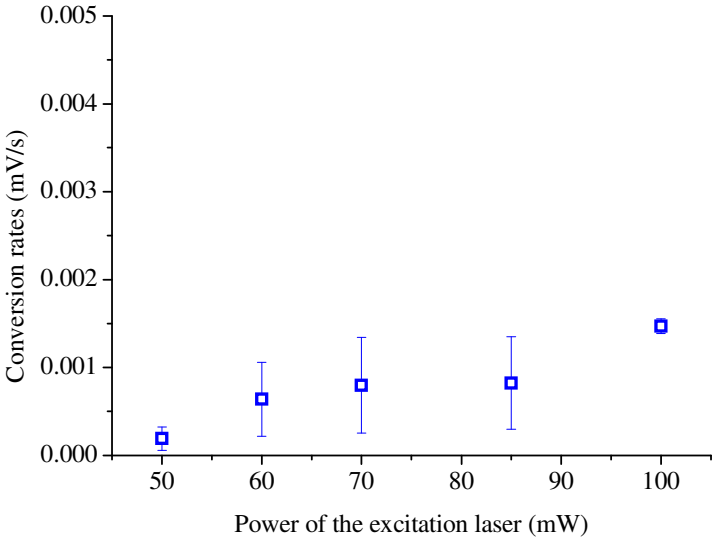

Figure 30: *Laser power influence on the stability of the reagent OPD (substrate dissolved in PBS).*

Moreover, the comparison of the absorbance spectra of the thermally changed solution and enzymatically change OPD solution showed some clear differences (Figure 31). The absorption peaks are shifted. It is possible that such shift is caused by different structure of absorbing molecules; leading us to the conclusion that thermally treated reagent might form a different product. Even if the product is not enzymatically formed it is sensed by the TLS because of the absorption band in the laser light region.

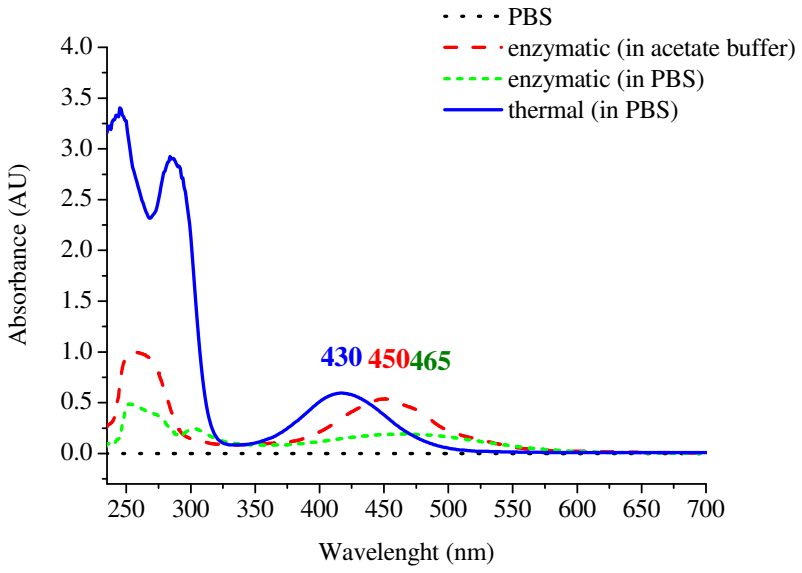

Figure 31: *Absorption spectra of the enzymatic product developed in different buffered solution and as the consequence of the thermal deterioration of the reagent.*

5.4.2 TLS signal perturbation

According to the literature, the best working pH for immobilized HRP is close to pH 5. The commonly used working solution for OPD reagent preparation is an acidic sodium acetate buffer (pH = 4.5), while the most suitable medium for an immunological interaction between the preincubated analyte and the immobilized antibodies is a PBS buffer (pH = 7.4). However, these two buffers are incompatible with TLS measurements in a FIA system. Their incomplete mixing results in the formation of concentration independent signals which have no optothermal origin and are superimposed to real TLS signal (Figure 32, left graph). After OPD injection into the FIA system, a signal perturbation is formed at the interface of the two solutions, due to incomplete mixing as confirmed by Pogačnik and Franko (Pogačnik and Franko 1999). This produces irregular double peak shaped TLS signals and this makes it difficult to evaluate the concentration of the analyte in the system.

Different combinations of acetate and PBS buffer solutions were tested in order to achieve unperturbed TLS signals. The combination of acetate buffer as a carrier and acetate as a solvent for OPD was soon rejected because of irreproducible measurements and high background signals (Figure 33).

More reproducible data were obtained with PBS buffers (Figure 33). The repeatable signals, corresponding to the amount of HRP labelled secondary antibodies bound to BLG captured in the bioanalytical column, were uniformly distributed, which made the evaluation of the BLG concentration more reliable (Figure 32, left). Although the HRP was found to work the best at pH 6.5, the lowest limits of detection were achieved in the combination of PBS buffers at pH 7.5. Despite of the lower activity of HRP under such conditions, the limit of detection was lowered to about 2 pM BLG (S/N=3), which can be attributed to lower background noise. The differences in slopes of calibration lines can be only related to the activity of HRP under different pH values (Figure 34 and Table 17). All buffers were prepared in 10 mM concentration, in order not to affect the thermooptical properties of solutions. Earlier reports (Franko and Tran 1991) have confirmed that signal enhancements of 35 – 70 % can only be expected at much higher electrolyte concentrations (1 M).

The results showed a tenfold improvement of LOD compared to the measurements performed in acetate-PBS system. These results confirmed the negative influence of improper mixing of the two buffers in a FIA-TLS detection system. The achieved LOD represents also a

significant improvement compared to ELISA detection kits (LOD = 100 pM). This is partly due to the constant rinsing of excess sample matrix in the case of FIA-ELISA-TLS, thus lowering the background signal, which is not possible in case of microtiter plates used in ELISA kits.

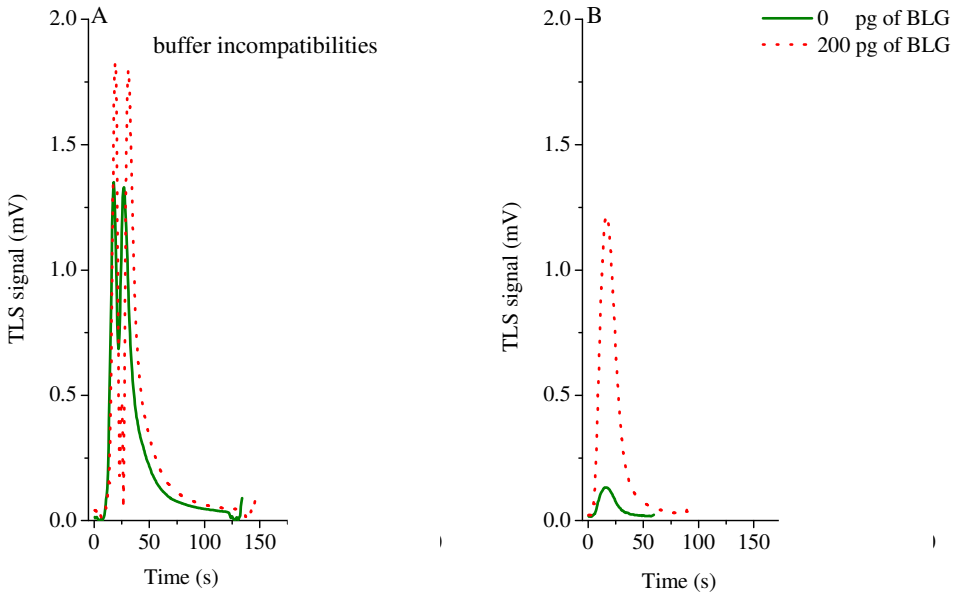

Figure 32: *Comparison of signal obtained with optothermal incompatible buffers (A) and signals obtained in PBS buffer for BLG sample (200 pg, red dotted line) and blank (green line).*

Table 17: *Sensitivity and RSD for the detection method performed in buffer of different pH.*

Buffer pH	Sensitivity (10^{-5}V/pg)	RSD (%)	LOD (pM)
4.5	0.05	//	//
5.5	1.03	33	11.5
6.5	2.31	20	10.9
7.5	0.71	3	2.5

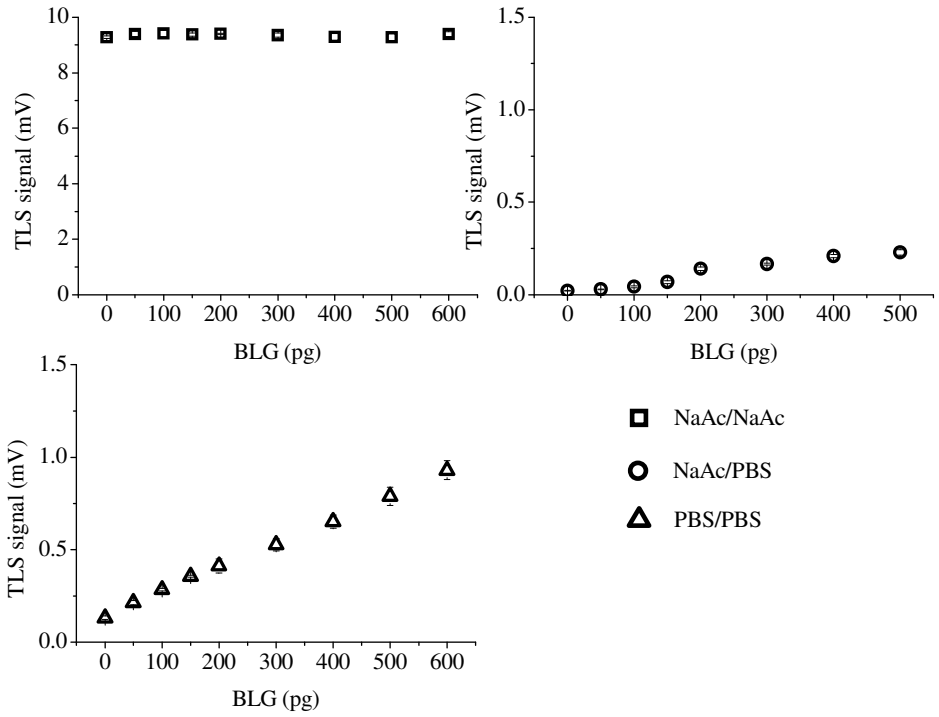

Figure 33: *Comparison of the FIA-ELISA-TLS calibration curve obtained with different combinations of buffered solution.*

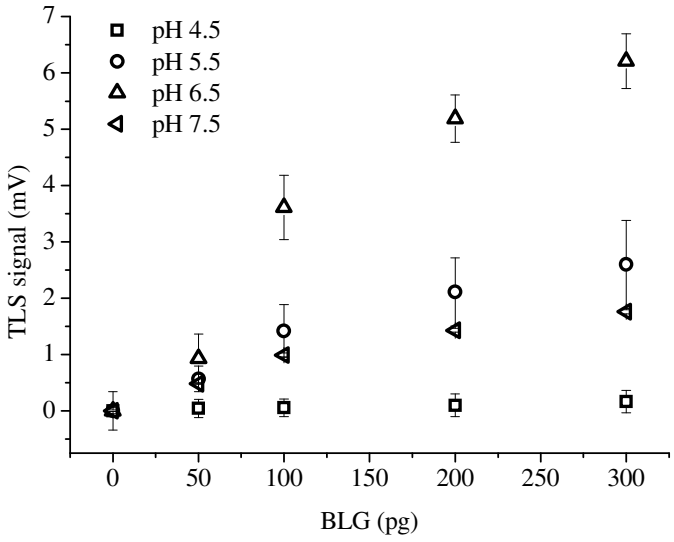

Figure 34: *Calibration curves for FIA-ELISA-TLS in phosphate buffer of different pH.*

5.4.3 Optimisation of the FIA system for the TLS detection

5.4.3.1 Flow rates for FIA-ELISA-TLS

Carrier solution and flow rates in FIA system have a great importance for the method performance. At the beginning, different flow rates of the carrier solution were tested. TLS detection needs a stable flow of the carrier buffer. This prevents the instability of the TLS signals. A limiting factor in the flow rate selection is the enzymatic activity of the marker enzyme bound to secondary antibodies. These secondary antibodies serve for the labelling of the allergen captured onto the immunocolumn. The enzyme needs time to turn over the reagent to the product. The flow rate was determined experimentally by checking flow rates ranging from 0.1 mL/min to 1 mL/min. The contact time between the enzyme and reagent varies from 37.6 seconds in the case of 0.1 mL/min flow rates to 3.8 seconds for the flow rates of 1 mL/min. The contact time influences the product formation and consequently the TLS signal formation. As already mentioned, the unchanged product is affected by photoconversion. As a consequence unwanted changing of the HRP substrate occurs. Thus, the flow rate of the carrier buffer has to be as fast as possible to avoid laser light influencing the stability of the reagent itself.

Three flow rates were chosen for further investigation (0.3, 0.4 and 0.5 mL/min). Having taken into account the sensitivity, relative standard deviation and LOD, the chosen flow rate for the FIA-ELISA-TLS system was equal to 0.4 mL/min (Figure 35). At the chosen flow rate the pumping did not affect the stability of the flow rate very much; therefore the signals were stable and repeatable. The contact time of the reagent with the enzyme captured inside the column was of 9.4 seconds. This was sufficient for the enzyme to produce detectable concentration of the measured product.

The flow rates affect the sensitivity of the method. At higher flows the sensitivity decreased. As already mentioned, the detection limits were lowest at the flow rate of 0.4 mL/min. The CIM support performed better at 0.4 mL/min, even though the difference was around 10 % in the limit of detection (Table 18). As shown in Figure 35, signals with CIM were smaller. This was attributed to lower contact time between the captured enzyme and the reagent. Moreover, CPG had a higher affinity for the HRP conjugated IgG, which resulted in a higher background signal, data not shown.

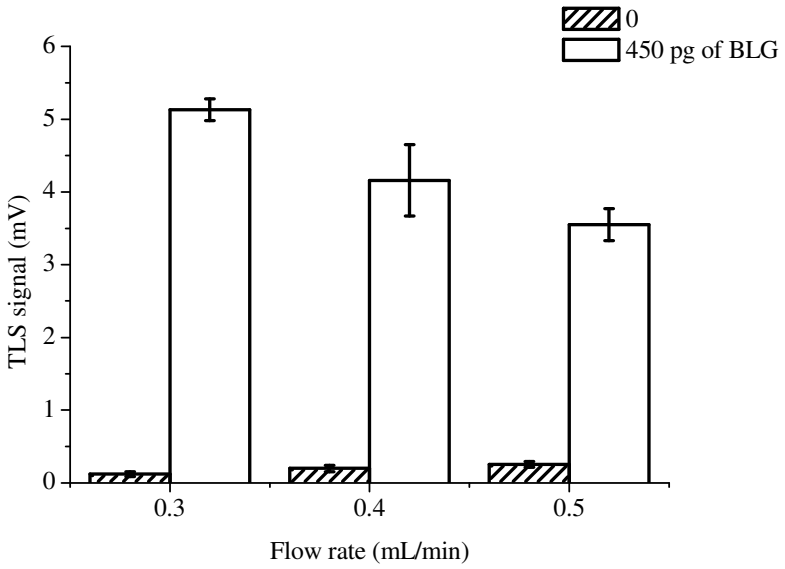

Figure 35: *Comparison of the product formation at different flow rates. The best ratio for FIA-ELISA-TLS is at 0.3 mL/min.*

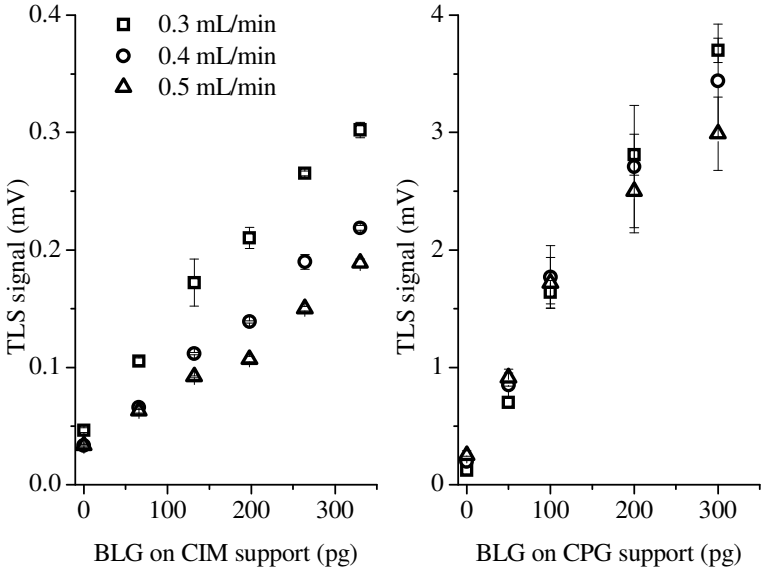

Figure 36: *Calibration curves for FIA-ELISA-TLS at different flow rates; comparison between the CPG and CIM support.*

Table 18: *Limits of detection at different flow rates for two different immobilization supports. Results before optimization of the TLS system obtained for a sample volume of 100 µL (36 Hz, 100 mW).*

	Flow rates (mL/min)		
Type of support	**0.3**	**0.4**	**0.5**
CIM micro disc	4.2 pg ± 0.16 pg	2.2 pg ± 0.08 pg	6.0 pg ± 0.06 pg
CPG	3.7 pg ± 0.99 pg	2.3 pg ± 0.48 pg	4.0 pg ± 0.60 pg

In order to achieve the highest detection signal with TLS the modulation frequency of the excitation laser beam had to be evaluated for the given flow rate. In the case of the flow rate of 0.3 mL/min the frequency of 36.5 Hz was preferred. The experimental data show that a frequency of 37.5 Hz gave higher signal to noise ratio for the flow rates of 0.4 mL/min, (data shown in the Figure 37).

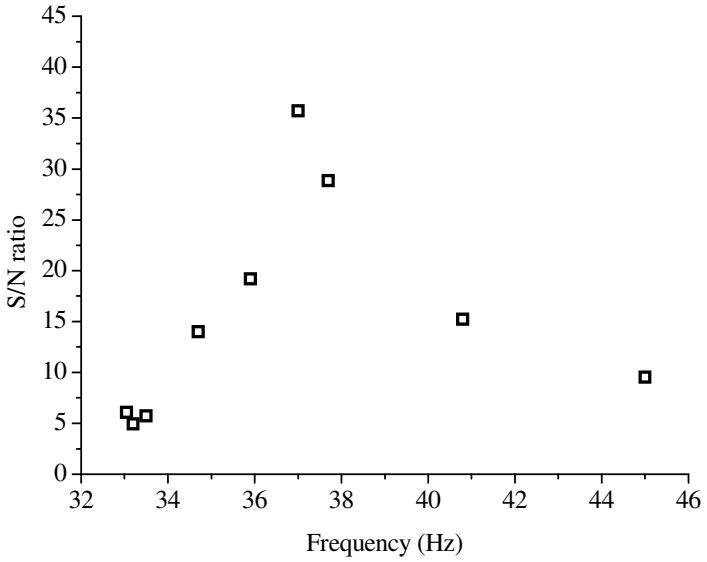

Figure 37: *Signal to noise ratio at different modulation frequencies for a flow rate of 0.4 mL/min. The best resulting frequency was in the range from 37.5 Hz.*

5.4.3.2 Reagent injection loops

In the performed assays two different solutions are injected into the FIA-ELISA-TLS system. The first solution is the sample. The second solution is the reagent for the HRP enzyme. Based on previous experience with immobilized enzymes (Boškin et al. 2009) we decided to

have two different injection valves, one for each solution. The residue of the marked antibodies in the injection loop caused an unspecific product formation prior arriving to the immunocolumn. This effect was noted visually already on the less sensitive spectrophotometer. To avoid the product formation due to residual immunoglobulins in the injection loop, the sample was injected onto the column through a separate injection valve equipped with 100 µL PEEK loop at flow rates of 0.1 mL/min. Slow flow rates assure a higher efficiency of binding of the present allergen onto the immunocolumn. The volume of the substrate for the HRP enzyme was established experimentally. Different volumes were checked. The injection loops varied from 20 µL to 5 µL. The signal formed from the 20 µL loop took a longer time to elute from the immunocolumn due to larger volume and diffusion of the reagent into the carrier stream. Consequently, the acquisition of the signal was prolonged (Table 19) due to signal broadening. Between two consecutive injections the signal has to drop back to the baseline level, therefore the longer is the elution time the longer is the time between two consecutive injections. At the end, the 5 µL sample loop was chosen for the system, which turned out to deliver enough OPD to detect the formed product. The maximum height of the signal formed from 5 µL loop was comparable to the signal formed with higher volumes of injection loops. Limited diffusion and broadening of the signal were observed. Positively, lower quantity of waste was released from the FIA system.

Table 19: *Times required between two consecutive injections of reagent for smaller injection volumes for the FIA-ELISA-TLS method (180 µg/mL of OPD).*

Injection loops (µL)	Average elution time (s) of the product
5	110 ± 8
10	140 ± 14
20*	160 ± 15

*experiment performed in a different experimental day with a newly refilled immunocolumn

5.5 Laser power influence on the calibration of the method

The thermal lens signal is directly proportional to the power of excitation beam. The experimental data obtained for the flow rate of the carrier buffer 0.3 mL/min showed that the sensitivity of the detection method doubles when the laser power is increased from 50 mW to 100 mW (Figure 38). However, the RSD of the blank was comparable for both laser powers. Accordingly the limit of detection (LOD) improved with higher laser powers. By doubling the

laser power, the LOD was 35 % lower. The excitation power of 100 mW was chosen for further work due to its higher sensitivity and enables lower detection limits.

Table 20: *Experimental data obtained at different excitation powers at the flow rate of the carrier buffer 0.3 mL/min.*

Excitation laser power (mW)	Sensitivity (10^{-6} V/pg)	RSD (%)	LOD (pM)
50	1.08	7.9	5.5
100	2.33	8.8	3.5

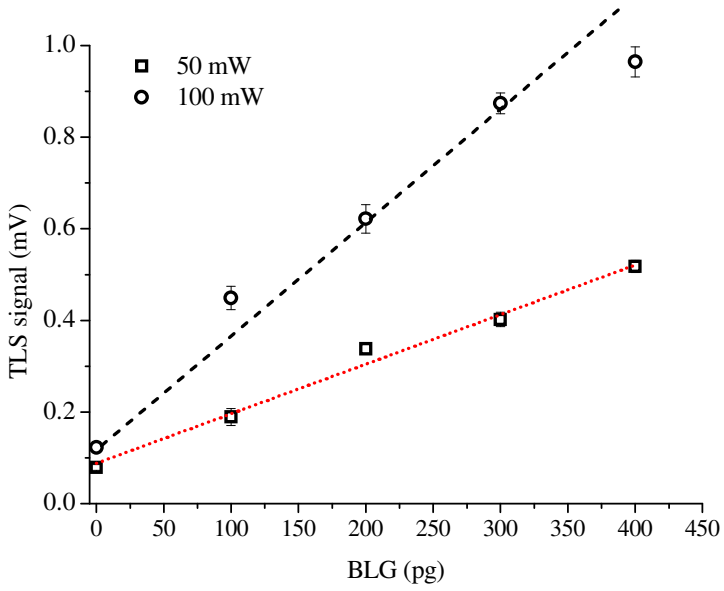

Figure 38: *Comparison of the calibration curves for the FIA-ELISA-TLS method (CPG support). At higher excitation laser powers the signal are higher.*

5.6 Influence of the support on the method sensitivity

Two different support materials for the columns were tested in order to evaluate the support influence on the detection procedure. One material was widely used controlled pore glass (CPG). The second chosen material was convective interaction monolith (CIM) discs. These supports serve as base material for antibody immobilization. When testing the performance of immunocolumns, high blank signals were observed, even though no beta-lactoglobulin was

present in the sample. A possible explanation for the origin of the blank signal could be non-specifically bound secondary antibodies, which are added in excess to the sample as detection antibodies. As reported in literature, the retention of free secondary antibodies in the bioanalytical column occurs because of non-specific protein-protein and protein-support interaction (Blincko et al. 1999). We observed the unwanted background signals on both support materials. To confirm the origin of the blank signal we compared signals formed with OPD before any sample injection on the column and the signal formed with OPD after the injection of a sample containing secondary antibodies, but without beta-lactoglobulin (blank).

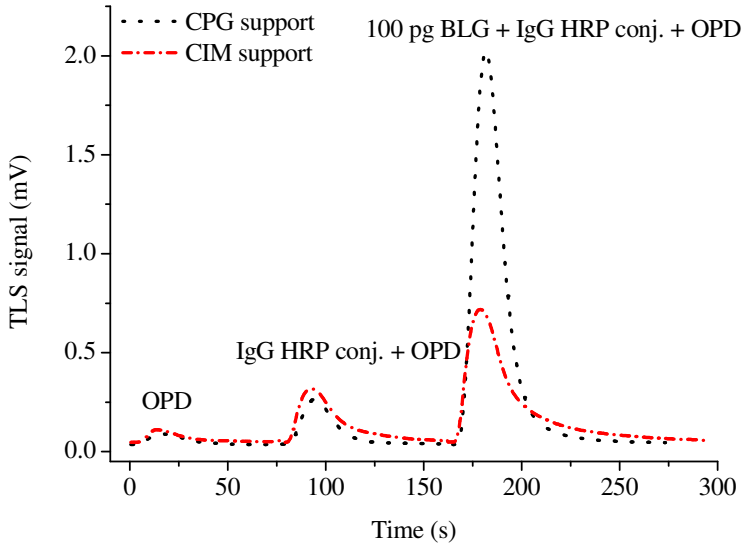

Figure 39: *Comparison of the signal obtained for FIA-ELISA-TLS on different immunocolumn supports. The dashed line refers to the CIM support, the dotted line refers to the CPG support.*

The differences in the responses are shown in Figure 39. It is clearly shown that the unwanted blank signal is mainly caused by nonspecific retention of antibodies. Even though, the absorbance spectra of OPD measured on a spectrophotometer showed no absorbance at the used excitation wavelength, we observed increases of the TLS signal after the injection of OPD in the flow injection system. The difference between the blank signal (IgG HRP conj. + OPD) and signal from OPD reagent passing through the system before injecting the HRP labelled secondary antibodies (OPD) is substantial. The blank signal is five times higher than the signal from OPD alone for both supports used. Even thought, the absorbance spectra of OPD measured on a spectrophotometer showed no absorbance at the used excitation wavelength, the increase of the signal was observed after the injection of OPD in the flow

injection system. As shown in Figure 40, the reagent itself produces a response in the detection system, which is otherwise hidden in the blank signal. The higher is the reagent concentration the higher is the contribution to the blank. Additional, each enzyme has a specific concentration range of the reagent, which is defined as the optimal working concentration. This optimum is changed in FIA system, where the inhibition at higher concentration is avoided because of the constantly flowing stream. Therefore, the optimal concentration of reagent, where the ratio between the analytical signal and the background signal from OPD was the highest for the given experimental condition, had to be found. Therefore, the optimal concentration in a stationary phase was 180 µg of OPD in a 1 mL buffered solution. As calculated from the experimental data, the ratio of analytical signal to signal from OPD varied only by about 10 % for OPD concentrations 140 – 180 µg/mL, (Figure 40). Therefore, all further experiments were performed using a reagent concentration of 180 µg OPD/mL solvent in order to have the same concentration performing a flow-injection experiment or an ELISA experiment.

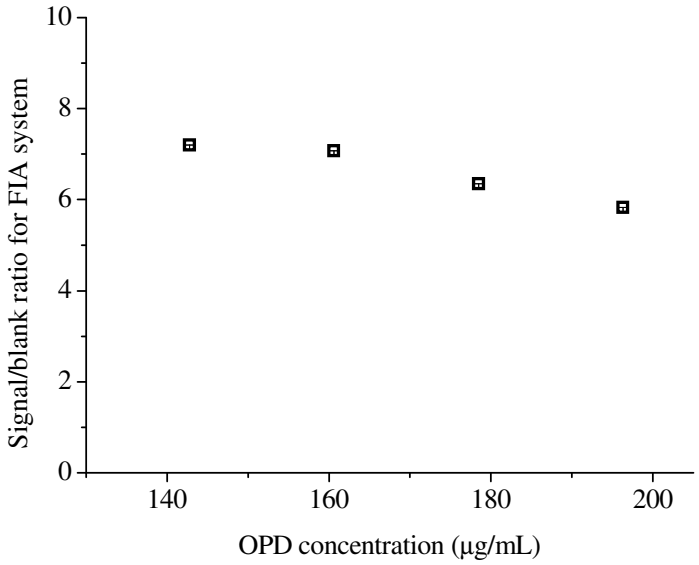

Figure 40: *The OPD concentration influence on the FIA-ELISA-TLS signal formation performed on CPG support.*

5.7 Influence of the support on IgG HRP retention

High background and rising signals were also a consequence of the retention of the HRP conjugated antibodies. The antibodies were retained onto the support in an un-specific manner. Figure 41 presents the retention of HRP conjugated antibodies on the naked (underivatised) support material. The rising signal is the result of non-specific interaction between the support and the HRP conjugated IgG, which causes the retention of the antibodies even though no BLG or any other protein is present in the column. The experiments revealed that the antibodies used for BLG detection has a greater affinity for CPG support (Figure 41). Most probably the cause of the antibody retention is the porosity of the CPG material and specific surface of the support material. CPG material has a specific surface area of 94 m^2/g, while CIM has a specific surface area of 7 m^2/g. In the case of porous CPG material the antibodies can enter the pores, where they are retained and cause the non-specific signal response. Therefore, when CPG is used great attention should be paid to the removal of the excess antibodies.

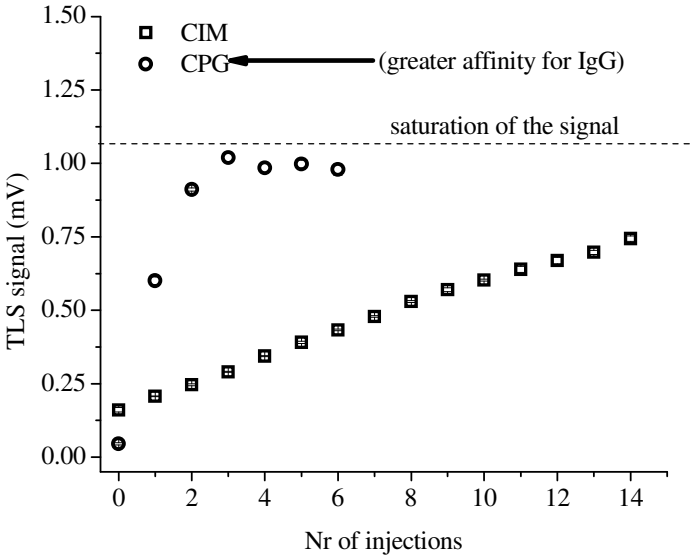

Figure 41: *Unwanted loading of IgG HRP on CIM and CPG support; the experiment was performed by injecting the antibody solution on the column without eluting them with Gly/HCl buffered solution.*

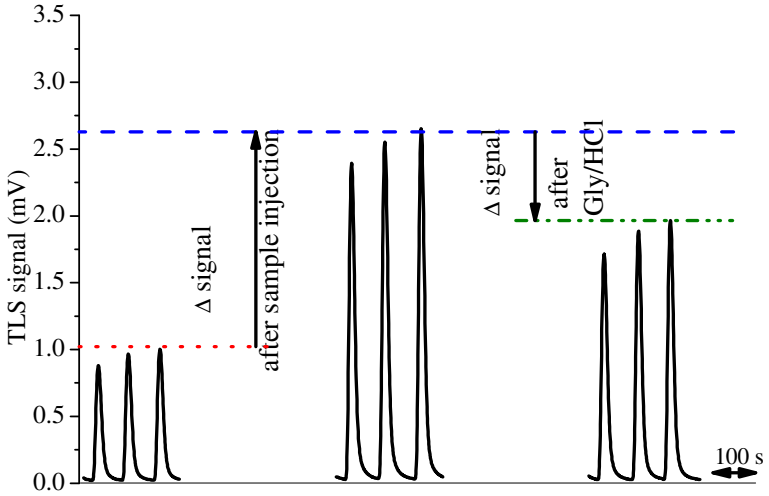

Figure 42: *Signal rising due to non-specific retention of HRP conjugated antibodies on CPG support.*

In order to avoid non-specific loading of HRP conjugate antibodies on the supports different additives were tested. Since the additives could not be chemically immobilized on the supports, they were added into the carrier solution or as a sample diluent (Table 21). Such additives are blocking agents, which are supposed to prevent the non-specific retention of HRP conjugate antibodies inside the immunocolumn by occupying empty spaces on the support surface or by changing ionic strength in order to prevent non-specific protein-protein interaction. However, none of them was very successful. If the additive was able to prevent the non-specific loading of the antibodies, it caused unstable of TLS signals. One of the additives, which did not result useful at all, was the globular protein bovine serum albumin, the BSA. This protein is commonly used as a blocking agent in ELISA experiments and the Western blotting detection. In the ELISA Quantitation Kit, the blocking agent is Tween 20. However, the addition of the said agent into the carrier solution provoked bubble formation and consequently TLS signal perturbation.

Additives such as BSA (0.05 and 0.5 %), Tween 20 (0.5 %), KCl (1 and 2 %) and SB3-10 (0.2 and 0.5 %) resulted in much higher standard deviations and RSD in comparison to the additives in Table 21.

Table 21: *Additives used for blocking surfaces of the support for the FIA-ELISA-TLS.*

Additive	Concentration	$Blank_{PBS}/blank_{additive}$	$SD_{PBS}/SD_{additive}$	RSD (%)
Tween 20	0.05 %	2.85 (lower)	0.07	15
Rice milk as sample diluent	100-time diluted and filtered	0.07 (higher)	0.06	18
Soy milk as sample diluent	100-time diluted and filtered	0.05 (higher)	0.68	1
KCl	0.8 %	0.95 (higher)	0.89	9
Ionic liquid SB3-10	0.1 %	1.35 (lower)	0.58	19

5.8 Enhancement of the TLS signal

In order to obtain lower LODs different approaches for TLS signal enhancement were tested. The TLS detection system demands an environment with high temperature coefficient of the refractive index and low thermal conductivity of the carrier buffer to gain the enhancement of the TLS signal. Water as a medium is the worst possible choice for such systems. On the contrary, organic solvents have the optothermal characteristics needed for high signal enhancement. Different combinations of FIA carrier solutions were tested in order to obtain a mixture suitable for TLS detection. The first set of experiments was performed in the one pump setup, where just different mixture of buffer and organic solvent were directly pumped through the immunocolumn. The carrier solutions tested were different mixtures of PBS buffer and organic solvents such as methanol, ethanol and acetonitrile. Among them the highest theoretical enhancement of the signal is obtained with acetonitrile. The experiment showed a low response, which was due to the impact of the organic solvent on the activity of the enzyme. Therefore, the FIA-ELISA-TLS setup was changed. An additional pump was added to the system and the tubing for the organic solvent was connected after the immunocolumn, as shown in Figure 44. However, the obtained signal was disturbed by the improper mixing of the two solutions and as a result a multiple peaks signal was recorded. The problem was overcome by the adding a mixing column after the junction of the tubings from both pumps. The results for acetonitrile/PBS systems are shown in Figure 45. The addition of acetonitrile resulted in higher signals, which is due to better optothermal properties of the mixture. As a consequence the detection limits improved as well. On the other hand, the addition of the mixing column occasionally resulted in exceedingly high

pressure due to clogging. Most probably the organic constituents of the sample coagulate at the filters (Figure 43) and are retained by the frits on the column causing occasional clogging.

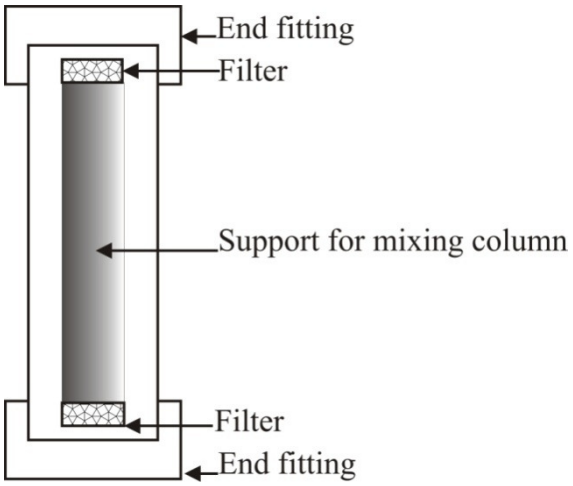

Figure 43: *Mixing column made in house.*

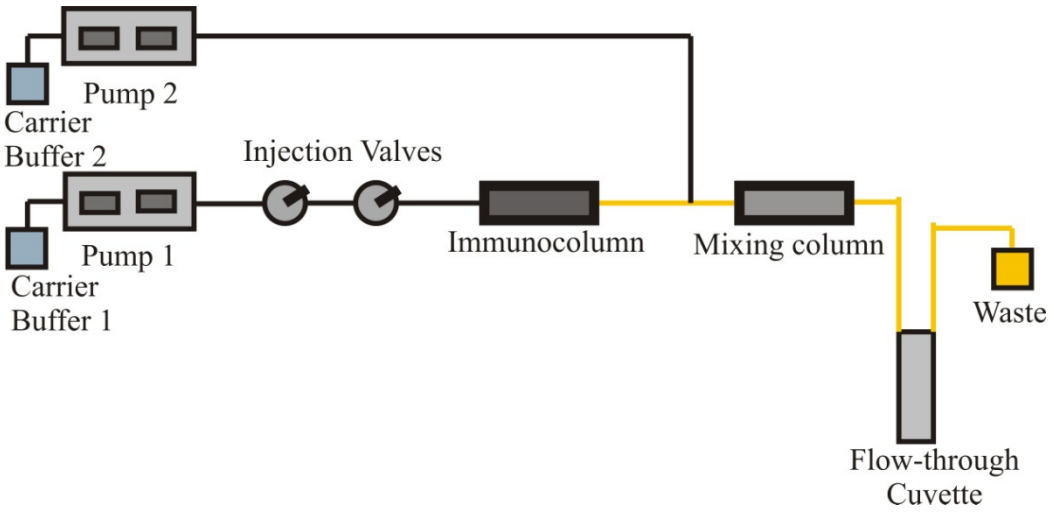

Figure 44: *Two pump FIA set-up used for the addition of organic solvent to the carrier buffer after the immunocolumn.*

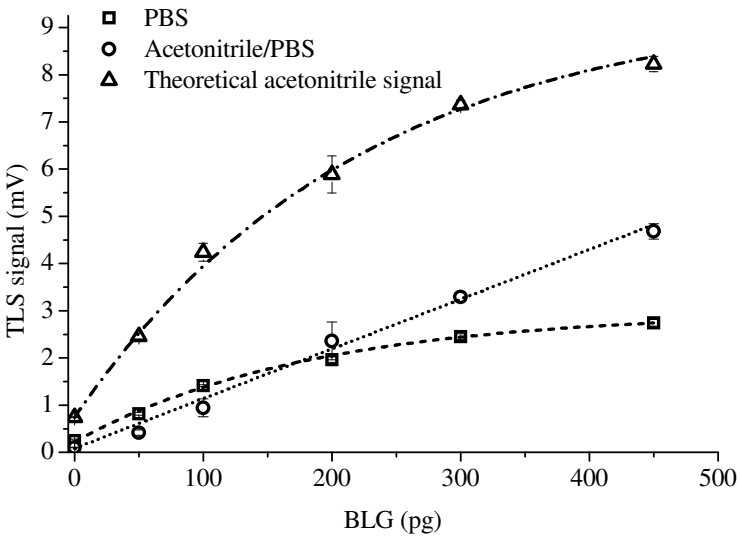

Figure 45: *Comparison of the calibration curve for the FIA-ELISA-TLS system. The enhancement of the signals is obtained by adding of the acetonitrile (dotted).*

In Table 22 are presented the LODs, obtained with a single pump system and PBS as a carrier buffer and for two pump FIA system, where mixing of acetonitrile and PBS occurred. The comparison of the detection limits achieved with equal flow rates at the flow-through cuvette showed an improvement of the LOD in case of added acetonitrile. The expected signals in the acetonitrile/PBS buffer should be 3-times higher in comparison to the signals in PBS alone. However, the signals obtained were 50 % lower than expected but the LOD was over 3-times lower compared to pure PBS eluent (if experiments were performed at the same flow rate) despite lower sensitivity than expected. Compared to the validated ELISA method for BLG detection, the improvement in both cases was evident. The detection limit in the TLS system was at least 25-times better. This confirmed that the implementation of the TLS detector makes it possible to detect pM concentrations of the analyte in question.

As already mentioned, the formation of the product is highly effected by the contact time between the enzymatic substrate and the enzyme in the immunocolumn. The optimal flow rate for the formation of the detectable product was equal to 0.4 mL/min. Therefore; the PBS buffer was pumped through the immunocolumn at that flow rate. The flow rate of acetonitrile was chosen experimentally by testing different flow rates between 0.1 mL/min and 0.8 mL/min. Different combinations of the flow rates resulted in instable signals due to oscillation of the flow rate from the two connected pumps. A stable flow rate contributes to

lower noise, better repeatability of the signals and consequently contributes to lower LODs. The experiments showed that the flow rate of 0.4 mL/min caused the lower oscillation of the TLS signal and therefore was chosen for following experiments. The perturbations due to solvent incompatibilities were removed by adding the mixing column. The final flow rate through the cuvette in a two pump FIA system was 0.8 mL/min.

However, higher flow rate remove the relaxation heat faster, therefore the temperature gradient formation was smaller. The evaluation of the heat loss due to changed flow rates and consequent impact on LODs was performed by comparing PBS signals at different flow rates with the results obtained for the two pump FIA system with acetonitrile addition. The impact of different combination of eluents and flow rates on LODs are presented in Table 22.

Table 22: *Limits of detection for the FIA-ELISA-TLS system and for validated sandwich ELISA achieved for BLG in 100 µL sample volume.*

Carrier buffer	**PBS (0.8 mL/min)**	**PBS (0.4 mL/min)**	**Acetonitrile/PBS (0.4 mL min^{-1}/ 0.4 mL min^{-1})**	**ELISA**
LOD (absolute quantity) BLG	7.6 pg	2.3 pg	2.1 pg	190 pg (Bethyl)
BLANK (10^{-4} V)	1.84	1.6	0.7	//
SD (10^{-5})	2.4	0.8	0.5	//
RSD (%)*	13	4	7	//

*for a set of experiments performed in the same experimental day

5.9 Optimised parameters for FIA-ELISA-TLS

For further analysis of food sample the subsequent parameters were established, shown in Table 23. The carrier buffer was PBS pumped through the FIA system at flow rates specific for each step of the analytical procedure. The samples were injected in to the stream of the carrier buffer through an injection loop of 100 µL at a flow rate of 0.1 mL/min. The enzymatic substrate prepared in PBS buffer was applied through a 5 µL injection loop at a flow rate of 0.4 mL/min. The enzymatic substrate was applied 3-5 times for each sample. Prior the application of the next sample the immunocolumn was washed with 100 µL glycine /HCl buffer at a flow rate of 0.4 mL/min.

Table 23: *Optimized parameters in FIA-ELISA-TLS*

	Buffered solution	Injection volume (µL)	Flow rate of carrier buffer (PBS) (mL/min)
Sample	PBS	100	0.1
Reagent (with 0.06 % H_2O_2)	PBS	5	0.4
Cleaning solution	Glycine/HCl	100	0.4

Figure 46 is the graphical representation of the detection procedure.

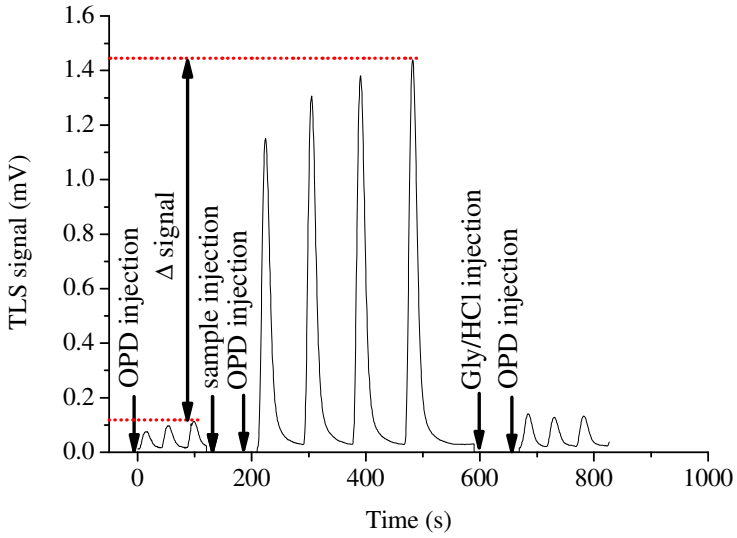

Figure 46: *Time sequence for allergen detection with FIA-ELISA-TLS.*

5.10 Matrix influence on calibration and real sample detection

One of the goals of this research was to develop a method for allergen detection in commercial products such as processed food and medicines. However, these products have a wide variety of different constituents such as fats, sugars and other proteins. These additional

constituents affect the detection procedures (Anklam and von Holst 2005; Wolf and Andrews 1995).

Two different sets of experiments were performed in order to evaluate the effect of the matrix. One set represents the comparison of calibrations using different matrixes which were spiked with the standards. The matrixes chosen for the experiments were soy milk, rice milk and sugar free orange juice. It was proven that the signals obtained with more complex samples are higher. Most probably, other constituents such us sugars, polypeptides and other proteins attach to the system. Even though, most of those constituents are washed away by the carrier flux in the FIA system.

Figure 47, presents calibration curves obtained in mentioned matrixes. As it can be seen the signals, obtained in spiked samples of soy milk and orange juice, were higher compared to signals for standards prepared in PBS. The most probable reason for this effect is the impact of the matrix constituents on the interaction of BLG with HRP labelled secondary antibodies in the preincubation process as well as on the interaction of preincubated BLG with primary antibodies in the bioanalytical column. The HRP conjugated antibodies may also interact with the matrix constituents producing agglomerates, which are non-specifically retained on the column entrance filters. These agglomerates are difficult to remove in a short time. Therefore, they are persistent in the detection system, which was shown in increased blank signals, which were however subtracted for the calibration curves present in Figure 47.

As a consequence of matrix effect, the limits of detection were changed. In the case of a complex matrix, such as orange juice and soy milk, the limits were higher. As shown in Figure 47 the sensitivity of the method changes in different matrixes. Even though the sensitivity of the measurements for orange juice and soy milk as matrix were 2.8-times and 1.2-times higher as for PBS, respectively, the LODs for orange juice and soy milk were 50 % and 20 % higher in comparison to the LOD of measurements performed in PBS. This could be attributed to the worsen repeatability of the measurements in complex matrixes. The blank signal for soy milk and orange juice increase for 45 % and over 300 %, respectively, in comparison to PBS blank. The RSD for PBS measurements was as low as 5 %, on contrary the RSD for orange juice and for soy milk were 11 % and 8 % respectively.

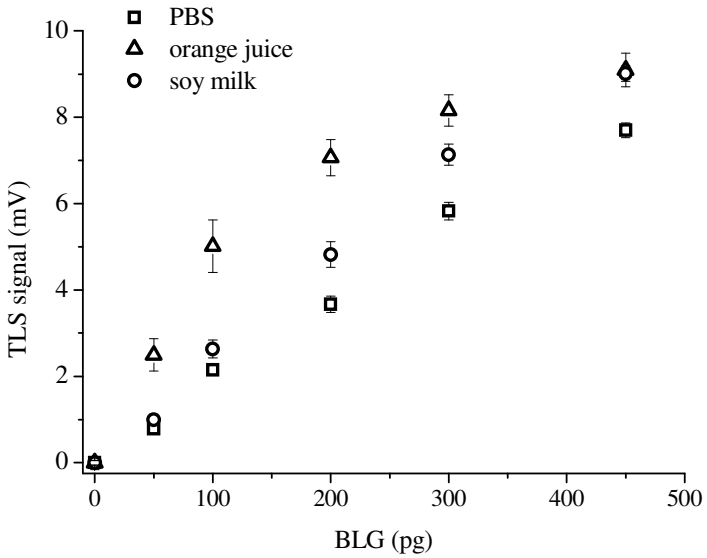

Figure 47: *Calibration curves obtained by spiking different matrices with the standard of BLG in different concentrations.*

Due to observed matrix effects the recovery experiments were carried out by analysing spiked samples based on calibration curves in PBS and corresponding sample matrixes. The results presented in Table 24 showed high deviations from the spiked concentrations when the calibration in PBS is used for quantification. The quantities of BLG for soy milk spiked with BLG and for orange juice spiked with BLG were found to be 37 % and 160 % higher (respectively) using PBS calibration curve. A much better result was obtained by preparing a calibration curve in the same matrix as expected for the sample. In the case of soy milk the observed recovery was 105 %. For orange juice the recovery was slightly higher at 113 %.

Also the repeatability of the measurements was investigated. As shown in Figure 48, the repeatability of the measurements was 15 %.

Table 24: *Recovery results for samples spiked with 100 pg of BLG in 100 µL sample.*

Sample		PBS calibration curve	Soy milk calibration curve	Orange juice calibration curve
100 pg spiked in soy milk	Expected quantity (pg)	100	100	//
	Determined quantity (pg)	135	105	//
100 pg spiked in orange juice	Expected quantity (pg)	100	//	100
	Determined quantity (pg)	270	//	113

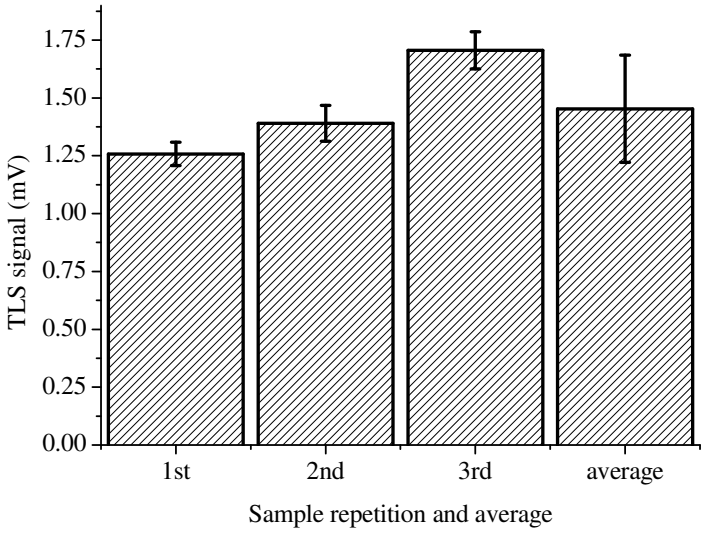

Figure 48: *TLS signals obtained for soy milk spiked with BLG.*

It can be concluded from presented results that the amount of BLG is difficult to evaluate accurately, when a complex matrix such as food is analyzed by the developed method. Still, at this stage of development, the method can be used for reliable fast screening of BLG in food products such as soy milk and orange juices as demonstrated by recovery tests. The matrix

effect however represents a serious limitation of the method, since it can results in large positive as well as negative analytical errors, depending on the sample matrix. This requires matrix matched calibration, which however makes the procedure more labour intensive. Additional pre-cleaning steps or extraction methods could resolve the mentioned problem. Moreover, implementation of highly specific monoclonal antibodies as immobilized immunoglobulins would confer additional specificity and enable more accurate identification of the allergen present in the sample (Nakano and Nagata 2003).

5.11 Method applicability for OVA

After application of the novel detection technique for the determination of the BLG, the technique was tested for other allergens. The same single-pump system was used for the detection of ovalbumin (OVA), protein form eggs' white, which may also cause allergic reaction. The difference in the system was in the selectivity of the immunocolumn. Instead of antibodies raised against BLG the immobilized antibodies were raised against OVA. The samples were pre-incubated with secondary antibodies raised against OVA and labelled with HRP. All the other parameters were kept unchanged. The system behaved similarly as the detection system for the BLG detection. The calibration curve showed deviation from linearity. OVA could be detected at higher concentration (Figure 49) compared to BLG. The sensitivity was ten thousand-times lower compared to the sensitivity obtained in case of BLG. The detection limit obtained was equal to 1 ng of OVA.

The poor performance, in terms of detection range, was investigated in more detail. It was demonstrated, that the antibodies used for the testing already performed worse compared to the anti-BLG IgG by checking the HRP conjugated antibody activity. The antibody activity was tested on the spectrophotometer. The results confirmed the lower enzymatic activity of the HRP bound on IgG raised against OVA (Figure 49). Therefore, the differences in sensitivities for BLG and OVA detection cannot be simply generalized to other allergens due to possible differences in the antibody activities.

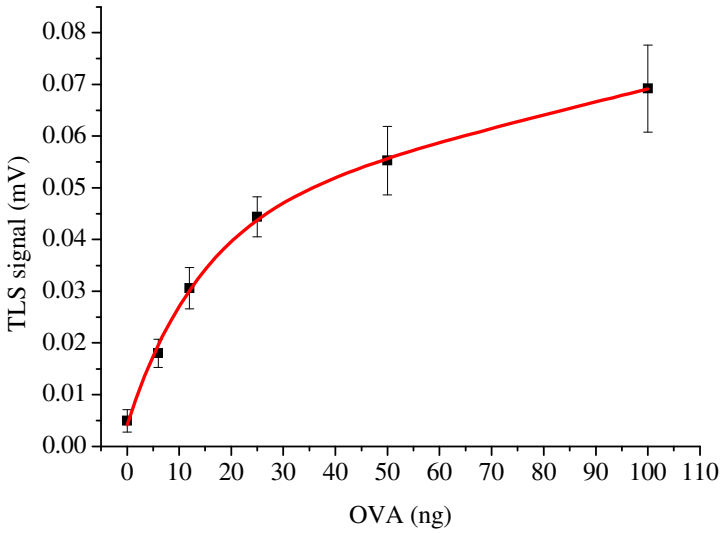

Figure 49: *Calibration curve for OVA obtained by FIA-ELISA-TLS (CPG support, PBS buffer 0.4 mL/min).*

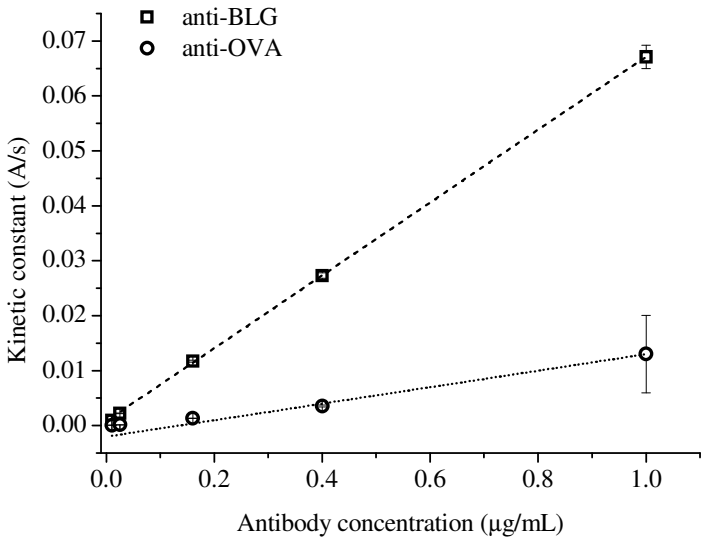

Figure 50: *Comparison of the enzymatic activity for HRP conjugated anti-BLG IgG (anti-BLG) and HRP conjugated anti OVA IgG (anti-OVA).*

5.11.1 Calibration in ELISA measurements

The newly developed method was compared with ELISA experiments. The experiments were performed according to the instructions of the Bovine Beta-Lactoglobulin ELISA Quantitation set from Bethyl laboratories. The only exception to the protocol was done during

the testing of the matrix effect on the ELISA experiments, where samples for standard curves were diluted in the chosen matrix (Figure 51). In this case, the influence of the matrix onto the availability of the analyte present in sample was tested. The matrix could inhibit the interaction between the allergen and the immobilized antibody.

The effect of the matrix was tested for FIA-ELISA-TLS and for common ELISA respectively and as a result calibration curves were obtained. Unlike in the developed TLS method, the matrix does not influence considerably the absorbance signal. Despite the presence of soy milk as a matrix material, the signals were not higher in comparison to the calibration curve performed in TBS buffer, seen in Figure 52. No statistical difference was observed between the signals for standards prepared in TBS or soy milk. However, the difference of matrix influence on ELISA and FIA-ELISA-TLS measurements is explainable by different protocols of analysis. While in case of FIA-ELISA-TLS the preincubation and binding of preincubated BLG cannot be carried our without the presence of sample matrix, the protocol for ELISA requires intensive washing of sample matrix from the ELISA plates before HRP labelled secondary antibodies are added. Therefore no effects of matrix on BLG-antibody interactions are possible,

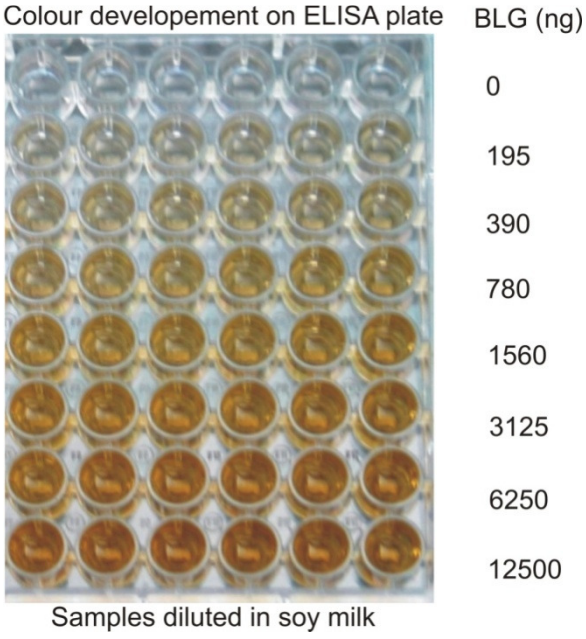

Figure 51: *ELISA experiment; calibration prepared in the soy milk.*

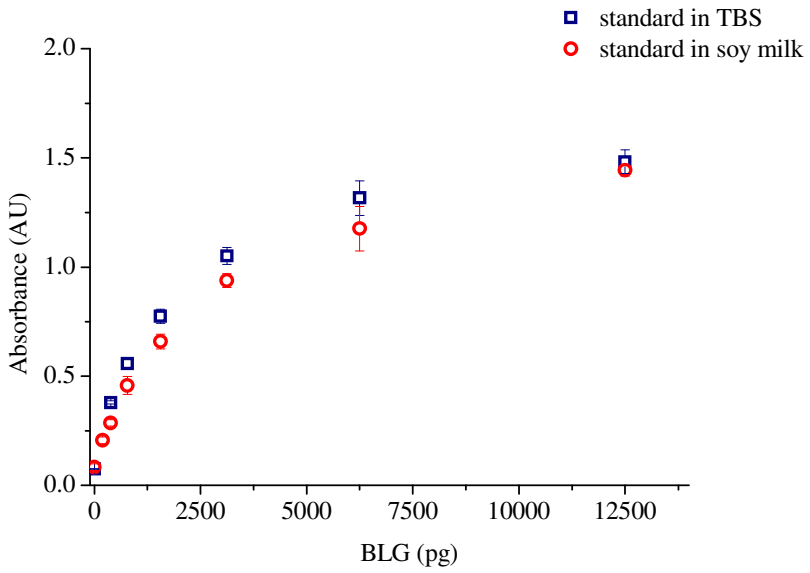

Figure 52: *Calibration curves obtained with ELISA; comparison of a standard ELISA curve with the calibration curve obtained in soy milk.*

5.12 Screening of BLG presence in randomly chosen food product

The main goal of this research was to develop a method for fast screening of allergen presence in products on the free market. In order to test the method different food sources were chosen.

Table 25: *Detection of BLG in spiked food samples with FIA-ELISA-TLS.*

Samples	**Coffee beverage**	**Orange juice**	**Baby food nr.1 (with rice)**	**Baby food nr. 2 (with wheat)**
	TLS signal (µV)	**TLS signal (µV)**	**TLS signal (µV)**	**TLS signal (µV)**
BLANK (in PBS)	4.32 ± 0.5	2.4 ± 0.6	4.32 ± 0.5	2.4 ± 0.6
Sample 1	10.2 ± 0.4	8.2 ± 0.5	14.7 ± 1.7	3.48 ± 0.3
Sample 2 (20 pg)	7.6 ± 0.6	9.2 ± 1.1	38.1 ± 4.2	5.9 ± 0.7

First, a simple extraction procedure was performed. PBS with the addition of ether was added to the samples and later the centrifugation was applied. This procedure was performed in

order to get rid of the fats present in the processed food and the bigger pieces of the fruit material. Sample 1 was the raw extract, which served as the starting material with 0 BLG present. Sample 2 was spiked with BLG after sample preparation.

The evaluation of the results was performed for the blank signal in PBS, where BLG was not present. The blank signal represents the signal obtained by incubating the immunocolumn only with a HRP conjugated antibody, where no BLG and any matrix component were present. In all samples the signals were higher in comparison to the blank signal of the calibration curve. This was expected since the matrix raises the TLS signals. The sample in real matrix had signals, which was 2-times higher than the blank, or even higher. In most of the samples, the presence of the BLG in Sample 2 was confirmed by comparing the signal obtained by the raw material with signals obtained with spiked samples.

Samples used for FIA-ELISA-TLS measurements were tested with the sandwich ELISA method. As expected, the allergen concentration was so small that no difference between the blank signal and the sample signal was observed in case of ELISA measurements. Therefore, it was concluded that ELISA could not distinguish between samples with the allergen in pg quantities. For this measurement two different blank signals were tested. The first blank sample was the binding buffer itself. The second blank sample was soy milk. In the case where soy milk was used as the material with no presence of the BLG, the signal was not higher than the usual blank signal.

On the contrary, in FIA-ELISA-TLS measurement we observed an increase in blank signal. This effect was explained by the matrix action on the immunocolumn. In ELISA measurements this increase was not evident, most probably because the method is not as sensitive as it is the FIA-ELISA-TLS.

The experiments presented in the Table 26, were performed with the same sample as presented in Table 25 for the FIA-ELISA-TLS. As already mentioned, the signal did not exceed the blank signal. This result was already expected, since the quantity of the BLG was under the limit of detection of the ELISA test used. However, the test was performed to prove, that the FIA-ELISA-TLS is more sensitive to the BLG in comparison to the common ELISA test.

Table 26: *Detection of BLG in spiked food samples with ELISA.*

Samples	Coffee beverage	Orange juice	Baby food nr.1 (with rice)	Baby food nr. 2 (with wheat)
BLANK (in PBS)	0.310 ± 0.005	0.310 ± 0.005	0.310 ± 0.005	0.310 ± 0.005
Sample 1	0.271 ± 0.05	0.195 ± 0.023	0.205 ± 0.013	0.2433 ± 0.018
Sample 2	0.297 ± 0.025	0.265 ± 0.034	0.291 ± 0.013	0.246 ± 0.026

FIA-ELISA-TLS is therefore more sensitive than the commercial ELISA, however great attention should be paid to the effects of the sample matrix and its effect on the calibration of the method.

5.12.1 Pigment release from column filter in screening of coffee samples in FIA-ELISA-TLS

The only exception was the beverage coffee, where other difficulties in the evaluation were encountered. Even though this sample has been considered as having the least complex matrix of them all, the evaluation was found to be difficult. The sample was coloured due to its naturally present pigments. These pigments were bound on the filter of the immunocolumn (confirmed visually). The elution of the pigments rinsed the background of the signal, even though an extensive washing of the column was performed prior to the reagent injection.

The signals with the high background are shown in the Figure 53, where comparison between the coffee signal and the signal obtained with the standard solution are presented. Both solutions contain 20 pg of BLG. There is an evident rise of the background caused by the elution of coffee pigments retained in the filters of the immunocolumn. Prior to the evaluation of the signals, the background had to be subtracted in order to obtain only signal caused by the reagent conversion. However, the pigments eluted off the immunocolumn made it difficult to evaluate the signals and as a consequence the BLG presence was hard to confirm.

The pigments and other matrix constituents of coffee seem to have a negative effect on the HRP activity causing lower signals (also shown in Figure 53). The effect of the pigment release from the column filters and the impact on the production of DAP are the cause of lower sensitivity and higher LOD for evaluation of BLG presence in coffee.

As also seen on Figure 53 the constituents of coffee sample do not just increase the baseline signal, which slightly decreases with time, due to a slow washing of the column, but also affect the conversion of OPD to DAP, which is reflected in about 2.7-times lower sensitivity.

However, the other samples can be easily evaluated despite the more complex matrix. As already mentioned the Signal 1 was the raw material. The supposition was that this material has no BLG present inside. Therefore, the Samples 1 represents the bases on which the BLG presence could be evaluated. As seen in Table 25, the increase in the signal of sample 2 confirms the presence of the allergen concerned.

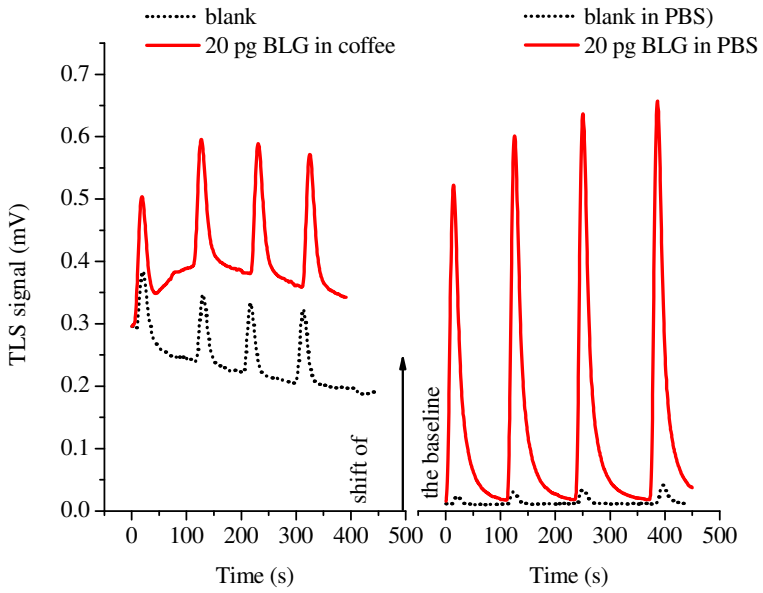

Figure 53: *Increase in the background signal of the coffee sample; elution of the pigments attached to the filters of the immunocolumn.*

5.13 FIA-ELISA-TLS features compared to ELIAC and immunometric method

The FIA-ELISA-TLS method shares some common features with ELIAC (Puerta et al. 2005) and immunometric method (Negroni et al. 1998). The methods are based on immunodetection by using two sets of immunoglobulins raised against BLG. Differently from FIA-ELISA-TLS and ELIAC, the immunometric method is exploiting monoclonal antibodies, which are more specific than polyclonal antibodies. Moreover, the immunometric method is not a flow injection method and is time consuming since each assay (96 wells plate) takes approximately 38 hours. However, the LODs achieved with this method (30 pg/mL) are comparable to the

FIA-ELISA-TLS method developed in this work (LOD = 60 pg/mL).

Differently from this, the ELIAC method is a flow injection method, which takes approximately 35 min for each sample. The ELIAC method is faster than the immunometric method, particularly when smaller numbers of samples are in question, but still not as fast as the FIA-ELISA-TLS method (~7 minutes per sample), which in principle enables analysis of over 300 samples in 38 hours time. The limit of detection for BLG achieved with ELIAC method is 370 pg/mL. In comparison with FIA-ELISA-TLS method for BLG detection, the LOD is about sixteen times higher. It must however be pointed out, that the linear range of the method (300 pg/mL to 2500 ng/mL) is much larger than the linear range for FIA-ELISA-TLS (20 pg/mL to 3500 pg/mL).

6 Conclusions

The comparison of CPG glass and CIM discs as support materials for immunoglobulin antibodies in bioanalytical detection of BLG demonstrated that CIM discs offer better performance regarding the linearity range, reproducibility and stability of immunocolumn, while the estimated LOD values do not differ significantly. Moreover, CPG derivatisation is a more time consuming procedure and additional packing step results in loss of material compared to CIM immobilization procedure. Additionally, the use of CIM discs results in lower eluent consumption and therefore lower volume of waste liquids. Therefore, CIM discs should be the support of choice for the described TLS detection of beta-lactoglobulin and other allergens in food products.

We demonstrated that non-specific background signals are present in case of both supports as the results of improper nonspecific binding of primary antibodies, and hinder the LOD of the method, which was estimated to be 60 pg/mL (3.3 pM) for BLG in case of CIM discs. The detection of beta-lactoglobulin at concentration levels substantially below those triggering allergic reactions (14 ng/mL for 100 g ingested food) can therefore be performed with a sample throughput of up to 8 samples per hour, which makes the described technique promising for sufficiently sensitive, reliable and fast screening of allergens in foodstuffs.

Even though, the FIA-ELISA-TLS method has been proved to be very sensitive, at this stage of the research still it could not be used for the quantification purpose of allergen present in samples with a complex matrix. The only output obtained would be a raw confirmation of the presence. Of course the interpretation of the results is totally subjected to the research as in all immunological experiments. If proper matrix, with confirmed absence of allergen, would be provide, the method could be used as a semi-quantitative method with RSD below 15 % and recovery between 97 and 103 % for tested matrixes such as soy milk and orange juice.

The FIA-ELISA-TLS technique was demonstrated to be a highly sensitive, rapid, as well as versatile detection method. The specificity of the system could be changed just by the substitution of the antibodies used. However, the performance of the methods strongly depends on the performance of antibodies used in the assay. For comparison the method was tested also for OVA detection. The FIA-ELISA-TLS method for OVA detection revealed 500-times lower sensitivity in comparison to FIA-ELISA-TLS for BLG detection. This difference in sensitivity is resulting also in higher LOD. The LOD for OVA was 10 ng/mL

(200 pM), which is 60-times higher in comparison with FIA-ELISA-TLS for BLG. Nonetheless the novel FIA-ELISA-TLS technique proved to be more sensitive compared to commercially available ELISA assays for BLG and OVA. The LODs achieved with FIA-ELISA-TLS were 100- and 1000-times lower compared to LODs of the respective ELISA detection kits.

The FIA-ELISA-TLS technique developed in this work shows advantages also compared to other non-commercial methods recently reported in the scientific literature. The sensitivities and LODs achieved by FIA-ELISA-TLS are for example over 10 times better compared to ELIAC method (Puerta et al. 2006), while in comparison to immunometric assay (Negroni et al. 1998) which provides comparable LODs, the FIA-ELISA-TLS gives the possibility of over three times higher sample throughput (for a set of up to about 90 samples). Even though the method is based on the ELISA detection principles, it provides much higher sensitivity and over 100-times lower LODs than the classical ELISA test, which is mainly due to the application of the highly sensitive TLS detection. All these features make the novel FIA-ELISA-TLS a promising analytical tool for screening and determination of minute concentrations of allergens in food products and drugs. However, matrix effects shall be carefully considered, particularly when applying the method for analysis of samples different than those tested within this research. In addition to matrix matched calibrations, development of appropriate sample preparation and purification procedures could be useful to overcome the problem of matrix influence on the FIA-ELISA- TLS detection. Additionally, the implementation of highly specific monoclonal antibodies should result in improved specificity of the method (Nakano and Nagata 2003).

Reference

Alvarez-Romero, G.A., Rojas-Hernandez, A., Morales-Perez, A., Ramirez-Silva, M.T., 2004. Determination of beta-D-glucose using flow injection analysis and composite-type amperometric tubular biosensors. Biosens Bioelectron 19(9), 1057-1065.

Anderson, R.J., Bendell, D.J., Groundwater, P.W., 2004. Organic Spectroscopic Analysis. The Royal Society of Chemistry, Cambridge.

Anibarro, B., Seoane, F.J., Mugica, M.V., 2007. Involvement of hidden allergens in food allergic reactions. Journal of Investigational Allergology and Clinical Immunology 17(3), 168-172.

Anklam, E., von Holst, C., 2005. Challenges and needs in food measurements. Analytical and Bioanalytical Chemistry 381, 102-105.

Apenten, R.K.O., Khokhar, S., Galani, D., 2002. Stability parameters for beta-lactoglobulin thermal dissociation and unfolding in phosphate buffer at pH 7.0. Food Hydrocolloid 16(2), 95-103.

Awade, A.C., Efstathiou, T., 1999. Comparison of the three liquid chromatographic methods for egg-white protein analysis. Journal of Chromatography B 723, 69-74.

Baines, D., 2001. Analysis of purity. In: Roe, S. (Ed.), Protein purification techniques: a practical approach, pp. 27-49. Oxford University Press, New York.

Baraguan, M.T., Laborda, F., Castillo, J.R., 2002. Reagent injection FIA system for lead determination by hydride generation - quartz-tube atomic absorption spectrometry. Analytical and Bioanalytical Chemistry 374, 115-119.

Becker, W.M., Reese, G., 2001. Immunological identification and characterization of individual food allergens. J Chromatogr B Biomed Sci Appl 756(1-2), 131-140.

Bell, K., 1967. The detection and occurrence of bovine beta-lactoglobulin C. Biochimica Et Biophysica Acta 147(1), 100-108.

Bellioni-Businco, B., Paganelli, R., Lucenti, P., Giampietro, P.G., Perborn, H., Businco, L., 1999. Allergenicity of goat's milk in children with cow's milk allergy. J Allergy Clin Immunol 103(6), 1191-1194.

Benčina, K., Benčina, M., Podgornik, A., Štrancar, A., 2007. Influence of the methacrylate monolith structure on genomic DNA mechanical degradation, enzymes activity and clogging. J Chromatogr A 1160(1-2), 176-183.

Benčina, M., Benčina, K., Štrancar, A., Podgornik, A., 2005. Immobilization of deoxyribonuclease via epoxy groups of methacrylate monoliths - Use of deoxyribonuclease bioreactor in reverse transcription-polymerase chain reaction. J Chromatogr A 1065(1), 83-91.

Besler, M., 2001. Determination of allergens in foods. Trac-Trend Anal Chem 20(11), 662-672.

Bialkowski, S.E., 1996. Photothermal Spectroscopy Methods for Chemical Analysis. John Wihley & Sons, New York.

Bičanič, D., Dhka, O., Luterotti, S., Bohren, A., Šikovec, M., van Veldhuizen, B., Berkessy, O., Chirtoc, M., Franko, M., Szabo, G., Sigrist, M., 2001. Assessing the extent of oxidation in thermally stressed vegetable oils. Part I: Optical characterization by photothermal and some conventional physical methods. Anal Sci 17, S547-S550.

Bičanič, D., Franko, M., Jalink, H., Dukić, R., Bozoki, Z., Linssen, J., 1995. Study of Saturated Triglycerides in Oil-Based on the Cw Transverse Co2-Laser Excited Photothermal Deflection Signals. Infrared Phys Techn 36(2), 617-622.

Biosca, M.Y., Ramis-Ramos, G., 1997. Optical saturation thermal lens spectrometry in non-polar solvents. Analytical Chimica Acta 345, 257-263.

Blincko, S., Rongsen, S., Decun, S., Howes, I., Edwards, R., 1999. Solid-phase supports. In: Edwards, R. (Ed.), Immunodiagnostics. A practical approach. Oxford University Press, Oxford.

Bonizzi, I., Buffoni, J.N., Feligini, M., 2009. Quantification of bovine casein fractions by direct chromatographic analysis of milk. Approaching the application to a real production context. J Chromatogr A 1216(1), 165-168.

Bordin, G., Raposo, F.C., de la Calle, B., Rodriguez, A.R., 2001. Identification and quantification of major bovine milk proteins by liquid chromatography. J Chromatogr A 928(1), 63-76.

Boškin, A., Tran, C.D., Franko, M., 2009. Oxidation of organophosphorus pesticides with chloroperoxidase enzyme in the presence of an ionic liquid as co-solvent. Environ Chem Lett 7(3), 267-270.

Boyano-Martinez, T., Garcia-Ara, C., Pedrosa, M., Diaz-Pena, J.M., Quirce, S., 2009. Accidental allergic reactions in children allergic to cow's milk proteins. J Allergy Clin Immunol 123(4), 883-888.

Bredehorst, R., David, K., 2001. What establishes a protein as an allergen? Journal of Chromatography B 756(1-2), 33-40.

Breiteneder, H., Mills, E.N.C., 2005. Molecular properties of food allergens. J Allergy Clin Immun 115(1), 14-23.

Brne, R., Lim, Y.P., Podgornik, A., Barut, M., Pihlar, B., Strancar, A., 2009. Development and characterization of methacrylate-based hydrazide monoliths for oriented immobilization of antibodies. J Chromatogr A 1216(13), 2658-2663.

Brownlow, S., Cabral, J.H.M., Cooper, R., Flower, D.R., Yewdall, S.J., Polikarpov, I., North, A.C.T., Sawyer, L., 1997. Bovine beta-lactoglobulin at 1.8 angstrom resolution - Still an enigmatic lipocalin. Structure 5(4), 481-495.

Buerk, D.G., 1992. Biosensors: Theory and applications. Technomic Publications, Lancaster.

Burks, A.W., Sampson, H., 1993. Food allergies in children. Curr Probl Pediatr 23(6), 230-252.

Darwish, I.A., Mahmoud, A.M., Al-Majed, A.R.A., 2007. Generic simple enzyme immunoassay approach to avert small molecule immobilization problems on solid phases - Application to the determination of tobramycin in serum. Talanta 72(4), 1322-1328.

de Luis, R., Lavilla, M., Sanchez, L., Calvo, M., Perez, M.D., 2009. Development and evaluation of two ELISA formats for the detection of beta-lactoglobulin in model processed and commercial foods. Food Control 20(7), 643-647.

Descotes, J., Ravel, G., Ruat, C., 2002. Vaccines: predicting the risk of allergy and autoimmunity. Toxicology 174, 45-51.

Dube, M., Zunker, K., Neidhart, S., Carle, R., Steinhart, H., Paschke, A., 2004. Effect of technological processing on the allergenicity of mangoes (Mangifera indica L.). J Agr Food Chem 52(12), 3938-3945.

Dzyabchenko, A.A., Proskurnin, M.A., Abroskin, A.G., Chashchikhin, D.V., 1998. Conjunction of thermal lens spectrometry and high-performance liquid chromatography - Approach to data treatment. J Chromatogr A 827(1), 13-20.

Edwards, R., Blincko, S., Howes, I., 1999. Principles of immunodiagnostic tests and their development; with specific use of radioisotopes as tracers. In: Edwards, R. (Ed.), Immunodiagnostics. A practical approach, second ed. Oxford University Press, Oxford.

Eigenmann, P.A., 2000. Anaphylactic reaction to raw eggs after negative challenges with cooked eggs. J Allergy Clin Immun 105, 587-588.

EUCouncil, 2000. DIRECTIVE 2000/13/EC. L109. European Parliament and Council, Offcial Journal of the European Communites.

EUCouncil, 2003. DIRECTIVE 2003/89/EC. L308. European Parliament and Council, Offcial Journal of the European Communites.

Fogolari, F., Ragona, L., Zetta, L., Romagnoli, S., De Kruif, K.G., Molinari, H., 1998a. Monomeric bovine beta-lactoglobulin adopts a beta-barrel fold at pH 2. FEBS Lett 436(2), 149-154.

Fogolari, F., Ragona, L., Zetta, L., Romagnoli, S., De Kruif, K.G., Molinari, H., 1998b. Monomeric bovine beta-lactoglobulin adopts a beta-barrel fold at pH 2. Febs Lett 436(2), 149-154.

Forge, V., Hoshino, M., Kuwata, K., Arai, M., Kuwajima, K., Batt, C.A., Goto, Y., 2000. Is folding of beta-Lactoglobulin non-hierarchic? Intermediate with native-like beta-sheet and non-native alpha-helix. J Mol Biol 296(4), 1039-1051.

Franko, M., 2009. Bioanalytical applications of thermal lens spectrometry. In: Moares, E.M. (Ed.), Thermal Wave Physics and Related Techniques: Basic Principles and Recent Developments. Transworld Research Network, Kerala.

Franko, M., Tran, C.D., 1991. Thermal Lens Effect in Electrolyte and Surfactant Media. J Phys Chem-Us 95(17), 6688-6696.

Franko, M., van de Bovenkamp, P., Bičanič, D., 1998. Determination of trans-beta-carotene and other carotenoids in blood plasma using high-performance liquid chromatography and thermal lens detection. Journal of Chromatography B 718(1), 47-54.

Gall, H., Kalveram, C.M., Sick, H., Sterry, W., 1996. Allergy to the heat-labile proteins alpha-lactalbumin and beta-lactoglobulin in mare's milk. J Allergy Clin Immun 97(6), 1304-1307.

Georges, J., 1994. A single and simple mathematical expression of the signal for cw-laser thermal lens spectrometry. Talanta 41(12), 2015-2023.

Girelli, A.M., Mattei, E., Messina, A., 2007a. Immobilized tyrosinase reactor for on-line HPLC application Development and characterization. Sensors and Actuators B 121, 515-521.

Girelli, A.M., Mattei, E., Messina, A., Papaleo, D., 2007b. Immobilization of mushroom tyrosinase on controlled pore glass: Effect of chemical modification. Sensor Actuat B-Chem 125(1), 48-54.

Gomez, V., Callao, M.P., 2007. Multicomponent analysis using flow systems. TrAC Trands in Analytical Chemistry 26(8), 767-774.

Goodman, R., Ebisawa, M., Hefle, S., Sampson, H., van Ree, R., Vieths, S., Wise, J., Taylor, S., 2007a. Allergen Online, an improved, curated protein database for assessing the potential allergenicity of genetically modified organisms and novel food ingredients. Allergy 62, 106-106.

Goodman, R.E., Taylor, S.L., Yamamura, J., Kobayashi, T., Kawakami, H., Kruger, C.L., Thompson, G.P., 2007b. Assessment of the potential allergenicity of a Milk Basic Protein fraction. Food Chem Toxicol 45(10), 1787-1794.

Gupta, R., Sheikh, A., Strachan, D.P., Anderson, H.R., 2007. Time trends in allergic disorders in the UK. Thorax 62, 91-96.

Halken, S., 1997a. Clinical symptoms of food allergy/intolerance in children. Environ Toxicol Phar 4, 175-178.

Halken, S., 1997b. Prevention of food allergy. Environ Toxicol Phar 4, 149-156.

Hall, E.A.H., 1991. Flow-Injection Analysis with Immobilized Reagents. Curr Opin Biotech 2(1), 9-16.

Hamada, D., Goto, Y., 1997. The equilibrium intermediate of beta-lactoglobulin with non-native alpha-helical structure. J Mol Biol 269(4), 479-487.

Hamada, D., Tanaka, T., Tartaglia, G.G., Pawar, A., Vendruscolo, M., Kawamura, M., Tamura, A., Tanaka, N., Dobson, C.M., 2009. Competition between Folding, Native-State Dimerisation and Amyloid Aggregation in beta-Lactoglobulin. J Mol Biol 386(3), 878-890.

Hansen, E.H., Miro, M., 2007. How flow-injection analysis (FIA) over the past 25 years has changed our way of performing chemical analyses. Trac-Trend Anal Chem 26(1), 18-26.

Hefle, S.L., Jeanniton, E., Taylor, S.L., 2001. Development of a sandwich enzyme-linked immunosorbent assay for the detection of egg residues in processed foods. Journal of Food Protection 64(1812-1816).

Hernandez-Ledesma, B., Ramos, M., Recio, I., Amigo, L., 2006. Effect of beta-lactoglobulin hydrolysis with thermolysin under denaturing temperatures on the release of bioactive peptides. J Chromatogr A 1116(1-2), 31-37.

Hianik, T., Nejdárková, M., Sokolíková, L., Meszár, E., Krivánek, R., Tvaroek, V., Novotný, I., Wang, J., 1999. Immunosensors based on supported lipid membranes, protein films and liposomes modified by antibodies. Sensors and Actuators B: Chemical 57(1-3), 201-212.

Hildebrandt, s., Steinhart, H., Paschke, A., 2008. Comparison of different extraction solution for the analysis of allergens in hen's egg. Food Chem 108, 1088-1093.

Hohensinner, V., Maier, I., Pittner, F., 2007. A 'gold cluster-linked immunosorbent assay': Optical near-field biosensor chip for the detection of allergenic beta-lactoglobulin in processed milk matrices. J Biotechnol 130(4), 385-388.

Hunt, L.T., Dayhoff, M.O., 1980. A surprising new protein superfamily containing ovalbumin, antithrombin-III, and alpha_1-proteinase inhibitor. Biochemical and Biophysical Research Communications 95(2), 864-871.

Huntington, J.A., Stein, P.E., 2001. Structure and properties of ovalbumin. Journal of Chromatography B 756, 189-198.

Janasek, D., Spohn, U., 1999. Chemiluminometric Flow Injection Analysis procedures for the enzymatic determination of L-alanine, alpha-ketoglutarate and L-glutamate. Biosens Bioelectron 14(2), 123-129.

Janeway, C.A., Travers, P., Walport, M., Shlomchik, M., 2001a. Immunobiology. The immune system in health and disease, Fifth ed. Garland publishing, New York.

Janeway, C.A.J., Travers, P., Walport, M.S., Mark J., 2001b. Immunobiology The immune system in heaalth and disease, Fifth edition ed. Garland Publishing.

Kadima, T.A., Pickard, M.A., 1990. Immobilization of Chloroperoxidase on Aminopropyl-Glass. Applied and Environmental Microbiology 56(11), 3473-3477.

Kahraman, M.V., Bayramoglu, G., Kayaman-Apohan, N., Gungor, A., 2007. alpha-Amylase immobilization on functionalized glass beads by covalent attachment. Food Chem 104(4), 1385-1392.

Karakus, E., Pekyardimci, S., 2009. Immobilization of apricot pectinesterase (Prunus armeniaca L.) on porous glass beads and its characterization. J Mol Catal B-Enzym 56(1), 13-19.

Kimber, I., Dearman, R.J., 2001. Food allergy: what are the issues? Toxicol Lett 120(1-3), 165-170.

Konig, A., Cockburn, A., Crevel, R.W.R., Debruyne, E., Grafstroem, R., Hammerling, U., Kimber, I., Knudsen, I., Kuiper, H.A., Peijnenburg, A.A.C.M., Penninks, A.H., Poulsen, M., Schauzu, M., Wal, J.M., 2004. Assessment of the safety of foods derived from genetically modified (GM) crops. Food Chem Toxicol 42(7), 1047-1088.

Kontopidis, G., Holt, C., Sawyer, L., 2002. The ligand-binding site of bovine beta-lactoglobulin: Evidence for a function? J Mol Biol 318(4), 1043-1055.

Kralj, M.B., Trebše, P., Franko, M., 2007. Applications of bioanalytical techniques in evaluating advanced oxidation processes in pesticide degradation. Trac-Trend Anal Chem 26(11), 1020-1031.

Kuramitz, H., Dziewatkoski, M., Barnett, b., Halsall, H.B., Heineman, W.R., 2006. Application of an automated fluidic system using electrochemical bead-based immunoassay to detect the bacteriophage MS2 and ovalbumin. Analytical Chimica Acta 561, 69-77.

Lagalante, A.F., Greenbacker, P.W., 2007. Flow injection analysis of imidacloprid in natural waters and agricultural matrixes by photochemical dissociation, chemical reduction, and nitric oxide chemiluminescence detection. Analytica Chimica Acta 590(2), 151-158.

Lee, W.E., Thompson, H.G., Hall, J.G., Bader, D.E., 2000. Rapid detection and identification of biological and chemical agents by immunoassay, gene probe assay and enzyme inhibition using a silicon-based biosensor. Biosensors and Bioelectronics, 14(10-11), 795-804.

Lindpaintner, K., Pfeffer, M.A., Kreutz, R., Stampfer, M.J., Grodstein, F., Lamotte, F., Buring, J., Hennekens, C.H., 1995. A Prospective Evaluation of an Angiotensin-Converting-Enzyme Gene Polymorphism and the Risk of Ischemic-Heart-Disease. New Engl J Med 332(11), 706-711.

Lodish, H., Berk, A., Baltimore, D., Zipursky, S.L., Matsudaira, P., Darnell, J., 1995. Molecular Cell Biology. W. H. Freeman Company, New York.

Logar, J.K., Malej, A., Franko, M., 2006. Double dual beam thermal lens spectrometer for monitoring of phytoplankton cell lysis. Instrum Sci Technol 34(1-2), 23-31.

Lozano, J.M., Giraldo, G.I., Romero, C.M., 2008. An improved method for isolation of beta-lactoglobulin. Int Dairy J 18(1), 55-63.

Luppa, P.B., Sokoll, L.J., Chan, D.W., 2001. Immunosensors - principles and applications to clinical chemistry. Clin Chim Acta 314(1-2), 1-26.

Luterotti, S., Franko, M., Bičanič, D., 1999. Ultrasensitive determination of beta-carotene in fish oil-based supplementary drugs by HPLC-TLS. J Pharmaceut Biomed 21(5), 901-909.

Luterotti, S., Franko, M., Šikovec, M., Bičanič, D., 2002. Ultrasensitive assays of trans- and cis-beta-carotenes in vegetable oils by high-performance liquid chromatography-thermal lens detection. Analytica Chimica Acta 460(2), 193-200.

Luterotti, S., Šikovec, M., Bičanič, D., 2000. Ultrasensitive determination of trans-beta-carotene in rat and beef livers by means of high-performance liquid chromatography coupled with thermal lens detection. Talanta 53(1), 103-113.

Madsen, C., 1997. Prevalence of food allergy/intolerance in Europe. Environ Toxicol Phar 4(1-2), 163-167.

Madžgalj, A., Baesso, M.L., Franko, M., 2008. Flow injection thermal lens spectrometric detection of hexavalent chromium. Eur Phys J-Spec Top 153, 503-506.

Male, K.B., Lyong, J.H.T., 1991. An Fia Biosensor System for the Determination of Phosphate. Biosens Bioelectron 6(7), 581-587.

Manderson, G.A., Creamer, L.K., Hardman, M.J., 1999a. Effect of heat treatment on the circular dichroism spectra of bovine beta-lactoglobulin A, B, and C. J Agr Food Chem 47(11), 4557-4567.

Manderson, G.A., Hardman, M.J., Creamer, L.K., 1999b. Effect of heat treatment on bovine beta-lactoglobulin A, B, and C explored using thiol availability and fluorescence. J Agr Food Chem 47(9), 3617-3627.

Mantyjarvi, R., Rautiainen, J., Virtanen, T., 2000. Lipocalins as allergens. Bba-Protein Struct M 1482(1-2), 308-317.

Marquette, C.A., Coulet, P.R., Blum, L.J., 1999. Semi-automated membrane based chemiluminescent immunosensor for flow injection analysis of okadaic acid in mussels. Analytica Chimica Acta 398(2-3), 173-182.

Mocak, J., Bond, A.M., Mitchell, S., Scollary, G., 1997. A statistical overview of standard (IUPAC and ACS) and new procedures for determining the limits of detection and quantification: Application to voltammetric and stripping techniques (technical report). Pure and Applied Chemistry 69(2), 297-328.

Monaci, L., van Hengel, A.J., 2008. Development of a method for the quantification of whey allergen traces in mixed-fruit juices based on liquid chromatography with mass spectrometric detection. J Chromatogr A 1192(1), 113-120.

Nakano, T., Nagata, A., 2003. ELISAs for free light chains of human immunoglobulins using monoclonal antibodies: comparison of their specificity with available polyclonal antibodies. J Immunol Methods 275, 9-17.

Negroni, L., Bernard, H., Clement, G., Chatel, J.M., Brune, P., Frobert, Y., Wal, J.M., Grassi, J., 1998. Two-site enzyme immunometric assays for determination of native and denatured beta-lactoglobulin. J Immunol Methods 220(1-2), 25-37.

Noyes, P.D., McElwee, M.K., Miller, H.D., Clark, B.W., Van Tiem, L.A., Walcott, K.C., Erwin, K.N., Levin, E.D., 2009. The toxicology of climate change: Environmental contaminants in a warming world. Environ Int 35(6), 971-986.

Ortolani, C., Pastorello, E.A., 2006. Food allergies and food intolerances. Best Pract Res Cl Ga 20(3), 467-483.

Pai, S.C., 2002. Evaluation of the temporal effect to the peak tailing in flow injection analysis. J Chromatogr A 950(1-2), 271-279.

Pan, Y., Shiell, B., Wan, J., Coventry, M.J., Roginski, H., Lee, A., Michalski, W.P., 2007. The molecular characterisation and antimicrobial activity of amidated bovine lactoferrin. Int Dairy J 17(6), 606-616.

Paulsen, P., Luf, W., Smulders, F.J.M., 2007. Different legislations on toxicants in foodstuffs. In: Pico, Y. (Ed.), Food toxicants analysis. Techniques, strategies and development. Elsevier, Amsterdam.

Pellegrini, A., Dettling, C., Thomas, U., Hunziker, P., 2001. Isolation and characterization of four bactericidal domains in the bovine beta-lactoglobulin. Bba-Gen Subjects 1526(2), 131-140.

Perez, M.D., Devillegas, C.D., Sanchez, L., Aranda, P., Ena, J.M., Calvo, M., 1989. Interaction of Fatty-Acids with Beta-Lactoglobulin and Albumin from Ruminant Milk. J Biochem-Tokyo 106(6), 1094-1097.

Pogačnik, L., Franko, M., 1999. Determination of organophosphate and carbamate pesticides in spiked samples of tap water and fruit juices by a biosensor with photothermal detection. Biosens Bioelectron 14(6), 569-578.

Pogačnik, L., Franko, M., 2001. Optimisation of FIA system for detection of organophosphorus and carbamate pesticides based on cholinesterase inhibition. Talanta 54, 631-641.

Puerta, A., Diez-Masa, J.C., de Frutos, M., 2005. Development of an immunochromatographic method to determine β-lactoglobulin at trace levels. Analytica Chimica Acta 537(1-2), 69-80.

Puerta, A., Diez-Masa, J.C., de Frutos, M., 2006. Immunochromatographic determination of beta-lactoglobulin and its antigenic peptides in hypoallergenic formulas. Int Dairy J 16(5), 406-414.

Punidadas, P., Rizvi, S.S.H., 1998. Separation of milk proteins into fractions rich in casein or whey proteins by cross flow filtration. Food Res Int 31(4), 265-272.

Qin, B.Y., Creamer, L.K., Baker, E.N., Jameson, G.B., 1998. 12-Bromododecanoi acid binds inside the calyx of bovine b-lactoglobulin. Febs Lett 438, 272-278.

Ragona, L., Pusterla, F., Zetta, L., Monaco, H.L., Molinari, H., 1997. Identification of a conserved hydrophobic cluster in partially folded bovine beta-lactoglobulin at pH 2. Fold Des 2(5), 281-290.

Ring, J., Brockow, K., Behrendt, H., 2001. Adverse reactions to foods. Journal of Chromatography B 756(3-10).

Robinson, P.J., Dunnill, P., Lilly, M.D., 1971. Porous Glass as a Solid Support for Immobilisation or Affinity Chromatography of Enzymes. Biochimica Et Biophysica Acta 242(3), 659-&.

Rogalski, J., Dawidowicz, A., Jozwik, E., Leonowicz, A., 1999. Immobilization of laccase from Cerrena unicolor on controlled porosity glass. J Mol Catal B-Enzym 6(1-2), 29-39.

Roukensa, A.H., Vossen, A.C., van Dissela, J.T., Vissera, L.G., 2009. Reduced intradermal test dose of yellow fever vaccine induces protective immunity in individuals with egg allergy. Vaccines 27, 2408–2409.

Ruzicka, J., Carroll, A.D., Lahdesmaki, I., 2006. Immobilization of proteins on agarose beads, monitored in real time by bead injection spectroscopy. Analyst 131(7), 799-808.

Sakaguchi, M., Nakayama, T., Inouye, S., 1996. Food allergynext term to gelatin in children with systemic immediate-type reactions, including anaphylaxis, to previous termvaccinesnext termstar, open. J Allergy Clin Immun 68(6), 1058-1061

Sampson, H.A., 1999. Food allergy. Part 2: Diagnosis and management. J Allergy Clin Immun 103(6), 981-989.

Sato, K., Yamanaka, M., Hagino, T., Tokeshi, M., Kimura, H., Kitamori, T., 2004. Microchip-based enzyme-linked immunosorbent assay (microELISA) system with thermal lens detection. Lab Chip 4(6), 570-575.

Sawyer, L., Kontopidis, G., 2000. The core lipocalin, bovine beta-lactoglobulin. Bba-Protein Struct M 1482(1-2), 136-148.

Scheurer, S.B., 2004. Modulation of gene expression by hypoxia in human umbilical cord vein endothelial cells: A transcriptomic and proteomic study (vol 4, pg 1737, 2004). Proteomics 4(9), 2822-2822.

Sell, M., Steinhart, H., Paschke, A., 2005. Influence of maturation on the alteration of allergenicity of green pea (*Pisum sativum L.)*. J Agr Food Chem 53, 1717-1722.

Shinohara, H., Horiuchi, M., Sato, M., Kurisaki, J., Kusakabe, T., Katsumi, K., Minami, Y., Aoki, T., Kato, I., Sugimoto, Y., 2007. Transition of avalbumin to thermostable structure entails conformational changes involving the reactive center loop. Biochimica et Biophysica Acta 1770, 5-11.

Sicherer, S.H., Leung, D.Y.M., 2005. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects. J Allergy Clin Immun 116(1), 153-163.

Skládal, P., Kaláb, T., 2005. A multichannel immunochemical sensor for determination of 2,4-dichlorophenoxyacetic acid. Analytica Chimica Acta 316(1), 73-78.

Snook, R.D., Lowe, R.D., 1995. Thermal Lens Spectrometry - a Review. Analyst 120(8), 2051-2068.

Sosnitza, P., Irtel, F., Ulber, R., Busse, M., Faurie, R., Fischer, L., Scheper, T., 1998. Flow injection analysis system for the supervision of industrial chromatographic downstream processing in biotechnology. Biosens Bioelectron 13(12), 1251-1255.

Soto, N.O., Horstkotte, B., March, J.G., de Alba, P.L.L., Martinez, L.L., Martin, V.C., 2008. An environmental friendly method for the automatic determination of hypochlorite in commercial products using multisyringe flow injection analysis. Analytica Chimica Acta 611(2), 182-186.

Stein, P.E., Leslie, A.G., Finch, J.T., Carrell, R.W., 1991. Crystal structure of uncleaved ovalbumin at 1.95 A resolution. J Mol Biol 221, 941-959.

Štrancar, A., Podgornik, A., Barut, M., Nečina, R., 2002. Short Monolithic Columns as Stationary Phases for Biochromatography. Advances in Biochemical Engineering/Biotechnology 76, 49-85.

Tag, K., Riedel, K., Bauer, H.J., Hanke, G., Baronian, K.H.R., Kunze, G., 2007. Amperometric detection of Cu2+ by yeast biosensors using flow injection analysis (FIA). Sensor Actuat B-Chem 122(2), 403-409.

Taylor, S.L., Hefle, S.L., Bindslev-Jensen, C., Bock, S.A., Burks, A.W., Christie, L., Hill, D.J., Host, A., Hourihane, J.O., Lack, G., Metcalfe, D.D., Moneret-Vautrin, D.A., Vadas, P.A., Rance, F., Skrypec, D.J., Trautman, T.A., Yman, I.M., Zeiger, R.S., 2002. Factors affecting the determination of threshold doses for allergenic foods: How much is too much? J Allergy Clin Immun 109(1), 24-30.

Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Cervel, R., Fitzpatrick, J., Mills, C., Privalle, L., Vieths, S., 2007. Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. Food Chem Toxicol 45, 1116-1122.

Tschmelak, J., Proll, G., Gauglitz, G., 2004. Sub-nanogram per litre detection of the emerging contaminant progesterone with a fully automated immunosensor based on evanescent field techniques. Analytica Chimica Acta, 519(2), 143-146.

Tzanavaras, P.D., Themelis, D.G., 2007a. Review of recent applications of flow injection spectrophotometry to pharmaceutical analysis. Analytica Chimica Acta 588(1), 1-9.

Tzanavaras, P.D., Themelis, D.G., 2007b. Validated high-throughput HPLC assay for nimesulide using a short monolithic column. J Pharm Biomed Anal 43(4), 1483-1487.

van Bommel, M.R., de Jong, A.P., Tjaden, U.R., Irth, H., van der Greef, J., 1999a. Enzyme amplification as detection tool in continuous-flow systems. I. Development of an enzyme-amplified biochemical detection system coupled on-line to flow-injection analysis. J Chromatogr A 855(2), 383-396.

van Bommel, M.R., de Jong, A.P., Tjaden, U.R., Irth, H., van der Greef, J., 1999b. Enzyme amplification as detection tool in continuous-flow systems. II. On-line coupling of liquid chromatography to enzyme-amplified biochemical detection after pre-column derivatization with biotin. J Chromatogr A 855(2), 397-409.

van Dijk, J.A.P.P., Smit, J.A.M., 2000. Size-exclusion chromatography-multiangle laser light scattering analysis of beta-lactoglobulin and bovine serum albumin in aqueous solution with added salt. J Chromatogr A 867(1-2), 105-112.

Van Es, R.M., Setford, S.J., Blankwater, Y.J., Meijer, D., 2001. Detection of gentamicin in milk by immunoassay and flow injection analysis with electrochemical measurement. Analytica Chimica Acta 429(1), 37-47.

Van Hengel, A., Anklam, E., Taylor, S.L., Hefle, S.L., 2007. Analysisi of food allergens. Practical applications. In: Pico, Y. (Ed.), Food Toxicants Analysis, First ed. Elsevier, Amsterdam.

van Hengel, A.J., 2007. Declaration of allergens on the label of food products purchased on the European market. Trends Food Sci Tech 18(2), 96-100.

Veitch, N.C., 2004. Horseradish peroxidase: a modern view of a classic enzyme. Phytochemistry 65, 249-259.

Vodopivec, M., Berovič, M., Jančar, J., Podgornik, A., Štrancar, A., 2000. Application of Convective Interaction Media disks with immobilised glucose oxidase for on-line glucose measurements. Analytica Chimica Acta 407(1-2), 105-110.

Vodopivec, M., Podgornik, A., BeroviČ, M., Štrancar, A., 2003a. Characterization of CIM monoliths as enzyme reactors. J Chromatogr B 795(1), 105-113.

Voet, D., Voet, J.G., Pratt, C.W., 1998. Fundamentals of Biochemistry. John Wiley & Sons, New York.

Walsh, M.K., Wang, X.W., Weimer, B.C., 2001. Optimizing the immobilization of single-stranded DNA onto glass beads. J Biochem Bioph Meth 47(3), 221-231.

Weimer, W.A., Dovichi, N.J., 1986. Simple-Model for the Time-Dependence of the Periodically Excited Crossed-Beam Thermal Lens. J Appl Phys 59(1), 225-230.

Wilson, R., Clavering, C., Hutchinson, A., 2003. Paramagnetic bead based enzyme electrochemiluminescence immunoassay for TNT. Journal of Electroanalytical Chemistry 557, 109-118.

Wolf, W.r., Andrews, k.W., 1995. A system for defining reference material applicable to all food matrices. Freseniu's Journal of analytical Chemistry 352(1-2), 73-76.

Wu, J., Fu, Z.F., Yan, F., Ju, H.X., 2007. Biomedical and clinical applications of immunoassays and immunosensors for tumor markers. Trac-Trend Anal Chem 26(7), 679-688.

Wu, L.C., Cheng, C.M., 2005. Flow-injection enzymatic analysis for glycerol and triacylglycerol. Anal Biochem 346(2), 234-240.

Yamasaki, M., Takahashi, N., Hirose, M., 2003. Crystal structure of S-ovalbumin as a non-loop-inserted thermostabilized Serpin Form. The Journal of Biological Chemistry 278(12), 35524-35530.

Yeniceli, D., Dogrukol-Ak, D., Tuncel, M., 2004. Determination of lansoprazole in pharmaceutical capsules by flow injection analysis using UV-detection. J Pharmaceut Biomed 36(1), 145-148.

Yu, H., Yan, F., Dai, Z., Ju, H., 2004. A disposable amperometric immunosensor for α-1-fetoprotein based on enzyme-labeled antibody/chitosan-membrane-modified screen-printed carbon electrode. Analytical Biochemistry 331(1), 98-105.

Zacharis, C.K., Kalaitzantonakis, E.A., Podgornik, A., Theodoridis, G., 2007. Sequential injection affinity chromatography utilizing an albumin immobilized monolithic column to study drug-protein interactions. J Chromatogr A 1144(1), 126-134.